Central antinociceptive activity of botulinum toxin A

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SREDIŠNJE ANTINOCICEPTIVNO DJELOVANJE BOTULINUM TOKSINA A

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Ova disertacija napisana je na engleskom jeziku temeljem članka 35. ***Pravilnika o doktorskim studijima*** *(Medicinski fakultet u Zagrebu, 2014.), te članka 15.* ***Pravilnika o doktorskim studijima na Sveučilištu u Zagrebu*** *(2010.).*

Disertacija je pisana kao skup objavljenih znanstvenih radova popraćen kritičkim preglednim poglavljem (tzv. skandinavski model), temeljem članka 35. ***Pravilnika o doktorskim studijima*** *(Medicinski fakultet u Zagrebu, 2014.), te članka 14.* ***Pravilnika o doktorskim studijima na Sveučilištu u Zagrebu*** *(2010.).*

This PhD thesis was made at the Department of Pharmacology, University of Zagreb School of Medicine. Majority of the experiments performed for this dissertation were done at the Laboratory of Molecular Neuropharmacology, Department of Pharmacology, University of Zagreb School of Medicine. ELISA and initial immunohistochemical experiments were performed at the Department of Psychiatry, University of Würzburg (under supervision of prof. Peter Riederer), while radioimmunoassay was performed at the Department of Pharmacology, University of Pécs (under supervision of prof. Zsuzsanna Helyes)

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Since present PhD thesis has been co-mentored by a foreign scientist (prof. Peter Riederer from Germany), it was written in English, in accordance with Regulations on Doctoral studies at the School of Medicine (Pravilnik o doktorskim studijima, čl. 35). The thesis has been written in the form of **a collection of published articles accompanied by a review** (Scandinavian type), in accordance with Regulations on Doctoral studies at the School of Medicine (Pravilnik o doktorskim studijima, čl. 35), and University of Zagreb (Pravilnik o doktorskim studijima Sveučilišta u Zagrebu, čl. 14). The collection of articles included in the thesis consists of **4 research articles** and **1 review article**.

List of published articles included in the thesis:

1. Matak, I., Bach-Rojecky, L., Filipović, B., Lacković, Z., 2011 **Behavioral and immunohistochemical evidence for central antinociceptive activity of botulinum toxin A**. Neuroscience 186, 201-207. *Research article*

2. Matak I., Riederer P., Lacković Z., 2012 **Botulinum toxin's axonal transport from periphery to the spinal cord**. Neurochem. Int. 61, 236-239. *Research article*

3. Matak, I., Stracenski, I., Lacković, Z. 2013 **Comparison of analgesic effects of single versus repeated injection of botulinum toxin in orofacial formalin test in rats**. J. Neural Transm. 120:141-144. *Research article*

4. Matak, I., Rossetto, O., Lacković, Z. 2014 **Botulinum toxin type A selectivity for certain types of pain is associated with capsaicin-sensitive neurons.** Pain 155, 1516-1526. *Research article*

5. Matak, I., Lacković, Z. 2014 **Botulinum toxin A, brain and pain.** Prog. Neurobiol. 119-120, 39-59. *Review article*

List of abbreviations

AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP, adenosine triphosphate

BoNT/A, botulinum toxin type A

CGRP, calcitonin gene-related peptide

CFA, complete Freund's adjuvant

ChAT, choline acetyltransferase

ELISA, enzyme-linked immunosorbent assay

GABA, γ-aminobutyric acid

GAP-43, growth-associated protein 43

GFAP, glial fibrillary acidic protein

GTP, guanosine triphosphate

H_C, C-terminal heavy chain domain of botulinum toxin

H_N, N-terminal heavy chain domain of botulinum toxin

i.a, intra-articular

i.g., intraganglionic

i.n., intraneural

INN, international nonproprietary name

IoNC, infraorbital nerve constriction

i.t., intrathecal

kDa, kilodalton (Mr=1000)

LC, light chain domain of botulinum toxin

MAP-2, microtubule-associated protein 2

m-RNA, messenger-ribonucleic acid

NeuN, neuronal nuclei

NMDA, n-methyl-D-aspartate

PC-12, pheochromocytoma-12

PREEMPT, Phase III Research Evaluating Migraine Prophylaxis Therapy

SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis

SNAP-25, synaptosomal-associated protein of 25 kDa

SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SV2, synaptic vesicle protein 2

TeNT, tetanus toxin

TNC, trigeminal nucleus caudalis

TRPV1, transient receptor potential vanilloid 1

USAN, United States adopted name

VAMP, vesicle-associated membrane protein;

CONTENTS:

1. INTRODUCTION AND BACKGROUND FOR PROPOSED RESEARCH

Botulinum toxin type A (BoNT/A) is a neuroparalytic bacterial exotoxin and a therapeutic protein. It is used for treatment of several hyperkinetic movement disorders, with recent expansion of its use in a wide array of other neurological disorders including chronic pain (Truong et al., 2009). Initially, the observed BoNT/A antinociceptive action was associated with reduction of muscle tone, e.g. in spasticity and dystonia (Mense, 2004). However, in the last 15-20 years, the use has been extended to non-muscular chronic pain disorders. After serendipitous discovery that it reduces symptoms of migraine headache in persons treated for reduction of facial wrinkles, BoNT/A has been clinically investigated in headache disorders (Silberstein et al., 2000). BoNT/A use for chronic migraine has been approved since 2010 (Dodick et al., 2010). Its off-label use has confirmed the BoNT/A efficacy in clinical trials of other types of pain, such as osteoarthritis, low back pain, trigeminal neuropathy, myofascial pain, temporomandibular joint disorders, etc. (Jabbari and Machado, 2011). The biggest advantage of BoNT/A use is that, due to the long-term survival of toxin's enzymatic part within neurons, the clinical effects may last for 3-6 months after a single peripheral application. Its efficacy in chronic pain disorders occurs without serious side effects related to repeated use of conventional analgesics, such as the tolerance and medication overuse (reviewed by Matak and Lacković, 2014; Appendix VI).

Surprisingly, although molecular mechanisms of BoNT/A action in peripheral cholinergic synapses have been well characterized, relatively little is known about the mechanism of its action in different pain disorders. In analogy with its well known effect on muscular paralysis, peripheral site of action and supposed local prevention of neurotransmitter release still dominate among current opinions (Aoki and Francis, 2011; Wheeler and Smith, 2013). In our laboratory, based on observed BoNT/A distant effect in bilateral pain models, and demonstrated necessity of axonal transport for BoNT/A efficacy, the idea of a central site of action has been conceived (Bach-Rojecky, 2006; Bach-Rojecky and Lacković, 2009; Bach-Rojecky et al., 2010). In present PhD thesis, to make further mechanistic insights into BoNT/A action on pain in CNS, we set out to investigate the axonal transport of enzymatically active BoNT/A in sensory neurons and to elucidate the sites of BoNT/A activity in central sensory regions.

1.1 BoNT/A, botulism and historical overview

BoNT/A and other serotypes of botulinum toxin (BoNT) are produced by a few species of sporogenous rod-shaped anaerobic bacteria from genus *Clostridium* (*C. botulinum, C. baratii, C. butyricum, C. argentinense)* (Popoff and Bouvet, 2005). BoNT exotoxin is a large protein complex formed inside the bacterial cell and released by secretion or bacterial lysis. It consists of neurotoxin part which invades neuronal terminals and blocks the vesicular release of neurotransmitters. Non-toxic part of BoNT complex formed by auxiliary proteins augments the stability of toxin prior to penetration into the systemic circulation (details explained in Section 1.2). Up to now, seven well known serotypes which induce a neuroparalytic disease called botulism in animals and humans have been characterized (A-G). These serotypes may also have several subtypes (for instance, BoNT/A has 5 subtypes designated as A1 through A5) (Dover et al., 2009; Kalb et al., 2011). Serotype H, which has been discovered only recently, has not yet been fully characterized and no antidotes have been produced up to now (Barash and Arnon, 2014; Dover et al. 2014). Due to their exquisite ability of to enter neurons and to block the neurotransmitter release in very low doses, BoNTs are among the most potent toxins known. Median intravenous lethal dose of serotype A in humans is only 1 ng/kg or 70 ng/70 kg person (Gill, 1982). This makes BoNT a potential threat if used as an inhalational bioweapon (Arnon et al., 2001; Bigalke and Rummel, 2005; Franz et al., 1997; Gill, 1982).

Botulism is a neuroparalytic disease caused by systemic intoxication with different BoNT serotypes in animals and humans. In humans, intoxication has been reported for serotypes A, B, E and F (Sobel et al., 2005). BoNT serotype H has been characterized for the first time in a child suffering from mixed BoNT/B and BoNT/H toxo-infection (Barash and Arnon, 2014). Although nowadays a very rare disease, botulism may have a fatal outcome in 5 to 10% of cases. Due to its ability to invade peripheral cholinergic terminals, typical BoNT/A symptoms include flaccid symmetric paresis of skeletal and smooth muscles, and autonomic nervous system impairment (WHO 2013). The effects on sensory system have also occasionally been reported in humans (Goode and Shearn, 1982; Kuruoğlu et al., 1996; Martínez-Castrillo et al., 1991). Relatively mild first symptoms include symmetric paresis of craniofacial muscles, followed by weakness and vertigo, dry mouth and difficulties in speaking and swallowing. In more severe cases, potentially fatal respiratory failure may occur due to the paralysis of respiratory muscles. Immediate treatment consists of early administration of neutralizing

antitoxin (useful only before BoNT enters the neurons), and subsequent respiratory support in the intensive care unit, if needed (WHO 2013). Respiratory support may be necessary for several months before the toxin's effects wear off.

Food-borne botulism was characterized for the first time by the early 19th century German physician Justinus Kerner, who described the symptoms of food poisoning caused by ingestion of contaminated smoked sausages. J. Kerner himself coined a name of the disease according to the suspected cause of food poisoning (*botulus* = lat. sausage) (Erbguth, 2008). Intoxication with toxin-contaminated food associated with different types of inadequately sterilized home-made conserved food continues to be the most common form of BoNT poisoning. *C. botulinum* endospores are extremely heat-resistant, while the toxin itself is destroyed by boiling or heating (WHO 2013). *Inhalational botulism*, occurring due to toxin inhalation and its subsequent penetration into the systemic bloodstream, can occur due to accidental toxin exposure during industrial production. BoNT use as an inhalational biological weapon has been regarded as a possible biological threat (Arnon et al., 2001). *Iatrogenic botulism* may occur due to misuse or incorrect dosage of therapeutically used protein, which has been reported after cosmetic use of high-dose of uncharacterized illegal BoNT/A preparation, and in children treated with high dose of commercial BoNT/A product for spasticity (Chertow et al., 2006; Crowner et al., 2007).

Apart from systemic intoxication with BoNT/A, botulism can also be caused by anaerobic toxo-infection with *C. botulinum* endospores germinating into viable bacteria, which generate the toxin inside the body. Due to initial anaerobic conditions and lack of normal gastrointestinal flora, after ingestion of toxin endospores the bacterium may grow in the child's intestinal tract and induce *infant botulism* (WHO 2013). *Intestinal toxemia botulism* in adults may occur if the normal gut flora has been altered by abdominal surgical procedures or antibiotic therapy (WHO 2013). In intravenous drug abusers, needle wound infection may induce spore germination and subsequent systemic intoxication (Sobel, 2005; Wenham 2008).

Development of clinical use. Nowadays, local use of purified BoNT/A in low doses has become a basis of its widespread use. Apart from BoNT/A, serotype B has been approved for clinical use, too. The long-term neuroparalytic activities of BoNT/A and BoNT/B in synapses lasting up to several months have been the basis of their clinical use in various neuromuscular and autonomous disorders. In the late 1960s and 1970s, an American physician and

ophthalmologist Allan B. Scott used low BoNT/A doses to treat strabismus in children by injecting small doses of purified BoNT/A into hyperactive lateral or medial rectus muscle (Scott et al., 1973; Scott, 1980). BoNT/A has been approved for this indication in 1989, and later in other types of muscular hyperactivity disorders like blepharospasm, hemifacial spasm, focal dystonia and upper limb spasticity (reviewed by Barnes 2003; Thengannat et al., 2012). Neuroparalytic effect on autonomic synapses has been employed for treatment of autonomic disorders such as primary axillar hyperhidrosis and urinary incontinence caused by neurogenic detrusor overactivity (Dressler 2013; Naumann et al., 2013; Seth et al., 2013). The ability of BoNT/A to paralyze neuromuscular junctions for a long period of time and induce the atrophy of mimic muscles has been turned into the most often cosmetic treatment of facial wrinkles, used yearly by millions of people worldwide. BoNT/A is approved for this indication since 2002.

Presently, BoNT/A is one of the most commonly used therapeutic proteins produced by over 20 manufacturers throughout the world (Truong et al., 2009). Potency units, based on mouse intraperitoneal LD50, are specific to each BoNT commercial preparation and cannot be compared or converted from one product to another. Therefore, BoNT preparations from different manufacturers have different generic names. European Medicines Agency (EMA) uses international nonproprietary names (INN) (EMA authorization and referral documents, www.ema.europa.eu), while in the United States, Food and Drug Administration uses United States Adopted Names (USAN) (FDA 2009; FDA 2013):

- Botox® (INN: Clostridium botulinum type A neurotoxin complex); USAN: onabotulinumtoxinA (produced by Allergan Inc)
- Xeomin® (INN: clostridium botulinum neurotoxin type A); USAN: incobotulinumtoxinA (produced by Merz Pharma GmbH & Co KGaA)
- Neurobloc® (marketed as Myobloc® in the USA) (INN: botulinum toxin type B); USAN: rimabotulinumtoxinB (produced by Solstice Neurosciences, LLC)

1.2 Structural, pharmacokinetic and pharmacodynamic properties of BoNT/A

1.2.1 Structure of BoNT/A complex

BoNT/A molecular complex released from *C. botulinum* consists of the toxic part (150 kDa) and auxiliary proteins (750 kDa). The neurotoxic part of the BoNT/A complex is composed of two polypeptide chains joint covalently by a disulfide bridge. The larger, 100 kDa heavy chain binds to specific BoNT/A membrane acceptors via distinct carboxy terminal binding domain (H_C), and mediates the toxin subsequent endocytotic internalization to nerve terminals. Translocation domain at the N terminal (H_N), mediates the light chain translocation from the endocytotic vesicle into the neuronal cytosol (Tighe and Schiavo, 2013). 50 kDa light chain (BoNT/A LC) is a proteolytic enzyme which, by targeting the synaptic release machinery, prevents synaptic neurotransmitter release. Other BoNT serotypes (B-H), along with tetanus toxin (TeNT), related toxin produced by *Clostridium tetani*, share a similar homologous structure of the 150 kDa neurotoxic part with BoNT/A.

Auxiliary proteins comprising haemaglutinins and non-haemaglutinins participate in the stabilization of BoNT/A complex and preservation outside the bacterial cell (Tighe and Schiavo, 2013). Throughout the GI tract, they protect the neurotoxic component from degradation by digestive proteolytic enzymes (Chen et al., 1998). 150 kDa neurotoxin by itself can be absorbed into the systemic circulation from the stomach, small intestine and lungs independently of auxiliary proteins. (Maksymowych et al., 1999; Al-Saleem et al., 2012).

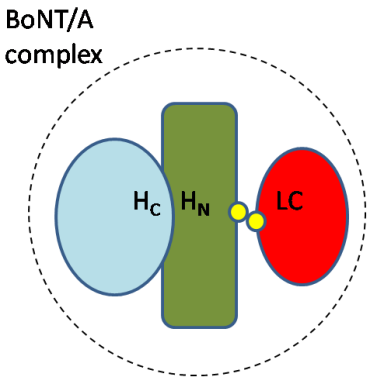

Figure 1 ***Schematic representation of structure of BoNT/A molecule.*** *BoNT/A 150 kDa neurotoxin molecule is composed of a heavy chain 50 kDa C-terminal domain (H_c), 50 kDa N-terminal domain (H_N), and 50 kDa light chain (LC). LC is attached to the heavy chain by a disulfide bridge (small yellow circles represent sulphur atoms). Dashed circle represents the auxiliary proteins of 750 kDa in total, not shown in details.*

1.2.2. Pharmacokinetics

After BoNT/A reaches the gut by ingestion or production by bacteria, 150 kDa neurotoxin

penetrates the gut epithelial lining, usually in the small intestine. Similarly, BoNT/A may penetrate the alveolar lining after its inhalation. After that, it is transcytosed across the basolateral surface of epithelial cells, from where it enters the blood capillaries and systemic circulation. It then reaches the extracellular fluid of peripheral tissues throughout the body (Simpson et al., 2013). Only a small part of orally-ingested BoNT/A reaches the systemic circulation due to degradation or inactivation by low-pH conditions and digestive proteases in the gut (Sugii et al., 1977; Sobel et al., 2005) (estimated median oral lethal dose is 70 μg vs. 0.07 μg i.v. dose, or 0.8-0.9 μg estimated inhalational dose) (Arnon et al., 2001, Gill, 1982).

Systemic pharmacokinetic data from rodents suggest that the elimination half-life of active toxin in the bloodstream, after its i.v. administration, is around 4 hours. In the blood, only 5-15% of toxin is bound to plasma proteins (Ravichandran et al., 2006). To investigate the time-course of poisoning, BoNT/A neutralization with antibodies was performed at different time points. Antitoxin administered 10 min after high dose i.v. BoNT/A only partially prolonged the animal survival, while after 20 min it was completely unable to prevent botulism (Ravichandran et al., 2006). The data suggest that the long elimination half-life in plasma and low binding to plasma proteins are sufficient to induce systemic poisoning. Low efficiency of post-challenge antitoxin administrations suggests that the toxin enters peripheral nerve terminals quickly after entrance into the bloodstream.

BoNT/A employed for therapeutic use may spread from the injection site, and induce localized side effects, such as the unwanted paralysis of nearby muscles (Brodsky et al., 2012; Majlesi et al., 2007). Appropriately chosen volume, dose and method of injection may reduce the incidence of side effects induced by toxin spread. BoNT/A auxiliary proteins, which quickly dissociate from 150 kDa neurotoxin under normal pH conditions, do not contribute significantly to the toxin spread in peripheral tissues (Brodsky et al., 2012; Carli et al., 2009). Although the circulating toxin may not penetrate the blood-brain barrier, it may reach the CNS by its axonal traffic in sensory and motor neurons (discussed in Section 5.1). BoNT/A pharmacokinetic is schematically shown in Fig. 2

BoNT/A enters neurons as a 150 kDa neurotoxic component alone. Both binding and endocytosis into neurons are mediated by H_c subunit, which binds the specific dual protein-sialoganglioside acceptors on the outer side of synaptic plasma membrane. BoNT/A establishes the initial contact with the outer side of plasma membrane by binding to

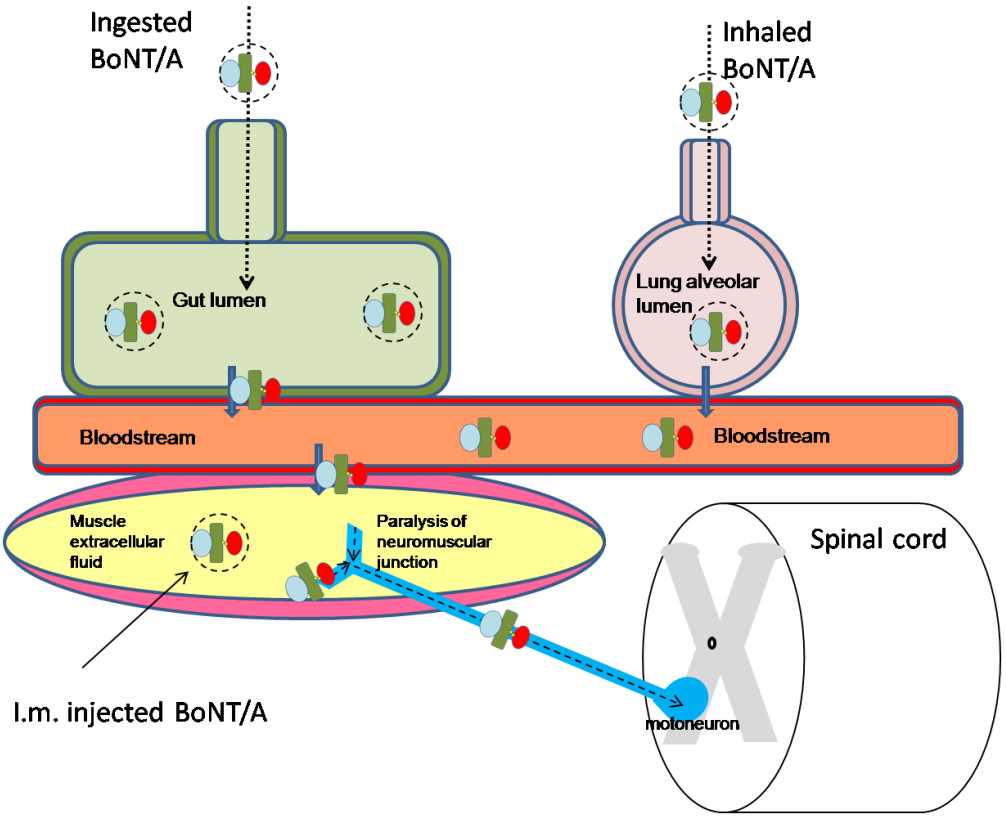

Figure 2 Pharmacokinetics of BoNT/A. *Ingested or inhaled BoNT/A complex reaches the lumen of intestines or lung alveoles, and the neurotoxic part enters the bloodstream by transcytosis across epithelial layers. This is followed by systemic distribution of toxin into the extracellular fluid of peripheral tissues, such as muscles (auxiliary proteins indicated by dashed circle line dissociate from 150 kDa neurotoxin). Systemically distributed or intramuscularly (i.m.) injected BoNT/A molecules then enter neuromuscular junctions and paralyses the muscles. Circulating BoNT/A cannot penetrate the blood-brain barrier, however, a portion of BoNT/A is retrogradely transported within peripheral nerves towards the CNS. Favorable pharmacokinetic properties for high BoNT/A toxicity are: the ability to survive harsh conditions of GI tract, to be able to cross the epithelial layersand enter the bloodstream, sufficient half-life in the systemic bloodstream and specific recognition of neuronal terminals to promptly enter the cytosol of neuronal terminals.*

polysialogangliosids (glycosphingolipids with sialic acid residues), which anchors the toxin from extracellular fluid (Simpson et al., 2013). In addition, the HC subunit binds to high affinity membrane protein acceptors, synaptic vesicle protein 2 (SV2) and fibroblast growth factor receptor 3 (Dong et al., 2006; Jacky et al., 2013; Mahrhold et al., 2006). SV2 protein is present in 3 isoforms (SV2A-C). BoNT/A binding and endocytotic entry into neurons can be mediated by all three SV2 isoforms, with the highest affinity for SV2C (Dong et al., 2006). By a dynamin-dependent process, these protein acceptors are internalized together with BoNT/A into the lumen of recycled synaptic vesicles (Colasante et al., 2013; Harper et al., 2011). The process is augmented by increased neuronal activity (Harper et al., 2011).

Subsequent BoNT/A internalization into neurons is followed by release of LC into the cytosol, mediated by the H_N domain which serves as a chaperone channel (Fischer and Montal, 2007; Kalandakanond and Coffield, 2001). H_N domain is inserted into the synaptic vesicle membrane and forms a pore, through which the unfolded LC is translocated into the cytosol. Under the influence of acidic pH in vesicles, disulphide bridge between the heavy and light chain is reduced during the translocation process (Fischer and Montal, 2007).

1.2.3 Pharmacodynamics

As the translocated LC polypeptide is refolded inside the cytosol, it becomes a Zn^{2+}-dependent metalloproteolytic enzyme. LC metalloprotease hydrolizes a single peptide bond 9 aminoacids away from the C-terminal of a membrane-bound synaptic protein Synaptosomal-associated protein of 25 kDa (SNAP-25) (Blasi et al., 1993). SNAP-25 is a 206 amino-acid (a.a.)-long palmitoylated membrane protein anchored to the cytosolic side of presynaptic plasma membrane, which, together with its binding partners: membrane-associated protein syntaxin, and vesicle-associated membrane protein (VAMP)/synaptobrevin, forms complexes which mediate synaptic vesicle fusion with presynaptic plasma membrane. This heterotrimeric complex, necessary for Ca^{2+}-dependent synaptic vesicular release of neurotransmitters, is known as the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Tighe and Schiavo, 2013). Each of the BoNT serotypes (A-G), together with tetanus toxin, targets a distinct peptide bond on one of the three SNAREs. BoNT/A, BoNT/E and BoNT/C1 cleave SNAP-25, while BoNT serotypes B, D,F,G and TeNT, cleave VAMP (Binz et al., 1994; Pelizzari et al., 1999; Schiavo et al., 1992). Apart from SNAP-25, BoNT/C1 cleaves syntaxin (Foran et al., 1996). Based on similarity of its primary sequence with BoNT/F5, it is predicted that newly discovered serotype BoNT/H cleaves VAMP (Barash and Arnon, 2013; Dover et al., 2014). By cleaving unique peptide bonds on SNARE-proteins, different BoNT serotypes and tetanus toxin (TeNT) prevent fusion of neurotransmitter-containing vesicles with presynaptic plasma membrane and subsequent neurotransmitter release.

BoNT/A unique therapeutic effects after single peripheral therapeutical application last for 3-6 months. In addition, minute amounts of toxin are needed to induce the therapeutic effect. Unique pharmacodynamic properties enable the high potency of BoNT/A at the synapse.

BoNT/A as a soluble enzyme may target many SNAP-25 molecules in the cytosol. Particular long-term efficacy and high potency at the synapse is due to remarkable longevity of BoNT/A LC in the cytosol, as well as the mechanistic consequences of BoNT/A-mediated SNAP-25 cleavage in synaptic SNARE complexes. Importantly, BoNT/A effects in synapses are reversible, i.e. the synapse regains its original function after completion of protease effects.

BoNT/A protease in the cytosol has a particularly long life: recent study indicated that proteolytic activities of different BoNT/A subtypes (1-5) in neuronal cultures may last for up to 10 months (Whitemarsh et al., 2014), while *in vivo* the protease may remain functional for up to 6 months in central neurons (Antonucci et al., 2008). Persistence of LC in the cytosol can be partially explained by its ability to evade ubiquitin-proteasome cellular protein degradation mechanism. Unlike BoNT/E, BoNT/A is not readily ubiquitinated by ubiquitin ligases. This property most likely mediates longer therapeutic effects of BoNT/A compared to BoNT/E (BoNT/E effects last for 2-3 weeks only) (Tsai et al., 2010). Dileucine motif in the primary sequence of LC appears to exibit a protective role on the protease longevity (Wang et al., 2011), which is associated with a newly-discovered LC interaction with cytoskeletal proteins called septins (Vagin et al., 2014). Another possible contributing mechanism is the unique mode of protein folding of LC at physiological conditions, which assumes a molten-globule conformation (partially folded intermediate protein state). Molten globule state is proposed to mediate a remarkable stability and flexibility of BoNT/A structure in physiological solutions lasting for many months (Kumar et al., 2014).

It seems that high potency of BoNT/A LC is also mediated by a small subset of synaptic SNAP-25 targeted by BoNT/A, however, highly relevant for synaptic function. Cleavage of only a small portion of synaptic SNAP-25 (10-20%) is sufficient to induce complete synaptic paralysis in muscles and cultured autonomic neurons (Kalandakanond and Coffield, 2001; Lawrence et al., 2013).

While the SNARE proteolysis induced by most other BoNT serotypes prevents the SNARE complex assembly, truncation of 9 C-terminal amino acids by BoNT/A does not impair the assembly of heterotrimeric SNARE complex (Lawrence et al., 2002; Meunier et al., 2003). Rather, the mechanism of BoNT/A-induced prevention of SNARE-mediated transmitter release is the impaired Ca^{2+}-induced triggering of vesicle membrane fusion with plasma membrane, which is mediated by interaction of Ca^{2+}-sensing protein synaptotagmin with C-

terminus of SNAP-25 (Gerona et al., 2000). Inactive SNARE complexes may persist for some time in the synapse and act as competitive inhibitors of the neurotransmitter release, thus, augmenting the BoNT/A-mediated synaptic paralysis (Keller and Neale, 2001). Following the inhibition of LC catalytic activity, the synapse regains its function in 4 days, which likely corresponds to the turn-over time of inactive SNARE complexes (Bartels et al., 1994; Keller and Neale, 2001). Several SNARE complexes forming a radial star-shaped oligomer (SNARE supercomplex) are necessary for the fusion of single synaptic vesicle with plasma membrane (Megighian et al., 2010). Hypothetically, occurrence of a single inactive SNARE complex within the oligomer may compromise the activity of the supercomplex.

Classical symptoms of botulism had lead to the initial assumption that BoNT action is selective for cholinergic transmission, only. However, in line with the ubiquitous role of SNAREs in neurotransmitter release and presence of membrane acceptors in other types of neurons, BoNTs may block the neurotransmitter release of most neurotransmitters. *In vitro* and *in vivo* studies demonstrated the BoNT/A prevents the release of serotonin, dopamine, noradrenaline, glutamate, gamma-aminobutyric acid (GABA), enkephalin, glycine, Substance P and calcitonin gene-related peptide (CGRP) (Nakov et al., 1989, Mc Mahon et al., 1992; Welch et al., 2000; Morris et al., 2002; Durham et al., 2004; Verderio et al., 2007).

BoNT/A exhibits a considerable selectivity for prevention of excitatory neurotransmission, such as cholinergic and glutamatergic, in comparison to GABA-ergic inhibitory neurotransmission. This is not due to different mechanism of neurotransmitter release in GABA-ergic neurons, since SNAP-25 mediates GABA release, and BoNT/A may also enter GABA-ergic neurons and cleave the SNAP-25. However, SNARE complexes containing BoNT/A-cleaved SNAP-25 (which lacks 9 C terminal aminoacids only) retain the ability to support the neurotransmitter release in GABA-ergic neurons. It seems that the functional ability of BoNT/A-cleaved SNAP-25 is mediated by higher Ca^{2+} concentrations in GABA-ergic neurons compared to excitatory neurons. The Ca^{2+} transient increase is higher in GABA-ergic neurons in comparison to glutamatergic neurons, which overcomes the BoNT/A-mediated paralysis (Grumelli et al., 2010). At low Ca^{2+} concentrations present in excitatory neurons, loss of 9 C-terminal aminoacids impairs the interaction between SNARE-complex and synaptotagmin, a Ca^{2+} sensor protein which triggers the neurotransmitter release,. In conditions of high Ca^{2+}, such as the ones present in GABA-ergic neurons, this interaction is restored and the neurotransmitter release remains functional despite the SNAP-

25 C-terminal truncation (Gerona et al., 2000; Grumelli et al., 2010; Lawrence et al., 2002). Reducing the Ca^{2+} levels by chelators confers GABA-ergic neurons more sensitive to BoNT/A (Grumelli et al., 2010; Verderio et al., 2004).

BoNT/A may also enter some non neuronal cells, and block the SNARE-dependent vesicular release from pancreatic beta cell lines (blockage of insulin release), astrocytes (glutamate) chromaffin cells and Schwann cells (acetylcholine) (He et al., 2008; Lawrence et al., 2002; Kanno and Nishizaki, 2012; Marinelli et al., 2012).

Recent study suggested that BoNT/A-mediated prevention of neurotransmitter release BoNT/A is not confined only to synapses, and that it may also prevent the ectopic neurotransmitter release away from synaptic active zones. BoNT/A inhibits the ectopic vesicular release of glutamate and ATP from axons of olfactory receptor neurons (Thyssen et al., 2010). Since SNAP-25, apart from synapses, is present in axons, it was proposed that SNAP-25 may mediate many different membrane fusion events in the axonal compartment (Galli et al., 1995; Duc and Catsicas, 1995)

SNAP-25 participates in the regulation of some additional processes other than vesicular neurotransmitter release. It modulates the activity of some voltage gated calcium channels, the effect which can be altered by BoNT/A (He et al., 2008; Ji et al., 2002; Pozzi et al., 2007; Zamponi, 2003). BoNT/A may prevent the SNARE-mediated translocation of receptors to plasma membrane, such as transient receptor potential vanilloid 1 (TRPV1) and N-methy-D-aspartate receptor (Cheng et al., 2013; Morenilla-Palao et al., 2004; Shimizu et al., 2012). BoNT/A may also prevent the G protein interaction with SNARE-dependent exocytotic machinery (Gerachshenko et al., 2005). In addition to BoNT/A effects on the activity of ion channels, BoNT/A may prevent the process of neurite extension by impairing the activity of axonal growth cones. Similar effect is observed in the process of dendrite extension (Groose et al., 1999; Morihara et al., 1999). In conclusion, BoNT/A *in vivo* effects might be much more complex than the simple prevention of neurotransmitter release, which requires to be examined (Matak and Lacković, 2014).

Several *in vitro* studies indicated a possibility that some of BoNT/A actions are not mediated by SNAP-25, at all. Several reports suggest that BoNT/A actions on some processes, such as phospholipase/arachidonic pathway-mediated neurotransmitter release, effect on apopotosis of prostate and breast cancer cells, and motoneuronal terminal sprouting, are not necessarily

mediated by SNAP-25 (Coffield and Yan, 2009; Ishida et al. 2004; Proietti et al., 2012; Ray et al., 1993; Ray et al., 1999; Zhang et al., 2013). In particularly, Coffield and Yan demonstrated that the binding activity of heavy chain subunit promotes the motoneuronal terminal sprouting similar to full-length BoNT/A neurotoxic complex. Observed connection of BoNT/A-mediated prevention of lysophosphatidic acid-promoted acetylcholine release with proteasomal degradation of a rho-GTP-ase enzyme RhoB (Ishida et al., 2004)., as well as the mechanism of promoted apoptosis in cell cultures of cancer cells lacking SNAP-25 is not fully understood (Bandala et al. 2013; Karsenty et al., 2009; Proietti et al., 2012). In prostate cancer cell lines BoNT/A promotes the phosphorylation of phospholipase A2, which might be associated with BoNT/A-induced apoptosis and inhibition of proliferation (Proietti et al., 2012). A newly-discovered LC interaction with GTP-binding cytoskeletal proteins called septins (septin-2 and septin-7) (Vagin et al., 2014), offer the possibility for some unknown additional BoNT/A effects yet to be discovered.

Few studies indicated that peripherally administered BoNT/A may promote distant changes in expression of different neuropeptides and neurotransmission-related elements. Bossowska and Majewski (2012) reported that BoNT/A injection into the pig bladder reduces the number of bladder-innervating dorsal root ganglion neuron somata expressing substance P, CGRP, calbindin, somatostatin, and neuronal nitric oxide synthase. In contrast to reduced protein expression in sensory ganglia, up-regulation of CGRP and enkephalin m-RNA expression in motoneurons has been observed after intramuscular BoNT/A injection. These changes have been interpreted as indirect consenquences of peripheral chemical denervation (Humm et al., 2000; Jung et al., 1997; Palomar 2012; Zhang et al., 1993). However, the evidence for BoNT/A retrograde axonal transport in motoneurons (Antonucci et al., 2008) allow the possibility for direct BoNT/A action on gene expression in motoneuronal cell bodies.

1.2.4 Comparison of BoNT/A and TeNT mechanisms of action

Genes for different BoNT serotypes and TeNT, a clostridial toxin derived from C. tetani which induces a paralytic disease called tetanus, are derived from a common ancestral gene (Eisel et al., 1986). TeNT shares a similar di-chain neurotoxin structure with BoNT/A and other BoNT serotypes, mechanism of entrance into neurons, and the action on synaptic paralysis mediated by SNARE cleavage. TeNT and BoNT serotype B proteolytically cleave

the same peptide bond on VAMP/synaptobrevin SNARE protein (Schiavo et al., 1994). Similarly to BoNT/A, the entrance of TeNT into neurons may be mediated by SV2 protein (Yeh et al., 2010). However, clinical manifestation of tetanus is markedly different than botulism. In motoneurons, the main action of TeNT is its retrograde axonal transport to motoneuronal bodies and apparently selective transcytosis to certain classes of inhibitory neurons within the ventral horn, such as Ia inhibitory neurons and Renshaw cells (Stöckel et al., 1977; Hassel 2013). Impairment of ventral horn inhibitory transmission results in motoneuron hyperactivity and consequent rigidity or spasticity of skeletal muscles. The most pronounced symptom of tetanus is the so-called "opisthotonus", the backward bent, arch-like posture produced by hyperextension of the head, neck limb and spinal muscles, and trismus or the "lockjaw"- reduced jaw opening. Tetanus poisoning may lead to spasm of laryngeal and respiratory muscles, resulting in respiratory failure (Hassel, 2013). While spastic paralysis is induced at low TeNT doses, only at high peripheral doses it translocates into the cytosol of neuromuscular junction terminals, and induces mild flaccid paralysis similar to BoNTs (Matsuda et al., 1982)

Both BoNT/A and TeNT influence autonomic nervous system. Symptoms of BoNT/A intoxication are associated with reduced function of peripheral parasympathetic synapses or smooth musculature leading to paralysis of excretion glands (salivatory, tear production), absence of peristalsis, ortosthatic hypotension etc.). TeNT intoxication can sometimes produce a fatal autonomic instability due to hyperactivation of sympathetic nervous system, leading to tachyarrhythmias, hypotension, sweating, constipation, cardiac arrest etc. (Hassel, 2013; Rodrigo et al., 2014). This might occur due to TeNT retrograde axonal transport via sympathetic neurons, and toxin action in sympathetic nuclei (Stöckel et al, 1975).

If injected directly into certain CNS regions *in vivo*, TeNT and BoNT/A have a considerably different action on neurotransmission, which induces behavioral and physiological effects related to function of injected regions. For instance, TeNT induced epilepsy if injected into the hippocampus (Ferecskó et al., 2014), or hyperalgesia after injection into the dorsal horn (Kryzhanovskii et al., 1975). Unlike TeNT, BoNT/A induces blockage of excitatory neuronal activity when injected into different central regions, like antiepileptogenic effect in experimental models of epilepsy (Kato et al., 2014, suppression of pathological rotation behaviors after striatal injection in a hemiparkinsonism model (Wree et al., 2011). In i.c.v.-treated rats, BoNT/A may induce slow onset, long-term cognitive impairment of spatial

memory (Lacković et al., 2009). The experimental *in vivo* data point to the more pronounced action of TeNT on inhibitory neurotransmission, in comparison to relatively spared (incompletely reduced) glutamatergic excitatory transmission (Ferecskó et al., 2014). Interestingly, *in vivo* study suggested that TeNT inhibits only the synaptic neurotransmitter release, while BoNTs may inhibit both synaptic and extrasynaptic (ectopic) neurotransmitter release (Verderio et al., 1999, Thyssen, 2010). Given the ubiquitous presence of VAMP/synaptobrevin and SV2 in both excitatory and inhibitory neurons, the exact mechanism of selective TeNT action on inhibitory neurotransmission, in contrast to BoNT/A, is presently not known. In addition, the exact cell sorting, axonal transport and transcytotic events explaining why TeNT is more efficient in inhibition of central synapses following transcytosis, than the peripheral nerve terminals, are yet uncharacterized.

1.3 BoNT/A and pain

1.3.1. Review of BoNT/A clinical use in chronic pain conditions

As previously mentioned, small doses of purified BoNT/A are clinically used for treatment of neuromuscular disorders characterized by increased tonicity or overactivity of certain muscles (Barnes, 2003; Thengannat and Fahn, 2012). The beneficial effect on muscular hyperactivity was accompanied by prolonged pain relief, initially believed to be associated with reduced contraction of affected spastic or dystonic muscles (Mense, 2004). However, the effect of BoNT/A on pain did not always co-occur simultaneously with beneficial effect on muscles, and in some cases it was longer than the muscular effect (Aoki, 2003; Freund and Schwartz, 2003; Jankovic et al., 1990; Relja and Klepac, 2002). Relja and Klepac (2002) observed that the BoNT/A antinociceptive effect in dystonic torticollis occurred before the beneficial muscular effect, and at a lower BoNT/A dose administered. The duration of analgesic effect in temporomandibular disorders extended the duration of decreased voluntary bite force (Freund and Schwarz, 2003). These observations suggested that BoNT/A effect on pain do not necessarily involve its neuromuscular effects (Giladi, 1997; Mense, 2004). Antinociceptive effect of BoNT/A was, up to now, reported in several non-muscular pain conditions, such as different types of neuropathic pain, migraine, arthritis etc (Jabbari and Machado, 2011). Experimental studies suggested that the BoNT/A effect on pain are mediated by its direct action on sensory neurons (Section 1.3.2).

Up to now, chronic migraine (migraine lasting >15 days per month) is the only non-muscular pain condition with approved BoNT/A use. The approval was based on the results of two large multicentric placebo controlled Phase III Research Evaluating Migraine Prophylaxis Therapy (PREEMPT) studies (Dodick et al., 2010). In mentioned trials BoNT/A was injected into fixed sites over several cranial and neck muscles (total dose of 155-195 units of onabotulinumtoxinA commercial preparation). Therapeutic outcome was a slight but significantly reduced mean number of migraine attacks per month and headache severity. Some other studies did not confirm the efficacy of BoNT/A for chronic migraine treatment (reviewed by Gady and Ferneini, 2013). Subpopulations which describe their pain as a pressure from outside may exhibit a higher benefit from BoNT/A treatment (Jakubowski et al., 2005; Burstein et al., 2009). In addition, it was suggested that the occurrence of pericranial allodynia, unilaterality of migraine pain and pericranial muscle tenderness in chronic migraine may be predictive markers for responsiveness to BoNT/A (Mathew et al., 2008).

Reports of off-label BoNT/A beneficial analgesic effects have demonstrated its efficacy in a wide array of painful conditions, such as interstitial cystitis (Kuo, 2013, Russell et al., 2013), chronic arthritis (Chou et al., 2010), residual limb pain (Wu et al, 2012), different types of neuropathic pain (Ranoux et al, 2008; Zuniga et al., 2008) including diabetic neuropathy (Relja and Miletić, 2005; Yuan et al., 2009; Chen et al., 2013), masticatory pain etc. Most of clinical reports on BoNT/A effectiveness are based on a small number of patients or individual case studies. Some rare conditions such as Parry Romberg syndrome (Borodic et al., 2013), Morton neuroma (Climent et al., 2013), painful legs and moving toes syndrome (Rodriguez and Fernandez, 2013), post-thoracotomy pain (Fabregat et al., 2013), post-amputation limb pain (Wu et al, 2012), etc. have been recently successfully treated with BoNT/A. Although BoNT/A seems to be a promising candidate for treatment of chronic pain, the results of systemic reviews and meta-analyses (Cochrane data base systemic reviews and other) are also mostly inconclusive (Table 1), and the majority of them concludes that more double blind placebo-controlled studies are needed to confirm BoNT/A efficacy. Apart from low sample size and limited number of randomized clinical trials, the reason for contradictory or negative findings might be the lack of standardized guidelines for BoNT/A application and dosage, and the inappropriate definition of study primary outcomes (Jabbari and Machado, 2011). Despite the inconsistencies, unique long-term efficacy after single BoNT/A application

presents an obvious advantage over classical analgesics. In addition, the repeated use of conventional analgesics is often associated with compliance issues and occurrence of side effects such as the development of tolerance and medication overuse. The lack of serious side effects associated with BoNT/A use is particularly useful in the treatment of some chronic pain conditions. Potential important use of BoNT/A is its reported efficacy in cases of refractory chronic pain where other treatments have failed (Matak and Lacković, 2014; Appendix VI). These favorable BoNT/A properties drive the need for further research and improvement of BoNT/A clinical use.

Table 1 Summary of systemic reviews and meta-analyses of BoNT/A efficacy in the treatment of different pain disorders (Matak and Lacković, 2014)

Clinical condition	*N (trials analyzed)*	*N (patients)*	*Outcome*	*References*
Myofascial pain syndromes	4	233	Inconclusive evidence for effectiveness	(Soares et al., 2012)
Subacute/chronic neck pain.	9	503	Lack of benefit	(Langevin et al., 2011)
low-back pain and sciatica	3	123	Low-quality evidence that BoNT/A is beneficial	(Waseem et al., 2011)
Shoulder pain due to spastic hemiplegia or arthritis	6	164	BoNT/A reduces pain and improves shoulder function	(Singh and Fitzgerald (2011)
Postoperative pain after subpectoral breast implants	7	427	Low-quality evidence that BoNT/A is beneficial	(Winocour et al., 2014)
Trigeminal neuralgia	6	101	BoNT/A may be beneficial in treatment of TN	(Hu et al., (2013)
Tension-type headache	7	675	No reduction in the number of headaches in comparison to placebo	(Jackson et al., 2012)
Episodic migraine	9	1838	No reduction in the number of headaches in comparison to placebo	(Jackson et al., 2012)
Chronic migraine	5	1508	Significant reduction in the number of headaches in comparison to placebo	(Jackson et al., 2012)
Chronic daily headache	3	1115	Significant reduction in the number of headaches in comparison to placebo	(Jackson et al., 2012)

1.3.2 Insights from *in vitro* studies and *in vivo* pain models

In cell cultures of sensory neurons and *ex vivo* models of bladder preparation it was demonstrated that BoNT/A reduces the evoked release of SP and CGRP, neuropeptides which

modulate inflammation and pain. In rat *ex vivo* bladder preparation BoNT/A reduced the spontaneous release of CGRP in animals with cyclophosphamide-induced cystitis, and evoked release of CGRP from HCl-treated naïve bladders (Rapp et al., 2006; Lucioni et al., 2008). In cultured sensory ganglia BoNT/A reduced the K^+ and capsaicin-evoked SP and CGRP release (Purkiss et al., 2000; Welch et al., 2000; Durham et al., 2004; Meng et al., 2007). BoNT/A effects were dependent on the presence of extracellular Ca^{2+} (Purkiss et al., 2000). In acutely isolated slices of caudal brainstem BoNT/A altered the basal CGRP drive on secondary neurons. The increase of Ca^{2+} concentration prevented the BoNT/A-mediated inhibition of K^+-stimulated CGRP release from trigeminal ganglion neurons (Meng et al., 2009). In cultured sensory ganglia BoNT/A reduces the plasma membrane expression of transient receptor potential vanilloid 1 (TRPV1). It was suggested that BoNT/A prevented the SNARE-mediated TRPV1 translocation to the plasma membrane (Morenilla-Palao et al., 2004; Shimizu et al., 2012; Yiangou et al., 2011).

Up to now, BoNT/A antinociceptive efficacy has been demonstrated in numerous models of inflammatory pain models (induced by formalin, carrageenan and capsaicin), CFA-induced knee arthritis, cyclophosphamide-induced prostatic pain, acetic acid-induced bladder pain and capsaicin-evoked prostatic pain. In addition, BoNT/A efficacy has been shown in peripheral nerve injury-induced neuropathic pain models, polyneuropathic pain models, mirror pain, postsurgical pain models (data and references summarized in Table 2).

Interestingly, it was noticed that BoNT/A has peculiar analgesic properties in comparison to conventional analgesics (Matak and Lacković, 2014; Appendix VI). Firstly, unlike classical opioid analgesics or local anesthetics, BoNT/A does not alter normal mechanical or thermal thresholds in humans or animals (Cui et al., 2004; Bach-Rojecky, 2006; Blersch et al., 2002). Its effect on pain seems to be limited to reduction of facilitated pain states associated with allodynia and hyperalgesia, mediated by central sensitization (defined by Wolf (2011) as „ a prolonged, reversible increase in the excitability and synaptic efficacy of neurons in central nociceptive pathways"). Secondly, there is a lack of dose-response at peripheral BoNT/A doses which do not induce neuroparalytic effects. Bach-Rojecky described a lack of dose response in a model of carrageenan-evoked hind-paw inflammation. BoNT/A applied at 3.5U/kg, the lowest effective dose, induces a similar analgesic activity at higher 5 and 7 U/kg BoNT/A doses. Similar conclusions were observed in models of neuropathic pain (Bach-Rojecky et al., 2005; Bach-Rojecky et al., 2010). Bach-Rojecky (2006) in her PhD

dissertation reported the lack of dose response of BoNT/A after its intrathecal application, too.

Table 2 BoNT/A antinociceptive efficacy in rodent experimental pain models. The pain type has been described in terms of etiology (inflammatory, neuropathic, etc.) and site (superficial somatic, deep somatic or visceral). Legend: s.c.=subcutaneous; i.c.v.=intra-cerebroventricular; i.t.=intrathecal; i.a.=intraarticular; ↓= reduction

Pain model & type	***Type of BoNT/A application***	***Effect on pain***	***Additional observations***	***References***
Formalin-induced pain (acute inflammatory, superficial somatic)	s.c.(hind-paw or whisker pad) i.c.v., i.t.; pretreatment	↓phase II pain behavior	↓ glutamate rise and edema in the inflamed paw, ↓c-fos second order neuronal activation	Cui et al., 2004; Luvisetto et al., 2006; Lee et al., 2011; Vacca et al., 2012; Drinovac et al., 2013)
Carrageenan-induced pain (acute inflammatory, superficial somatic)	s.c.(hind-paw) pretreatment	↓mechanical and thermal hyperalgesia	No effect on paw edema or inflammatory cell infiltration	(Bach-Rojecky and Lacković, 2005; Favre-Guilmard et al., 2009; Shin et al., 2013
Capsaicin-evoked hind-paw or facial pain (acute inflammatory, superficial somatic)	s.c.(hind-paw or. whisker pad) pretreatment	↓mechanical and thermal hyperalgesia (paw); Reduced nocifensive behavior	No effect on neurogenic plasma protein extravasation	(Bach-Rojecky and Lacković, 2005, Shimizu et al., 2012
Prostate pain evoked by capsaicin	Intraprostatic (visceral)	↓pain behavior and hypolocomotion		Chuang et al., 2007
Freund's adjuvant–induced knee arthritis (deep somatic)	i.a.	↓joint tenderness, improved spontaneous wheel running		Krug et al., 2009.
Partial sciatic nerve transection injury (peripheral neuropathic, somatic)	s.c.(hind-paw) post-treatment	↓mechanical and thermal hyperalgesia, ↓ mechanical and cold allodynia		Bach-Rojecky et al., 2005; Drinovac et al., 2013)
Spinal nerve ligation (peripheral neuropathic, somatic)	s.c.(hind-paw) post-treatment	↓mechanical and cold allodynia		Park et al., 2006
Sciatic nerve constriction injury (peripheral neuropathic, somatic)	s.c.(hind-paw) post-treatment	↓mechanical allodynia	↓upregulation of pronociceptive opioid neuropeptides and SNAP-25 in the sensory ganglia, ↓markers of glial activation in the	Luvisetto et al., 2007; Marinelli et al, 2010; Mika et al., 2011 Vacca et al., 2013

			spinal cord; ↑ functional recovery of injured nerve	
Infraorbital nerve constriction injury (peripheral neuropathic, somatic)	s.c. (whisker pad) post-treatment	↓mechanical allodynia (bilaterally), thermal allodynia	↓evoked vesicular release of neurotransmitters within sensory ganglia;	Kitamura et al., 2009; Filipović et al., 2012; Kumada et al., 2012
Ventral root transection (neuropathic, somatic)	s.c.(hind-paw) post-treatment	↓Mechanical allodynia (bilaterally)	↓expression of P2X3 and TRPV1 receptors	Xiao et al., 2011; Xiao et al., 2013
Streptozotocin-induced diabetic pain (polyneuropathic)	s.c.(hind-paw): i.t. post-treatment	↓mechanical hyperalgesia (bilaterally), and thermal hyperalgesia, formalin-induced hypersensitivity		Bach-Rojecky et al.,, 2010)
Chemotherapeutic-induced pain (polyneuropathic)	s.c.(hind-paw) post-treatment	↓mechanical hyperalgesia (bilaterally)		Favre-Guilmard, (2009)
Hyperalgesia induced by repeated acidic saline gastrocnemius injections (mirror pain, somatic)	s.c.(hind-paw) pre- and post-treatment	↓mechanical hyperalgesia (bilaterally)		Bach-Rojecky and Lacković, 2009)
Hyperalgesia evoked by gastrocnemius incision (postsurgical pain, somatic)	s.c.(hind-paw) pre-treatment	↓mechanical hyperalgesia		Filipović et al., 2010

1.4.4. Peripheral theory of BoNT/A action

According to a still dominating theory, BoNT/A, similarly to its effect in peripheral cholinergic synapses, reduces the neurotransmitter release from sensory nerve endings. This, in turn, mediates the BoNT/A action on pain and peripheral inflammation (Aoki and Francis, 2011; Wheeler, 2013). This hypothesis was based primarily on the initial experimental study on BoNT/A antinociceptive activity from Cui et al. (2004). The authors reported the effect of BoNT/A on the second phase of formalin-induced flinching/licking behavior. In addition, they observed reduced peak increase of glutamate in the hind-paw tissue, and slight reduction

of hind-paw edema. At the time, it seemed logical to assume that BoNT/A local effects on peripheral neurotransmitter release and inflammation are causally associated with its antinociceptive effects. The observed reduced activation of second-order neurons (interpreted as a reduced central sensitization) was regarded as an indirect consequence of BoNT/A peripheral effects (Aoki, 2005; Cui et al., 2004). In some human experiments, BoNT/A mediated reduction of neurogenic flare was observed in normal human subjects (Gazerani et al., 2006, 2009)

1.4.5. Dissociation between anti-inflammatory and antinociceptive actions of BoNT/A

The antinociceptive efficacy of BoNT/A was confirmed in models of capsaicin and carrageenan-induced inflammatory pain. However, no anti-inflammatory effect on tissue inflammatory cell infiltration, edema, or neurogenic inflammation were observed, despite significant reduction or full reversal of mechanical and thermal hyperalgesia (Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2008; Favre-Guilmard et al., 2009). In the study of Cui et al. (2004) the discrepancy between the minimal anti-inflammatory and antinociceptive doses was observed (7 U/kg vs. 3.5 U/kg). The study of Krämer et al. (2003) reported that BoNT/A reduces neurogenic flare evoked by capsaicin in human subjects, however, with very limited analgesic effects. These studies demonstrated that the BoNT/A peripheral anti-inflammatory effect is not necessarily connected to its analgesic activity.

1.5. Central effects of BoNT/A

1.5.1 Insights from bilateral and polyneuropathic pain models

Dissociation between peripheral and anti-inflammatory effects of BoNT/A indicated that the mechanism of BoNT/A action on pain might be more complex that the simple prevention of peripheral neurotransmitter releaser. Strong evidence that BoNT/A antinociceptive efficacy involves a central activity has been suggested by bilateral pain models.

Bach-Rojecky and Lacković (2009) investigated the effect of BoNT/A in a mirror pain model evoked by two intramuscular acidic saline injections into the gastrocnemius. Mentioned pain model is characterized by reduction of mechanical thresholds on both hind paws after two unilateral acidic saline gastrocnemius injections, without any detectable peripheral nerve or

tissue injury. In this model, ipsilateral injection of a local anesthetic lidocain does not reduce the experimental pain on the contralateral side, suggesting that the occurrence of distant contralateral pain is centrally mediated (Sluka et al., 2001). Da Silva et al. (2010) suggested the involvement of descending facilitatory pathways in the occurrence of acidic saline-induced mirror pain.

Bach-Rojecky and Lacković (2009) reported that BoNT/A applied unilaterally to the acidic saline-injected leg reduces the pain on both ipsilateral and contralateral hind-limb, the effect which is difficult to explain only by BoNT/A local effects. Interestingly, BoNT/A applied contralaterally to acidic saline injections prevented the pain only on that side, which demonstrated that the bilateral effect is not mediated by systemic BoNT/A diffusion.

Similar bilateral effect was also observed in polyneuropathic models. BoNT/A reduced the mechanical hyperalgesia evoked by a chemotherapeutic drug paclitaxel (Favre-Guilmard et al., 2009). The authors ruled out a systemic effect by demonstrating that BoNT/A is not effective in carrageenan-induced inflammatory pain when injected contralaterally to the side of inflammation. Bach-Rojecky et al. (2010) demonstrated BoNT/A bilateral effect on mechanical hyperalgesia in a model of streptozotocin-evoked diabetic pain. Since the mechanism of pain induction in polyneuropathic pain models is related to bilateral peripheral and central changes leading to widespread sensitivity, bilateral effect of BoNT/A seems to be necessarily centrally mediated. Interestingly, BoNT/A contralateral effect on mechanical hyperalgesia had a similar intensity in both mirror and polyneuropathic pain models.

More recently, BoNT/A bilateral effect was observed in ventral root transection-induced neuropathic pain, and infraorbital nerve constriction-induced trigeminal pain (Filipović et al., 2012; Xiao et al., 2011, 2013,). It seems that BoNT/A bilateral effect in mirror or polyneuropathic pain models is a general phenomenon not dependent on the mechanism of pain induction.

1.5.2. Evidence that the axonal transport is necessary for BoNT/A action in pain

The above-mentioned experiments suggested that BoNT/A action in bilateral pain models involves a distant toxin activity, not mediated by systemic toxin diffusion. The only logical explanation is that BoNT/A action might be mediated by toxin's movement to CNS. Early experiments suggested that radioactively labeled BoNT/A applied into the cat's

gastrocnemius muscles may spread via sciatic nerve and ventral roots into the corresponding spinal cord segment (Habermann 1974; Wiegand et al., 1976). However, up to recently, it was believed that the axonal transport to CNS of BoNT/A is very limited or non-existent, and does not appear to involve the active toxin molecules (Tang Liu et al., 2003).

In a model of mirror pain evoked by intramuscular acidic saline, the involvement of axonal transport in the toxin's antinociceptive activity was conclusively demonstrated for the first time (Bach-Rojecky, 2006; Bach-Rojecky and Lacković 2009). The autors reported that injecting the low toxin dose (7 times lower than the smallest peripherally effective dose) into the stump of distally transected nerve reduces the mechanical pain on contralateral side. In addition, the bilateral effect of peripherally injected BoNT/A was prevented by injecting the axonal transport blocker colchicine into the sciatic nerve ipsilateral to acidic saline and BoNT/A treatment. Colchicine injected into the contralateral side sciatic nerve did not prevent BoNT/A bilateral effect. These experiments demonstrated the necessity of axonal transport for BoNT/A analgesic efficacy. The antinociceptive effect of BoNT/A on mechanical hyperalgesia was also prevented by colchicine in a model of carrageenan-induced inflammatory pain, suggesting that the BoNT/A action on pain in general is axonal transport-dependent (Bach-Rojecky, 2006).

In models of mirror pain and diabetic polyneuropathic pain, the authors also demonstrated that BoNT/A injected intrathecally induces a faster analgesic effect (within 24 h), in comparison to the delayed BoNT/A action after peripheral application (3-7 days after peripheral application). The authors explained the observed effect by progressive toxin axonal traffic into the CNS which takes several days (dependently on the distance of injected site from CNS).

2. HYPOTHESIS AND AIMS OF THE RESEARCH

2.1 Hypothesis

BoNT/A antinociceptive activity is dependent on retrograde axonal transport within primary sensory neurons. BoNT/A cleaves SNAP-25 at central afferent nerve endings, but not affecting fast transmission which transmits normal pain sensation, but rather slow pain transmission which mediates hyperalgesia and allodynia.

2.2. Aims of research:

-To investigate the presence of BoNT/A molecule and its activity in central sensory regions after its axonal transport from periphery

- To examine the necessity of axonal transport in sensory neurons for BoNT/A antinociceptive activity

- To examine the possibility of BoNT/A transcytosis to second order neurons in central sensory regions.

- To characterize the cellular and regional sites of BoNT/A central antinociceptive activity

- To examine the possible role of capsaicin-sensitive nociceptive neurons which mediate central sensitization and pain hypersensitivity without transmitting acute nociceptive pain, in BoNT/A antinociceptive action.

-To examine the effect of BoNT/A on neuropeptide transmitters which participate in pain transmission, central sensitization and hyperalgesia.

3. MATERIALS AND METHODS

3.1 Animals and BoNT/A treatment

3.1.1 Animals

Male Wistar rats (University of Zagreb School of Medicine, Croatia), weighing 300-400 g, kept on 12 h/12h light and dark cycle, were used in all experiments. The experiments were conducted according to the European Communities Council Directive (86/609/EEC) and recommendations of the International Association for the Study of Pain (Zimmerman, 1983). All efforts were made to reduce the number of animals used and to reduce their suffering. Animal procedures were approved by the Ethical Committee of University of Zagreb School of Medicine (permit No. 07-76/2005-43), and performed according to the Croatian law on animal protection (Zakon o zaštiti životinja NN 135/06).

3.1.2 BoNT/A injections

For peripheral administration, conscious, restrained rats were injected unilaterally with 20-30 µl of saline-diluted BoNT/A therapeutic preparation (Botox®, INN: Clostridium botulinum type A neurotoxin complex, Allergan Inc., Irvine, CA, USA) using a $27^{1}/_{2}$-gauge needle via different routes: 1. into the whisker pad, 2. Subcutaneously (s.c.) into the plantar hind paw side 3. Intramuscularly (i.m.) into the gastrocnemius. 1 unit (1 U) of mentioned BoNT/A preparation contains 48 pg of 900 kDa BoNT/A complex. 3.5 U/kg, 5 U/kg, and 15U/kg doses were chosen based on previous experiments by Cui et al., (2004) and from our laboratory (Bach-Rojecky and Lacković 2005, Bach-Rojecky et al., 2005).

For intraganglionic (i.g.) and intraneural (i.n.) injections, animals were anesthetized with chloral hydrate (300 mg/kg i.p.). Trigeminal ganglion was injected similarly as described by Neubert et al. (2005) (Figure 3). 0-10 µl Hamilton syringe needle (Hamilton Microliter #701, Hamilton, Switzerland) was inserted through the skin overlying the medial part of zygomatic process, and inserted into the infraorbital foramen. The needle was then advanced (Figure 3) through the infraorbital canal and foramen rotundum directly into the trigeminal ganglion.

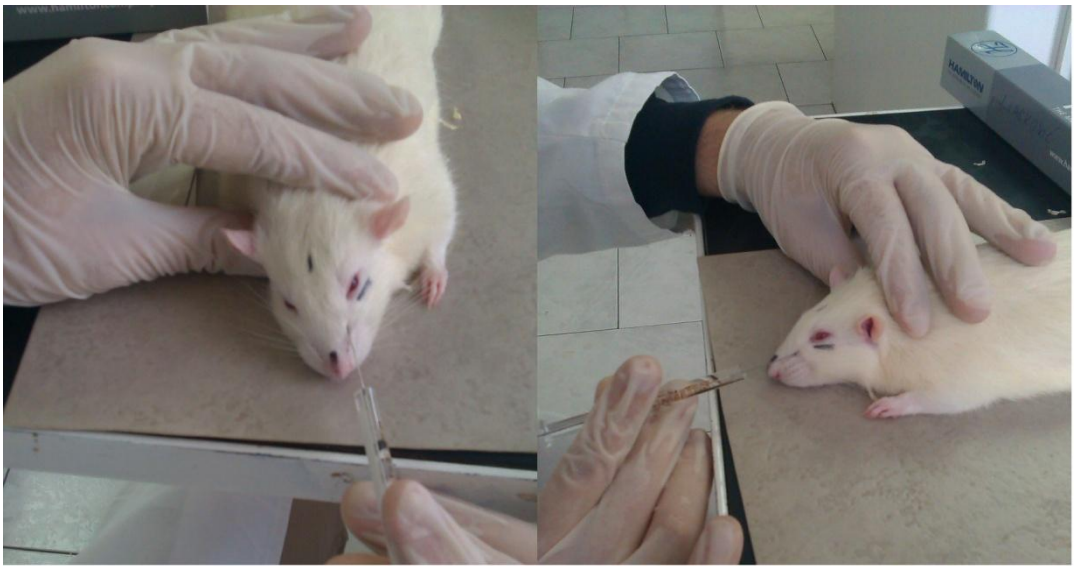

Figure 3 Percutaneous injection into the rat trigeminal ganglion. *Hamilton syringe needle is inserted into the infraorbital foramen at an angle inclined 10º relative to the mediosagittal plane and passing in a direction 2-3 mm below the eye when viewed from profile. After advancement through the infraorbital canal and foramen rotundum, the needle tip stops at the medial wall of Meckel's cave at the skull base, where the trigeminal ganglion is situated.*

Saline-diluted BoNT/A (1 U/kg, 2 µl) was slowly injected into the ganglion. Dose of 1 U/kg was chosen based on preliminary experiments and on the dose needed for antinociceptive effect after intrathecal application (Bach-Rojecky et al., 2010). Site of injection was verified by injecting 2 µl of methylene blue to 5 animals. The dye resided only in trigeminal ganglion. For i.n. injection sciatic nerve was exposed after skin incision at mid-femoral level and blunt dissection through the thigh muscles. Special care was made to check for possible leakage by placing piece of parafilm under the nerve prior to i.n. injection. 0-10 µl Hamilton needle (Hamilton, Bonaduz, Switzerland) was used to inject 2 µl of BoNT/A into the nerve (10 U/kg dose). 3 minutes following the treatment, parafilm was removed, the nerve returned to previous position and the skin sutured.

3.2. Behavioral studies

3.2.1 The role of axonal transport in sensory neurons

The role of axonal transport within sensory neurons for BoNT/A antinociceptive activity was studied in rat trigeminal region. To selectively prevent the axonal transport in trigeminal sensory neurons, microtubule polymerization inhibitor colchicine (Sigma, St Louis, MO, USA; 5 mM) was injected percutaneously via infraorbital canal into the trigeminal ganglion

as described. Colchicine was injected 24 h prior to BoNT/A 3.5 U/kg into the whisker pad, or 1 U/kg i.g. injection into the trigeminal ganglion.

The effect of BoNTA was assessed in a model of formalin-induced orofacial pain. Prior to behavioral measurement, the rats were allowed to accommodate to testing cage environment. The rats were then briefly restrained and injected with 50 µl of saline-diluted 2.5% formalin (0.92 % formaldehyde) into the whisker pad ipsilateral to BoNT/A pretreatment and returned to cages for observation period of 45 min. The number of seconds of formalin-induced ipsilateral facial rubbing/grooming was measured in 3 min periods during phases I and II of formalin-induced pain (Rabboison and Dallel, 2004). Phase I (0-12 min) behavior represents the immediate pain response characterized by direct chemical stimulation of peripheral nerve endings with formalin. Phase II (12-45 min) behavior is characterized by delayed hyperalgesic response maintained by ongoing afferent input and central sensitization. Formalin test was performed 1 and 2 days after BoNT/A i.g. injection, and 3 days after the BoNT/A whisker pad injection.

3.2.2 Involvement of capsaicin-sensitive neurons in BoNT/A antinociceptive efficacy

To examine the possible role of TRPV1-expressing sensory neurons in BoNT/A action on pain, the antinociceptive activity of BoNT/A was examined in animals desensitized with i.g. capsaicin. Anesthetized animals (chloral hydrate, 300 mg/kg) were administered percutaneously into the trigeminal ganglion (~1 µl/min) with 10 µl 2% capsaicin (Sigma, St. Louis, MO, USA) or vehicle (0.9% saline + 10% ethanol + 10% Tween-80). Following day the procedure was repeated. Four days after the completion of capsaicin i.g.-induced desensitization, animals were injected into the whisker pad with saline/5 U/kg BoNT/A. Desensitization of capsaicin-sensitive neurons was confirmed by capsaicin eye-wipe test. Orofacial formalin test was then performed 5-6 days after peripheral saline/BoNT/A treatment. Formalin test was employed as described in section 3.2.1.

We examined the possibility that BoNT/A does not alter acute tactile or nociceptive sensitivity by its selective action on TRPV1-expressing sensory neurons. Therefore, we compared the effects of BoNT/A and the effect of selective denervation of TRPV1-expressing neurons on acute sensory responses. The acute innocuous and noxious sensitivity was measured in animals pretreated with BoNT/A, and/or submitted to different denervation

procedures: animals with selectively denervated TRPV1 neurons only, or the animals with non-selectively ablated all populations of trigeminal neurons. 5 days following the peripheral BoNT/A (15 U/kg) or saline injection into the whisker pad, rats were injected intraganglionically (i.g.) with either vehicle, 2% capsaicin (double vehicle or capsaicin treatment separated 24 h), or formalin (10 µl of 100% formalin, single i.g. treatment). Mechanical sensitivity of the facial area was examined 3-4 days after ganglion treatments. Prior to behavioral measurements, the rats were allowed to accommodate to testing cage environment until normal sniffing/no locomotion posture was assumed. The observer was blinded to the animal treatment.

Whisker pad mechanical sensitivity was first monitored with Von Frey filaments (2 and 8 g bending forces), and then followed by pin-prick test (5-10 min. interval between each stimulus). Pin prick test was performed with a sterile 27 1/2 gauge needle pressed gently against the whisker pad without penetrating the dermis.

Response to innocuous and nociceptive mechanical stimuli in the facial area was quantified by a behavioral scoring paradigm, originally devised by Vos et al. (1994). Aversive behavior was semi-quantified by descriptive categories consisting of one or more response elements (in brackets):

0 = no response (no detection);
1 = non-aversive response (detection);
2 = mild-aversive response (detection + withdrawal)
3 = strong aversive response (detection + withdrawal + escape/attack);
4 = prolonged aversive behavior (detection + withdrawal + escape/attack + facial grooming);

Capsaicin eye-wipe test was employed to validate the denervation of TRPV1-expressing neurons. The test consists of counting the number of ipsilateral eye wipe movements after application of a small drop (~10 µl) of 0.01% saline-diluted capsaicin to the rat corneal surface. The eye wiping response and eye closing in naïve animals lasts for 0.5-1 min, after which the animal resumes its normal behavior. The neurons are considered desensitized if the wiping movements are largely reduced or prevented. Prior to eye-wipe test, corneal reflex was examined bilaterally by assessing a blinking response after briefly applying a tipped sterile cotton wisp to the cornea. Cotton tip was applied 5 times (>30 second interval between consecutive applications), and the percentage of elicited blinking responses was used as a

measure of behavioral response. Normal blinking response suggested normal sensitivity to acute tactile stimuli of the corneal surface.

3.2.3 Behavioral comparison of BoNT/A efficacy after single and repeated injection

Repeated use of BoNT/A is the basis of its clinical use in pain treatment, however, in a recent study (Piovesan et al., 2011) it was reported that BoNT/A loses its efficacy in animals after repeated injection. We examined the BoNT/A antinociceptive action in animals pretreated once or twice with BoNT/A. In one animal group, BoNT/A 5U/kg was injected twice into the whisker pad with a separation period of 40 days. Single control group was injected with saline. After 6 days formalin orofacial test was performed as described previously. Period of 42 days between the two BTX-A injections was chosen based on study of Piovesan et al. (2011).

3.3 Immunohistochemical localization of BoNT/A antinociceptive action in CNS

3.3.1 Antibody characterization for cleaved SNAP-25 detection in central neurons

The antibody used for immunohistochemical detection of BoNT/A-cleaved SNAP-25 (a kind gift from Assist. Prof. Ornella Rossetto, University of Padua, Italy) was used previously in study from Antonucci et al. (2008). The polyclonal antibody raised specifically against the C terminus of truncated SNAP-25 was used to demonstrate the presence of BoNT/A.protease in sensory regions.

To characterize the specific detection of 24 kDa truncated SNAP-25 product in central neurons, the direct injection of BoNT/A into the rat hippocampus was employed. Rats were anesthetized, and the midline scalp incision was made to expose the frontal, parietal and occipital bone, so that bregma and lambda landmarks are visible. Small hole was drilled through the parietal bone overlying the cortex and dorsal hippocampus at position -4 mm relative to bregma, and -3 mm relative to midline, so that the cortical surface was exposed. 2 µl of saline-diluted BoNT/A was slowly injected into the rat dorsal hippocampus by using a 0-10 ml Hamilton syringe. The rats were then sutured and allowed to recover from anesthesia. One day following the BoNT/A injection rats were sacrificed and hippocampus was excised. Protein isolation, sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots were performed as described previously (Antonucci et al., 2008, Constantin

et al., 2005).

The nitrocellulose membranes were blocked and incubated firstly with rabbit anti-cleaved SNAP-25 (1:500) in blocking solution overnight at 4°C, and then the next day with goat-anti-rabbit HRP -conjugated secondary antibody (Biosource, Invitrogen, USA). After visualization of 24 kDa fluorescent signal in chemoluminescent (Super Signal West Femto, Pierce, USA) by electrochemiluminescence camera (Biorad), membrane was washed and incubated with the mouse monoclonal antibody to total SNAP-25 (1:5000, overnight at 4°C). SMI-81 Sternberger Monoclonals, USA). This antibody recognizes both intact and BoNT/A-cleaved SNAP-25 (Jurasinski et al., 2001). Then, the membrane was incubated with goat anti-mouse secondary antibody (BD Pharmingen, USA) and visualized. In a separate experiment, the membrane was incubated only with monoclonal antibody to total SNAP-25.

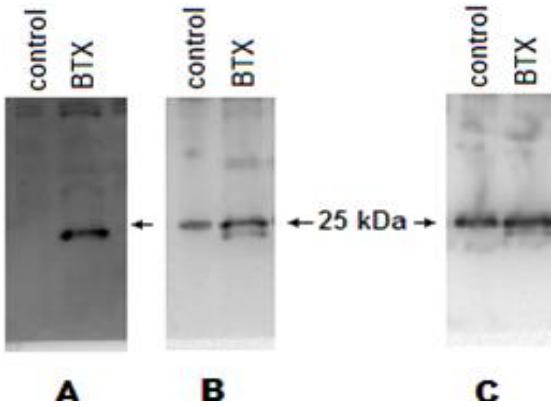

Figure 4 Characterization of the antibody specificity for BoNT/A-cleaved SNAP-25 in central neurons by Western blot (Matak et al., 2011). *Staining of saline vs BoNT/A-injected hippocampus A.) with polyclonal antibody to cleaved SNAP-25 B.) subsequent staining with SMI-81 monoclonal antibody to total SNAP-25 C.) separate experiment with SMI-81 staining only.*

Cleaved SNAP-25 (24 kDa band) was visible only in BoNT/A-injected hippocampus (A-C) and positioned under non-cleaved SNAP-25 (25 kDa band) (B,C). The intact and cleaved SNAP-25 band was also visible in membranes incubated only with total-SNAP-25 monoclonal antibody (C) (Figure 4). This experiment suggests that the polyclonal antibody binds only the cleaved SNAP-25 in toxin-injected brain tissue.

3.3.2 BoNT/A enzymatic activity in sensory nociceptive nuclei after its peripheral application

Botulinum toxin's enzymatic activity in CNS was assessed using immunofluorescent detection of its cleaved substrate synaptosomal-associated protein 25 (SNAP-25) following injection

into the rat whisker pad, hind-paw, and intramuscular injection into the gastrocnemius.

Rats were injected with BoNT/A into the whisker pad and sacrificed by perfusion at different time points (1, 3 or 5 days after BoNT/A), or at different doses (3.5, 5, 15, 30 U/kg). For hind-limb injections BoNT/A was injected at 5 and 30 U/kg. Animals were anesthetized and transcardially perfused with physiological saline followed by 4% formaldehyde in phosphate-buffered saline (PBS). Brain or lumbar spinal cord tissue was excised and cryoprotected with sucrose. Then, the tissue was placed at -80ºC until further use. Tissue was cut in a freezing cryostat in coronal slices (40 µm) and processed for free-floating immunohistochemistry. All washing steps and incubating was performed with PBS. Brainstem or spinal cord sections were blocked in 10% goat serum and incubated overnight at room temperature with the polyclonal antibody to cleaved SNAP-25 dissolved in 1% goat serum (1:400 – 1:1500 concentrations). Next day the sections were incubated with 1:400 fluorescently labeled goat anti rabbit secondary antibody (Alexa 555 dye), washed and mounted on glass slides with Fluorogel mounting medium. Optionally, the tissue was processed further for neuronal counterstaining (mouse anti-NeuN antibody, 1:500 dilution, incubation overnight at 4 ºC, goat anti-mouse Alexa 488secondarries), or nuclear dye diamidinophenylindole (DAPI). The slides were visualized by Olympus BX-51 microscope equipped with appropriate filters and DP-70 camera.

3.3.3 Characterization of toxin's axonal transport in sciatic nerve

BoNT/A transport in axonal compartments of peripheral nerve was examined by employing a intraneural injection of BoNT/A (10 U/kg) into the sciatic nerve, as described in Section 3.1.2. To prevent the axonal transport of BoNT/A, a group of animals was injected with 2 µl of 5 mM colchicine into the more proximal part of sciatic nerve (2 cm apart). The animals were perfused for immunohistochemistry 3 days after intraneural BoNT/A. Occurrence of cleaved SNAP-25 was examined in dorsal and ventral lumbar horn.

3.3.4 Cellular localization of truncated SNAP-25 in sensory and motor regions after toxin's peripheral application

We employed confocal study to examine the cellular sites of BoNT/A enzymatic activity. After BoNT/A injection into the trigeminal region, its occurrence was examined in relation to

markers of synapses (synaptophysin), dendrites (microtubule-associated protein 2 (MAP-2), astrocytes (glial-fibrillary acidic protein (GFAP)), and neuronal bodies (NeuN). Similarly, in ventral horn region BoNT/A-cleaved SNAP-25 was co-stained with antibodies to synaptophysin, MAP-2, GFAP, and additionally to total SNAP-25 (present in synapses and axons). Antibody to choline-acetyltransferase (ChAT) was employed to examine the position of BoNT/A relative to cholinergic neurons in ventral horn.

BoNT/A-cleaved SNAP-25 staining was performed as previously described (Section 3.3.2). Co-staining procedures with monoclonal antibodies were performed with goat serum and goat secondary antibodies, while the incubation with goat anti- ChAT antibody was performed in donkey serum and secondary antibodies raised in donkey. All stainings were performed as a two-step procedure – cleaved SNAP-25 staining was completed first (overnight at room temperature), and then the second antibody (overnight at 4ºC).Images were taken with Leica SP2 AOBS confocal microscope.

Table 3 Cellular structures and antibodies employed for co-staining with BoNT/A-cleaved SNAP-25.

Cellular/neuronal structure	Marker	Antibody clonality and dilution	commercial supplier
Presynaptic complexes	Synaptophysin	monoclonal, 1:500	Sigma
Neuronal dendrites	MAP-2	monoclonal, 1:1000	Sigma
Neuronal bodies	NeuN	monoclonal, 1:500	Millipore
Astrocytes	GFAP	monoclonal, 1:1000	Sigma
Axons and synapses	SNAP-25	monoclonal, 1:2000	Covance
Cholinergic neurons	ChAT	polyclonal, 1:100-1:200	Millipore

3.3.5 Study of toxin's transcytosis in CNS

To examine the possible transcytosis of BoNT/A to second-order neurons in nociceptive nuclei, we examined the occurrence of cleaved SNAP-25 staining in trigeminal nucleus caudalis (TNC) after trigeminal nerve ablation procedure. Rats were injected into the whisker pad with 15 U/kg BoNT/A. After 5 days, the animals were anesthetized and injected with 10 μl saline or formalin (37% formaldehyde) into the trigeminal ganglion, as described in section 3.2.2. After additional 5 days the animals were perfused for immunohistochemistry. To confirm the ablation of primary afferents, nociceptive testing was performed as previously described (Section 3.2.2). In addition, staining of TNC with calcitonin gene-related peptide (CGRP) was performed, since at the examined brainstem level CGRP is present exclusively in

primary afferents (Jeffry et al., 2009)

In addition, BoNT/A transcytosis and traffic to distant sensory regions was assessed by immunohistochemistry. The animals were injected with 5 and 15 U/kg BoNT/A, and perfused for immunohistochemistry after 5-15 days. Possible occurrence of BoNT/A cleaved SNAP-25 was examined in thalamus, hypothalamus, sensory cortex, locus coeruleus and periaqueductal gray.

3.3.6 BoNT/A activity in capsaicin-sensitive central afferent terminals

To further assess the role of capsaicin-sensitive neurons in BoNT/A antinociceptive efficacy, the occurrence of BoNT/A in capsaicin-sensitive neurons was examined at the level of TNC. BoNT/A-cleaved SNAP-25 occurrence in TRPV1-expressing neurons was examined by colocalization using the antibody to cleaved SNAP-25 and goat anti-TRPV1 antibody (Santa Cruz). The staining was performed in donkey serum and donkey secondary fluorescent antibodies (anti-goat Alexa 555 and anti-rabbit Alexa 488) were used.

The possibility that BoNT/A protease activity is located within capsaicin-sensitive primary afferents was studied by employing capsaicin-induced trigeminal desensitization. 5 days following the peripheral BoNT/A (15 U/kg) or saline injection into the whisker pad, rats were injected intraganglionically (i.g.) with either vehicle or 2% capsaicin (double vehicle or capsaicin treatment separated 48 h). Animals were perfused for immunohistochemistry 3 days after the completion of denervation procedure. The desensitization was confirmed by using the capsaicin eye-wipe test.

3.3.7 BoNT/A action on pain-evoked neural activity in different sensory regions

Orofacial formalin test was employed to examine the effect of BoNT/A on pain-evoked neural activity. Animals were ijnected with saline/5 U/kg BoNT/A into the whisker pad, and orofacial formalin test was performed as described before 5-6 days after BoNT/A injection. Animals were perfused for immunohistochemistry 2 h after formalin injection. Immunohistochemistry was performed as previously described in different levels of brainstem, mesencephalon and diencephalon. Expression of c-Fos immediate early gene was employed as a marker of neuronal activity. Immunostaining of c-Fos was performed by using

a rabbit polyclonal antibody to c-Fos (Santa Cruz, 1:500, overnight at room temperature). Brain regions were identified in coronal sections using the rat stereotaxic atlas and appropriate landmarks for each region (central canal, obex, aqueduct, ventricles, etc.). C-Fos-positive neuronal fluorescent profiles were automatically counted using cellSens Dimension software (Olympus, Tokyo, Japan).

3.4 BoNT/A antinociceptive activity and neuropeptides

We examined if the BoNT/A may modulate the central CGRP release in basal and painful conditions by measuring CGRP concentration in cerebrospinal fluid (CSF). CGRP concentrations were studied by employing the enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay.

BoNT/A effect on central CGRP release was studied in models of inflammatory pain (carrageenan-induced paw inflammation, orofacial formalin-induced pain and complete Freund's adjuvant (CFA)-induced temporomandibular joint inflammation (TMJ), and neuropathic pain (infraorbital nerve constriction injury-induced trigeminal neuropathy).

Approximately 100 µl of CSF was withdrawn from cisterna magna after induction and full development of experimentally-induced pain. CSF was immediately frozen in liquid nitrogen and kept on -80 until further use. In a model of CFA-induced TMJ inflammation, apart from CSF, tissues of trigeminal ganglia, caudal brainstem and cerebral dura were harvested for measurement of CGRP by radioimmunoassay.

ELISA was performed with an ELISA kit (SPI Bio) according to manufacturer's instructions. Preparation of tissue samples and radioimmunoassay was performed as previously described (Pozsgai et al., 2012).

3.5 BoNT/A action on markers of synaptic plasticity and neural growth

Dependently on the site of BoNT/A application, the toxin may promote the neurite sprouting (neuromuscular junction), or inhibit the axonal or dendritic outgrowth (neuronal cell culture). We hypothesized that similar changes may potentially affect the expression of growth and synaptogenesis-associated molecules in central sensory regions.

We analyzed the expression of growth-associated protein-43 (GAP-43) which is highly

expressed in the axonal growth cones during neural growth and synaptophysin as a marker of presynaptic terminals by Western blot in excised tissue of trigeminal nucleus caudalis in control and BoNT/A (15 U/kg) – injected animals.

3.6 Statistical analysis

Parametric data were represented as mean ± standard error mean (SEM), and analyzed by unpaired t-test (for comparison between two groups) or one-way ANOVA followed by Newman-Keuls post hoc test (multiple group comparisons). Non-parametric data (response scores of aversive behavior to mechanical stimuli) were represented by scatter plot and median, and analyzed by Kruskall-Wallis test, followed by Dunn's post hoc. $p<0.05$ was considered significant.

4. RESULTS

4.1 Attempts to detect the BoNT/A molecule in nerve tissue and CNS after peripheral injection

In the PhD study of Bach-Rojecky (2006), the direct immunodetection of 150 BoNT/A neurotoxin or its light chain (LC) fragments was attempted in the tissues of sciatic nerve, lumbar dorsal root sensory ganglia, and spinal cord, however, with irreproducible results and non-specific staining of the tissue belonging to both control and BoNT/A-treated animals. In this PhD study, we initially tried to continue these experiments by employing the antibodies to BoNT/A LC fragment. In one of the experiments, the animals were tightly ligated with a suture around the sciatic nerve at the mid-thigh level, and injected with high dose BoNT/A into the periphery. We expected that axonally transported BoNT/A might build-up in the nerve segment distant to the ligature, however, there was no specific staining corresponding to BoNT/A LC in distal sciatic nerve segments (Fig. 5, unpublished data). In this experiment and several other experiments (not shown), our attempts to detect the BoNT/A LC in neural tissue by Western blot or immunohistochemistry were unsuccessful, most likely due to the very small amounts of BoNT/A which were retrogradely transported towards the CNS.

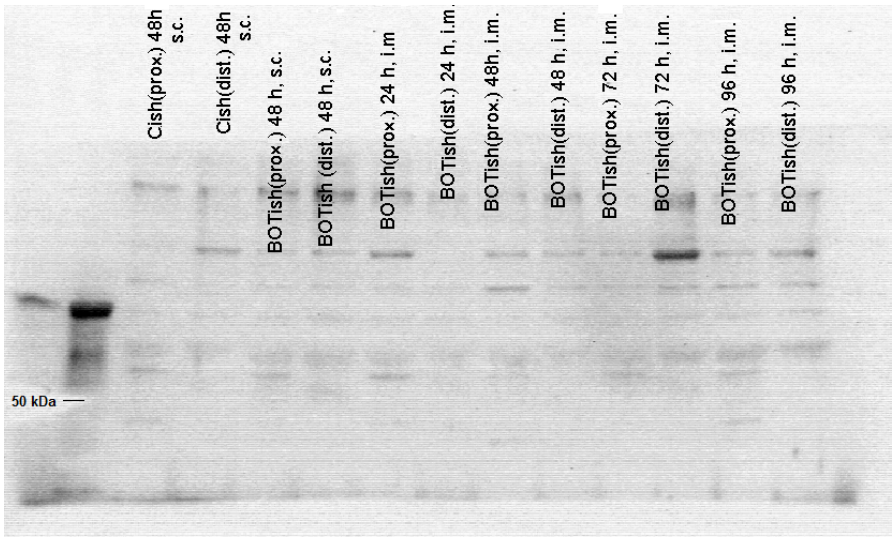

Figure 5 *Study of BoNT/A axonal transport in sciatic nerve.* ***BoNT/A (30 U/kg) was injected into the hind paw or into the gastrocnemius (i.m.). Sciatic nerve was tightly ligated with a single suture at the mid-thigh level. Detection of BoNT/A light chain was attempted in the sciatic nerve fragment near the ligation site at different time points (24-96h). Homogenized sciatic tissue was analyzed by Western blot using the primary antibody to LC (Anti BoNT/A LC polyclonal (Acris, 1:500 dilution). C, control (saline-injected); BOT; BoNT/A-injected; ish(prox.), proximal sciatic nerve fragment; ish(dist.), distal sciatic nerve fragment. 50 kDa – notch on the membrane corresponding to 50 kDa, which was the expected position of BoNT/A light chain (LC).***

4.2 Detection of cleaved SNAP-25 in central nociceptive nuclei

We examined the occurrence of BoNT/A-cleaved SNAP-25 in different sensory regions after different peripheral applicationsies. Following the toxin application into the whisker pad, BoNT/A was visible in TNC (Fig. 6) at different peripheral doses applied (3.5, 5, 15 and 30 U/kg), starting from day 3 after toxin injection (Matak et al., 2011; Appendix I). The immunoreactivity of cleaved SNAP-25 was localized predominantly in laminae I and II of TNC, in the area of termination of maxillary branch of trigeminal nerve located in the middle part of TNC. This is in line with somatotopical organization of TNC and the site of toxin injection. BoNT/A-cleaved SNAP-25 was seldom visible contralaterally at higher dose applied (15 U/kg), possibly due to some toxin diffusion across the whisker snout into the area of contralateral trigeminal nerve. Although not quantified, the amount of cleaved SNAP-25 was higher at higher doses applied (Matak et al., 2011; Appendix I). Few fibers of BoNT/A cleaved SNAP-25 were visible also at C1-C2 levels, and in trigeminal nucleus oralis (not shown).

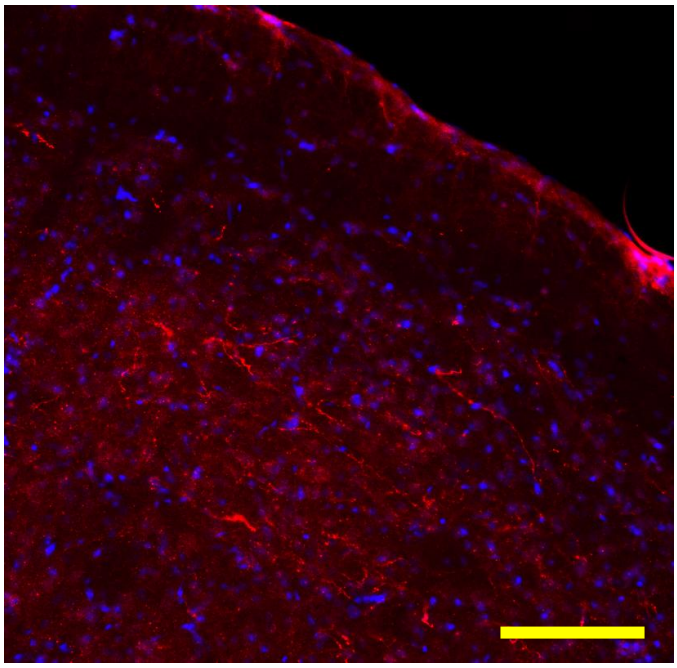

Figure 6 Occurrence of BoNT/A-cleaved SNAP-25 in TNC 5 days after BoNT/A (15 U/kg) injection into the rat whisker pad. *Red immunofluorescence = cleaved SNAP-25; blue fluorescence=DAPI nuclear counterstain. Scale bar =100 μm.*

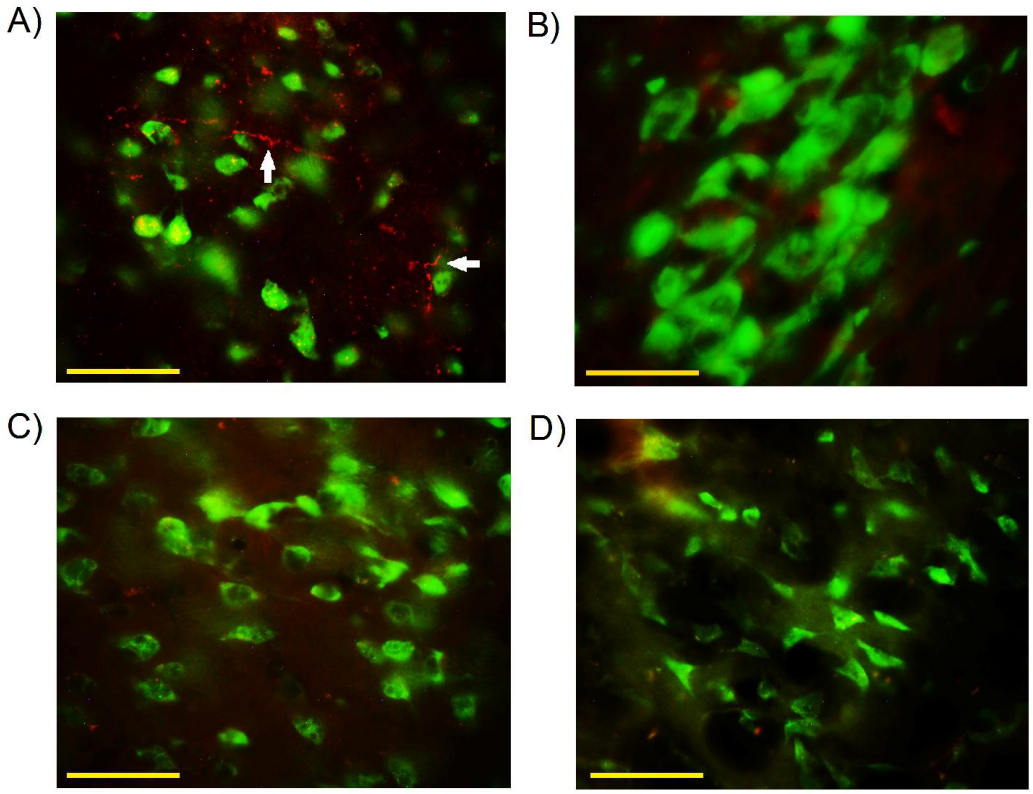

Figure 7 BoNT/A-cleaved SNAP-25 occurence in TNC and lack of BoNT/A activity in sensory regions upstream from TNC. *Cleaved SNAP-25 was examined 6 days after peripheral BoNT/A injection (5 U/kg). Immunoreactivity of cleaved SNAP-25 (red fibers marked by arrows) was visible in TNC (A). Cleaved SNAP-25 was not visible in ipsilateral locus coeruleus (B), periaqueductal gray (C), or contralateral ventral posteromedial nucleus of thalamus (D). Green represents NeuN neuronal counterstaining. Scale bar = 50 μm.*

BoNT/A- cleaved SNAP-25 was not visible in trigeminal nucleus interpolaris (not shown). At higher levels examined (locus coeruleus, thalamus, hypothalamus, sensory cortex), BoNT/A cleaved SNAP-25 was not observed (Fig. 7). These data suggest the occurrence of BoNT/A proteolytic activity in the area of termination of central afferent terminals of trigeminal sensory nociceptive neurons, but not in supramedullary (supraspinal) sensory nuclei.

After toxin intramuscular injection into the gastrocnemius, or subcutaneous injection into the hind-paw, BoNT/A-cleaved SNAP-25 occurred also in corresponding segments of the lumbar dorsal and ventral horn, demonstrating the axonal transport in dorsal horn sensory neurons and ventral horn motoneurons. BoNT/A-cleaved SNAP-25 was never observed in the contralateral dorsal or ventral horn (Matak et al., 2012; Appendix II).

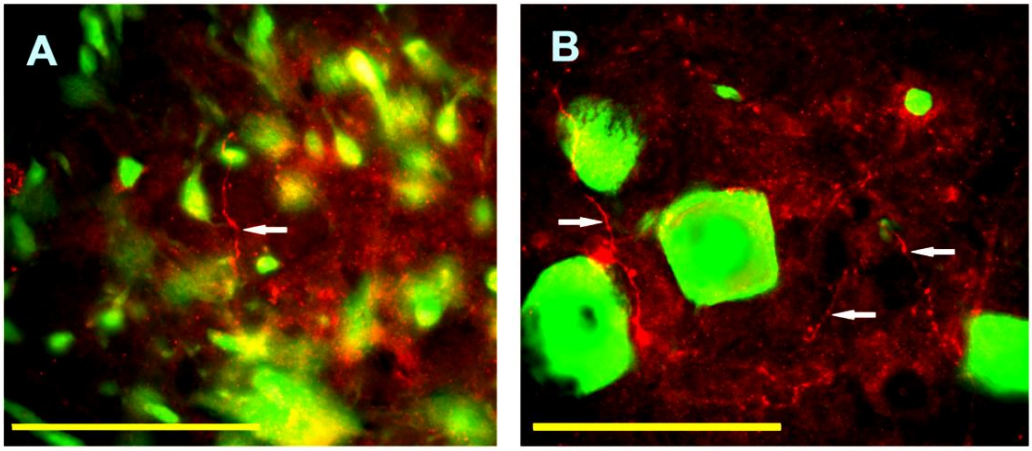

Figure 8. Occurrence of BoNT/A-cleaved SNAP-25 in A. ipsilateral dorsal horn and B. ipsilateral ventral horn after 5 U/kg toxin injection into the rat gastrocnemius muscle (Matak et al., 2012). *Red fibers (arrows) represent cleaved SNAP-25, green represents NeuN neuronal counterstaining. Scale bar = 100 μm.*

4.3. BoNT/A is transported to CNS via peripheral nerves by a microtubule-dependent mechanism

We studied the BoNT/A axonal traffic via peripheral nerves by employing BoNT/A intrasciatic injection, and proximal application of colchicine. 10 U/kg BoNT/A injected into the nerve induced the cleaved SNAP-25 occurrence in dorsal and ventral lumbar horns. Truncated SNAP-25 occurrence in the spinal cord was prevented by microtubule depolymerizer colchicine (Fig. 9, similar to published in Matak et al, 2012, Appendix II). These observations confirm the presumed BoNT/A axonal transport within peripheral nerves.

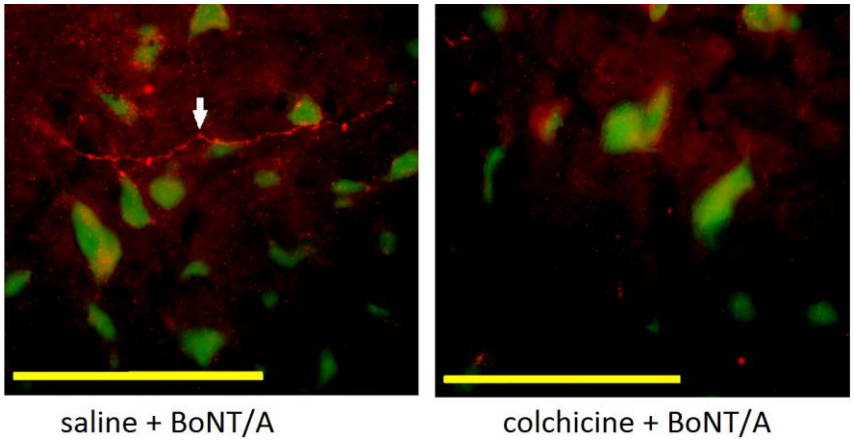

Figure 9. BoNT/A is axonally transported in peripheral nerve. *Colchicine prevents the occurrence of BoNT/A-truncated SNAP-25(red) in ventral horn. BoNT/A and colchicine were injected into the same sciatic nerve at the mid-thigh level. Injection sites were spaced 2 cm in between, with colchicine injected more proximally. Scale bar = 100 μm.*

4.4 Necessity of axonal transport in sensory neurons for BoNT/A antinociceptive efficacy

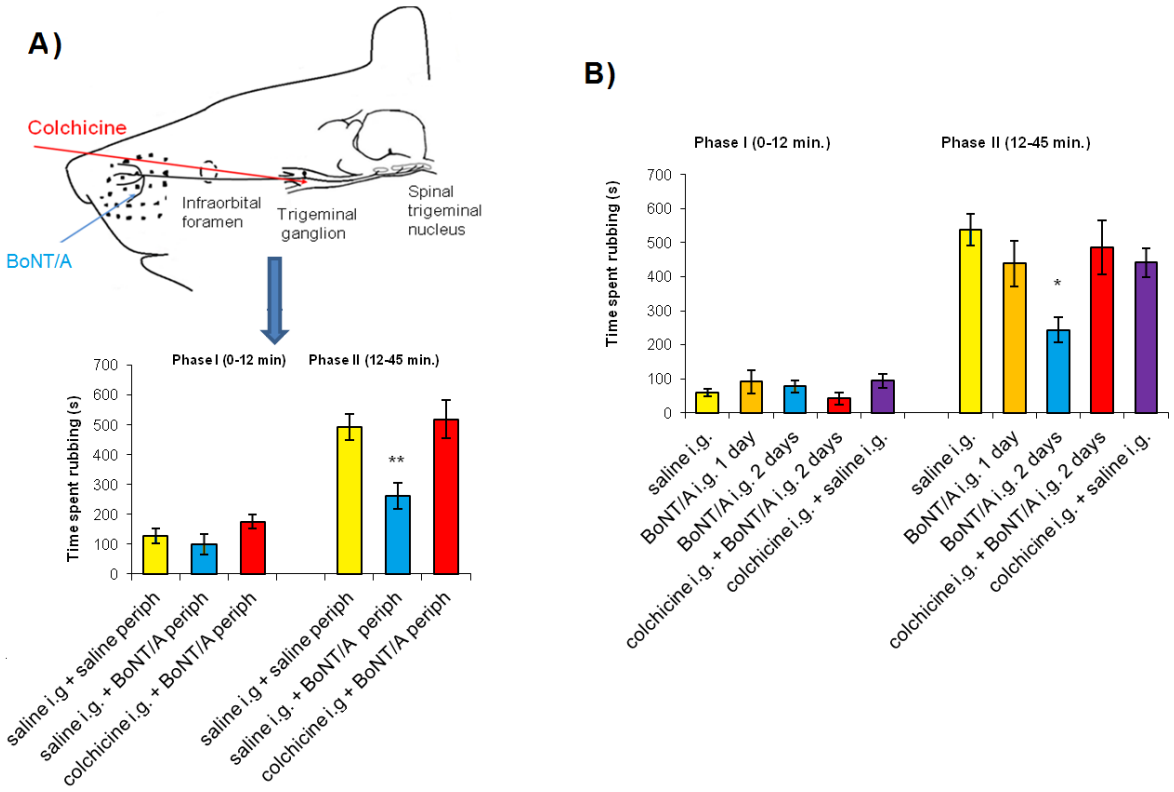

Figure 10. BoNT/A antinociceptive action depends on axonal transport within sensory nerve (similar to Matak et al., 2011). *Axonal transport blocker colchicine injected into the trigeminal ganglion prevents the antinociceptive activity of A.) peripherally applied BoNT/A (3.5 U/kg) and B.) intraganglionic BoNT/A (1 U/kg) in orofacial formalin test. Mean ± SEM; *-p<0.05 **-p<0.01 in comparison to saline control (One way ANOVA followed by Newman-Keuls post-hoc)*

In a model of formalin-induced orofacial pain, we studied the effect of axonal transport prevention on the antinociceptive activity of BoNT/A. We took advantage of the unique anatomy and exclusively sensory character of the maxillary branch of trigeminal nerve- Therefore, BoNT/A was injected into the whisker pad, and colchicine was injected directly into the sensory trigeminal ganglion. Peripherally applied BoNT/A (3.5 U/kg) did not reduce the acute immediate nociceptive response occurring within the first 12 min of formalin-induced pain. In line with previous findings in a model of peripheral hind-paw formalin-induced inflammation, BoNT/A reduced the phase II delayed facial rubbing behavior associated with central sensitization. In animals injected with axonal transport blocker colchicine into the trigeminal ganglion, the antinociceptive activity of BoNT/A was completely prevented (Fig. 10A). This study suggested that the antinociceptive activity of BoNT/A depends on the axonal transport within trigeminal sensory nerve (Matak et al., 2011;

Appendix I)

In line with proposed role of sensory neurons, injections of low-dose BoNT/A into the trigeminal ganglion (1 U/kg) 2 days prior to nociceptive testing reduced the phase II hyperalgesic behavior in formalin test, again without any effect on phase I. The BoNT/A analgesic effect was delayed: it was not observed 1 day after BoNT/A injection. Again, the colchicine prevented BoNT/A antinociceptive activity even after intraganglionic BoNT/A application. The duration of formalin-induced nocifensive behavior was not altered by colchicine itself (Fig. 10B) (Matak et al., 2011; Appendix I)

4.4. Cellular localization of cleaved SNAP-25 in central sensory and motor regions after BoNT/A peripheral application

To study the cellular localization of toxin's enzymatic activity we employed a double label study of cleaved SNAP-25 with various cellular and neuronal markers. Localization of BoNT/A-truncated SNAP-25 in sensory region was performed in TNC after toxin's peripheral application into the whisker pad. We also performed a double label study of spinal cord ventral horn to characterize the occurrence of cleaved SNAP-25 after toxin injection into the hind-paw.

In the TNC, BoNT/A-cleaved SNAP-25 colocalized partially with synaptophysin, the marker of presynaptic terminals. Punctate cleaved SNAP-25 immunoreactivity, most likely corresponding to synapses, co-occurred with synaptophysin. On the contrary, elongated cleaved SNAP-25 fibers did not colocalize with synaptophysin, which is most likely due to axonal occurrence of mentioned immunoreactivity. This suggests that BoNT/A-cleaved SNAP-25 occurs within both synaptic terminals and axons. We additionally examined the possible occurrence of BoNT/A-cleaved SNAP-25 in dendrites of secondary neurons in the TNC. We did not observe any colocalization of truncated SNAP-25 and dendritic marker MAP-2. BoNT/A-truncated SNAP-25 also did not occur within GFAP-immunoreactive astrocytes (Fig. 11A, Matak et al., 2014; Appendix IV). Since Marinelli et al., (2012) reported the occurrence of cleaved SNAP-25 in GFAP-immunoreactive astrocytes in mice subjected to nerve injury, we additionally examined the colocalization of cleaved SNAP-25 and GFAP in animals subjected to infraorbital nerve constriction, prepared as described previously (Filipović et al., 2012). In these animals we also did not observe any colocalization with

GFAP in the TNC (not shown).

In the spinal cord, BoNT/A-cleaved SNAP-25 did not colocalize with MAP-2 (Fig. 11B left). It colocalized with total SNAP-25, and ChAT, marker of cholinergic neurons (Fig. 11B middle and right). It did not colocalize with GFAP, the marker of astrocytes (not shown). These data suggest that the BoNT/A enzymatic activity in the ventral horn, after its axonal transport from periphery, occurred in spinal cord axons (The data on cleaved SNAP-25 colocalization with ChAT and GFAP were published in Matak et al. (2012); Apendix II), while the data on MAP-2 and total SNAP-25 in ventral horn are unpublished)

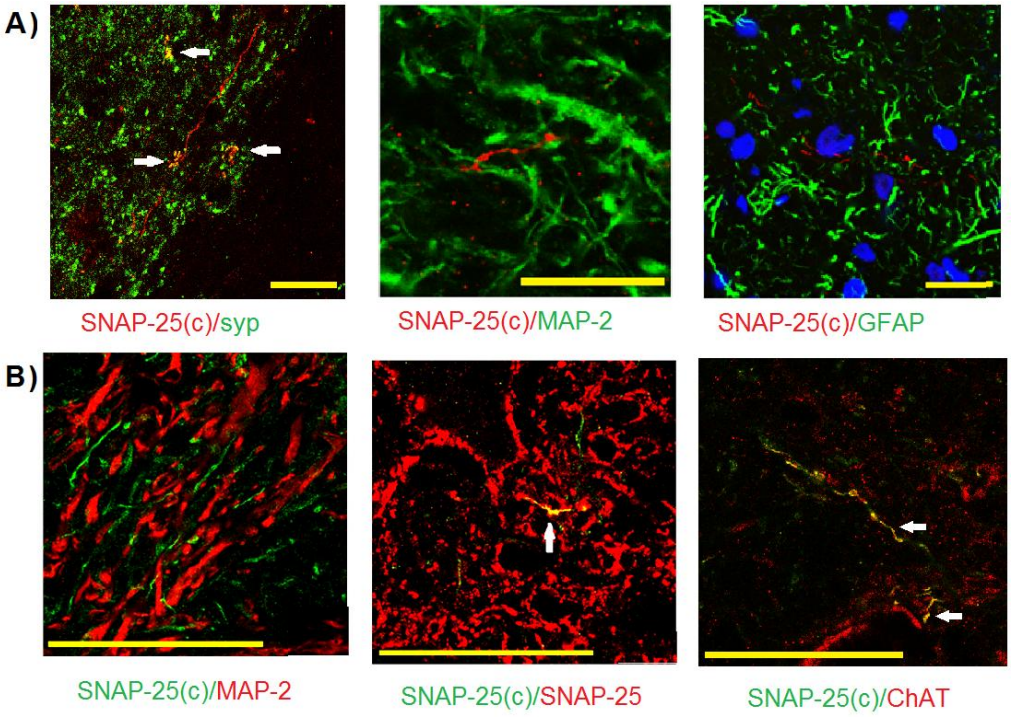

Figure 11 Colocalization of cellular and neuronal markers with cleaved SNAP-25 (SNAP-25(c) in A.) TNC (Matak et al., 2014) and B.) spinal cord ventral horn. *Syp=synaptophysin (synapses); MAP-2=microtubule associated protein 2 (dendrites); GFAP=glial fibrillary acidic protein (astrocytes), SNAP-25 = total (cleaved + uncleaved SNAP-25); ChAT=cholin-acetyltransferase (cholinergic neurons). Overlap of green and red resulting in yellow staining represents the sites of colocalization (indicated by arrows) Scale bar in A.) =20 μm; scale bar in B.)= 50 μm.*

4.5 BoNT/A is enzymatically active in central afferent terminals

Cellular colocalization study indicated that BoNT/A is present in synapses and along the axons of nerve terminals in the TNC. By employing the ablation procedure involving denervation of trigeminal ganglion with formalin, we studied the possible occurrence of enzymatically active BoNT/A in central afferent terminals (Matak et al., 2014; Appendix IV). The animals did not respond to ipsilateral noxious stimulation of whisker pad with pin prick, suggesting the denervation of trigeminal sensory nerve. Ganglion treatment did not affect the atonic position of rat whiskers induced by peripheral neuromuscular paralysis of whisker pad muscles. Cleaved SNAP-25 immunoreactivity disappeared after formalin-induced ganglion denervation. In in line with its occurrence in primary afferents, the immunoreactivity of calcitonin gene-related peptide (CGRP) disappeared, as well (Fig. 12, Matak et al., 2014). Automatically quantified area covered by CGRP in ipsilateral side TNC was almost completely abolished by formalin ($p<0.001$ in comparison to vehicle treatment, t test for dependent samples) (Matak et al., 2014; Appendix V-Supplementary data). The immunoreactivity for dendrites and neuronal nuclei of the second order neurons in TNC was unchanged on the denervated side compared to control side (not shown) – indicating that the second order neurons were unaffected by the denervation procedure. (Matak et al., 2014; Appendix V – Supplementary data).

These data suggest that the formalin injection into the trigeminal ganglion induced a denervation of primary trigeminal afferents, which was confirmed behaviorally and immunohistochemically. This procedure, applied 5 days following the BoNT/A peripheral treatment, induced the disappearance of cleaved SNAP-25 immunoreactivity in the TNC, suggesting that BoNT/A enzymatic activity is located in central afferent terminals. In addition, the complete disappearance of cleaved SNAP-25 immunoreactivity after nerve ablation indicates the lack of evidence for toxin's transcytosis to central second order neurons (Matak et al., 2014; Appendix IV).

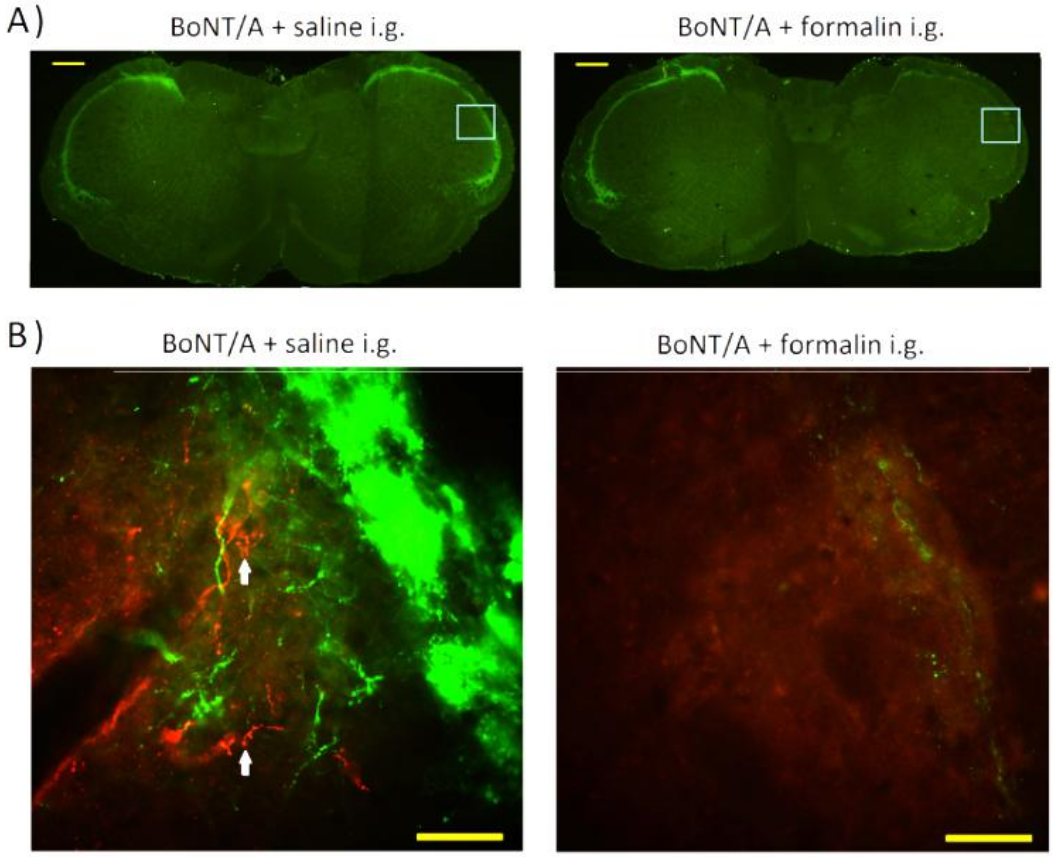

Figure 12 Proteolytic activity of BoNT/A in TNC is located in central afferent terminals of primary sensory neurons (Matak et al., 2014). *A.) Immunoreactivity for CGRP (green), marker of peptidergic primary afferents, is almost completely eliminated from TNC ipsilaterally to formalin intraganglionic (i.g.) treatment, in comparison to i.g. saline treatment (right sides of coronal sections). Scale bar=200 µm. B.) Formalin i.g. abolishes cleaved SNAP-25 staining in TNC (red immunofluorescence, arrows) and CGRP terminals (green) . Saline or formalin (10 µl) was administered into the trigeminal ganglion 5 d following peripheral BoNT/A injection into the whisker pad (15 U/kg). N(animals per group)=4 (15-25 sections were examined per each animal). Scale bar=50 µm*

4.6 Involvement of vanilloid-1 receptor expressing neurons in BoNT/A antinociceptive action

We examined possible involvement of capsaicin-sensitive (transient receptor potential vanilloid 1-expressing) neurons in the BoNT/A antinociceptive efficacy by employing capsaicin-induced desensitization of trigeminal nerve. Firstly, we examined the effect of BoNT/A pretreatment and capsaicin-induced denervation on normal sensory function (Matak et al., 2014; Appendix IV).

Sensory testing of responsiveness to acute mechanical stimuli in whisker pad area performed

using Von-Frey filaments (2 g and 8 g bending force) demonstrated normal sensitivity to mechanical stimuli of vehicle and capsaicin treated animals, while the formalin-induced denervation produced lack of response to mechanical stimuli. Similarly, pin prick test evoked a normal nocifensive behavior, except in formalin-treated animals which did not respond to noxious stimulus. Sensitivity of corneal surface to cotton touch was also preserved in capsaicin and vehicle treated animals. Capsaicin (0.01%)-evoked ipsilateral eye wiping was strongly reduced in capsaicin-treated, and prevented in formalin-injected animals. BoNT/A pretreatment did not alter the mechanical sensitivity, similarly to capsaicin-induced denervation (Table 4, Matak et al., 2014; Appendix IV).

Table 4 Sensory testing of animals injected with BoNT/A, and/or denervated with capsaicin or formalin (Matak et al., 2014; Appendix IV). BoNT/A 15 U/kg was injected into the whisker pad 5 days before the formalin or 2% capsaicin injection into the trigeminal ganglion. Testing was performed 3-4 days following the completion of denervation procedure. Statistical analysis for non-parametric data was performed by employing Kruskal Wallis test followed by Dunn's post hoc (scores), while parametric data were analyzed by ANOVA followed by Newman-Keuls post hoc. $p<0.05$ was considered significant. The p values or n.s. (non significant) refer to comparison with saline + vehicle group. N(animals/group)=5-6

Animal treatment	***Von Frey filament 2g***	***Von Frey filament 8 g***	***Pin-prick test***	***Corneal reflex***	***Capsaicin eye-wipe test***
saline + vehicle i.g.	non-aversive response (Median score =1)	non-aversive response (Median score =1)	strong aversive response (Median score = 3)	100% response	No. of eye wipes= 30±1
BoNT/A + vehicle	Score =1 (n.s.)	Score =1 (n.s.)	Score =3 (n.s.)	100% response (n.s.)	35±3 (n.s.)
Saline + capsaicin i.g.	Score =1 (n.s)	Score =1 (n.s.)	Score =3 (n.s.)	100% response (n.s.)	6±3 (p<0.001)
BoNT/A + capsaicin i.g.	Score =1 (n.s.)	Score =2 (n.s.)	Score =3 (n.s.)	100% response (n.s.)	4±2 (p<0.001)
Saline + formalin i.g.	Score = 0 (p<0.05)	Score = 0 (p<0.05)	Score = 0 (p<0.001)	lack of response (p<0.001)	0±0 (p<0.001)
BoNT/A + formalin i.g.	Score = 0 (p<0.05)	Score = 0 (p<0.05)	Score = 0 (p<0.001)	lack of response (p<0.001)	0±0 (p<0.001)

Capsaicin i.g. –induced denervation prevented the antinociceptive activity of BoNT/A in the phase II of orofacial formalin-induced pain, while the denervation itself did not influence the duration of nocifensive behavior in formalin test (Fig. 13). These data suggest that the BoNT/A antinociceptive efficacy is dependent on TRPV1-expressing sensory neurons

(Matak et al., 2014; Appendix IV).

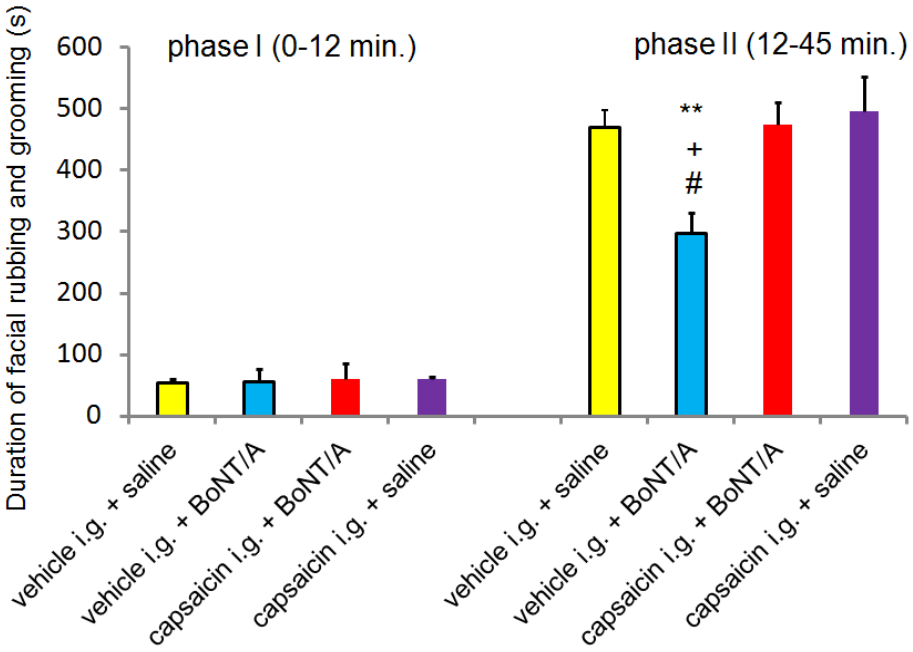

Figure 13 Chemical denervation with 2% i.g. capsaicin prevents BoNT/A's antinociceptive activity in the phase II of orofacial formalin-induced pain (Matak et al., 2014). *Capsaicin/vehicle pretreatment was completed 4 days prior to peripheral saline or BoNT/A (5 U/kg) injection, and formalin test was performed 5-6 days after saline/BoNT/A injection. Number of animals per group = 4-6. Results are represented as mean ±SEM. ** - p<0.01 in comparison to vehicle control; + - p<0.05 in comparison to capsaicin i.g. + BoNT/A; # - p<0.05 in comparison to capsaicin i.g.+ vehicle (one way ANOVA followed by Newman-Keuls post hoc).*

We examined if the central cleaved SNAP-25 in TNC is present in capsaicin-sensitive neurons. Double labeling of cleaved SNAP-25 and TRPV1 in TNC demonstrated the occurrence of products of BoNT/A enzymatic activity in TRPV1-expressing neurons (Fig. 14). Similarly to formalin-induced ablation (Figure 12), animals subjected to chemical denervation with capsaicin 5 days following peripheral BoNT/A lacked the immunoreactivity for cleaved SNAP-25 in TNC (Matak et al., 2014, Appendix IV). This observation suggests that BoNT/A enzymatic activity occurs in capsaicin-sensitive central afferent terminals. Animals subjected to i.g. capsaicin-induced denervation prior to BoNT/A injection lacked the BoNT/A-cleaved SNAP-25 in TNC, suggesting that the occurrence BoNT/A enzymatic activity in the TNC is dependent solely on capsaicin-sensitive neurons (not shown). Area covered by CGRP in ipsilateral side TNC was reduced by i.g. capsaicin ($p<0.001$ in comparison to vehicle i.g. treatment, t test for dependent samples). (Matak et al., 2014, Appendix V – Supplementary data)

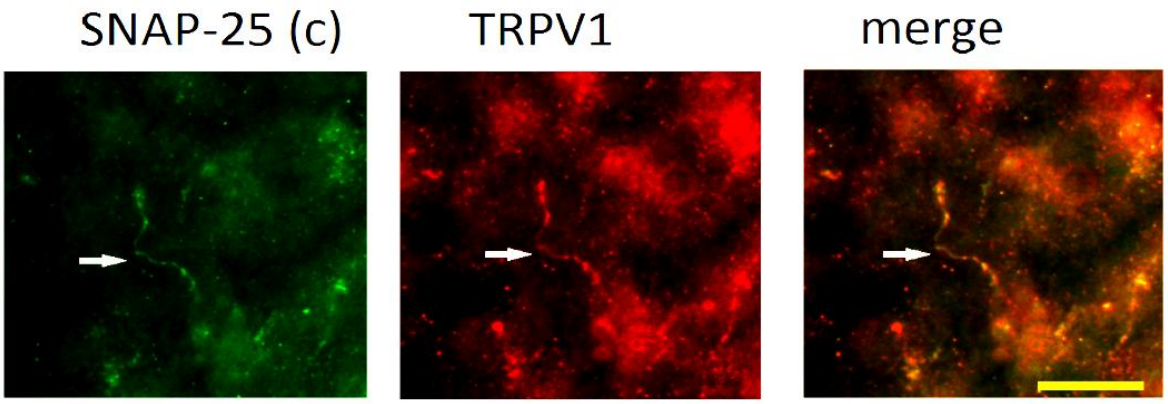

Figure 14 Enzymatic BoNT/A activity in the TNC is present in TRPV1-expressing neurons (Matak et al., 2014). *Colocalization of cleaved SNAP-25 (green) and TRPV1 (red immunofluorescence) in TNC. Scale bar=20 μm*

4.7 BoNT/A reduces the pain–evoked neuronal activation in certain brain regions

In a model of formalin-induced orofacial pain we examined the effect of BoNT/A on regional neuronal activation measured by c-Fos expression. In comparison to control, animals subjected to formalin-induced pain had an increased c-Fos expression in all sensory regions examined (Table 5). BoNT/A reduced the c-Fos expression in trigeminal dorsal horn (trigeminal nucleus caudalis), bilateral locus coeruleus, and periaqueductal gray, while the pain-evoked c-Fos expression was unaffected in thalamus (paraventricular nucleus), hypothalamus and central amygdaloid nucleus (Table 5). These data indicate a selective BoNT/A action only in certain sensory nociceptive nuclei (Matak et al., 2014; Appendix IV).

Table 5 BoNT/A effect on neuronal activation in orofacial formalin-induced pain (Matak et al., 2014)**.** Data represent the number of automatically counted c-Fos-positive profiles in each region (mean± SEM; one-way ANOVA followed by Newman-Keuls post hoc, $p<0.05$ was considered significant); n.s. = non-significant.Values of p in green were shown for comparison with saline group, while p values in red were shown for comparison with saline + formalin group. N=(number of animals/group)

	saline (N=3)	saline + formalin (N=4)	BoNT/A + formalin (N=4)
trigeminal nucleus caudalis (ipsilateral)	14.7±0.7	138.5±14.0 (p<0.001)	75.7±9.3 (p<0.01)
locus coeruleus (ipsilateral)	4.7±2.8	21.2 ±2.4 (p<0.01)	13.7±1.7 (p<0.05)
locus coeruleus (contralateral)	3.0±1.5	24.6±3.3 (p<0.001)	15.3±1.5 (p<0.05)

periaqueductal gray	90.7±26.4	290.9±20.4 (p<0.001)	149.7±8.9 (p<0.001)
hypothalamus (ipsilateral)	40.7±5.4	342±15.6 (p<0.001)	338.2±24.3 (n.s.)
hypothalamus (contralateral)	44.7 ±16.1	341.8±27.3 (p<0.001)	294.9 ±20.7 (n.s.)
paraventricular thalamic nucleus	19.2±2.5	132.5±17.7 (p<0.01)	110.1±11.8 (n.s.)
central amygdaloid nucleus (contralateral)	7.4±2.3	36.0±6.3 (p<0.01)	45.9±3.9 (n.s.)

4.8 BoNT/A effect on central CGRP transmission

By employing ELISA, we examined the release of CGRP into the cerebrospinal fluid after pain induction. In a model of paw inflammation induced by carrageenan, no significant increase of CGRP in CSF withdrawn from cistern magna was observed (data not shown). In addition, we did not observe the increased CGRP expression in models of orofacial formalin-induced pain or infraorbital nerve constriction-induced trigeminal neuropathy. BoNT/A by itself or in combination with pain did not alter the CGRP levels in the CSF. (Fig. 15, unpublished data)

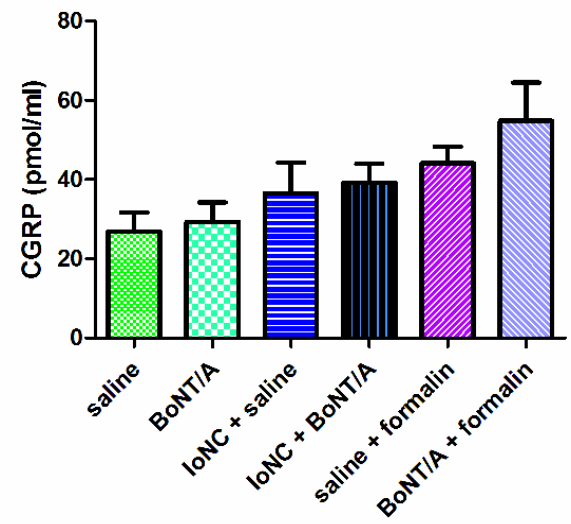

Figure 15 ***No significant changes of CGRP concentrations in the cerebrospinal fluid of animals subjected to infraorbital nerve constriction (IoNC) or orofacial formalin test.*** *CGRP immunoreactivity was measured by enzyme-linked immunosorbent assay (ELISA). Animals were treated with either saline or 5U/kg BoNT/A. Data were analyzed by one-way ANOVA followed by Newman-Keuls post hoc.*

In a model of orofacial formalin-induced pain and CFA-induced temporomandibular joint inflammation, we examined the immunoreactivity of CGRP by radioimmunoassay. In the model of orofacial formalin test, no significant increase of CGRP was observed in CSF, dura mater, trigeminal ganglion or trigeminal nucleus caudalis. However, in a model of TMJ inflammation, CGRP levels were significantly increased in TNC and cerebral dura. Slight, but

not significant increase was seen in trigeminal ganglion and CSF. BoNT/A significantly counteracted the increased CGRP expression in cranial dura. Small but non-significant reduction of CGRP levels by BoNT/A were observed in the TNC, trigeminal ganglion and CSF (Table 6). Although the data studying the CGRP release into the CSF are inconclusive, it seems that BoNT/A may prevent the increase of CGRP in the trigeminovascular system, particularly in dura mater (unpublished data).

Table 6 BoNT/A effects on the CGRP levels in trigeminovascular system of animals injected with complete Freund's adjuvans (CFA) measured by radioimmunoassay. Data are represented as mean±SEM; ($p<0,05$; $p<0.01$) – in comparison to saline; ($p<0,01$) in comparison to saline + CFA (ANOVA followed by Newman Keuls post hoc).

	saline (N=6)	saline + CFA (N=6)	BoNT/A + CFA (N=6)
trigeminal nucleus caudalis (ipsilateral) (fmol/mg)	96±6.7	131.2±7.7 ($p<0,05$)	109.6±7.5 (n.s.)
dura mater cerebri(fmol/mg)	3.6±0.3	6±0.4 ($p<0,01$)	4±0.3 ($p<0,01$)
trigeminal ganglion (ipsilateral) (fmol/mg)	31.3±1.5	37.6±3 (n.s.)	31.5±2.1 (n.s.)
temporomandibular joint (ipsilateral) (fmol/mg)	4.7±0.2	4.7±0.6 (n.s.)	6±0.4 (n.s.)
cerebrospinal fluid (fmol/ml)	49.7±7.2	66.6±7.5 (n.s.)	52.9.2±7.7 (n.s.)

In addition, we examined the colocalization of BoNT/A-cleaved SNAP-25 in trigeminal nucleus caudalis with CGRP. Except for in few neuronal terminals, majority of BoNT//A-truncated SNAP-25 did not colocalize with CGRP peptide. These data suggest that BoNT/A effect on pain is not necessarily mediated by prevention of central CGRP release (Fig.16 (Matak et al., 2014; Appendix IV).

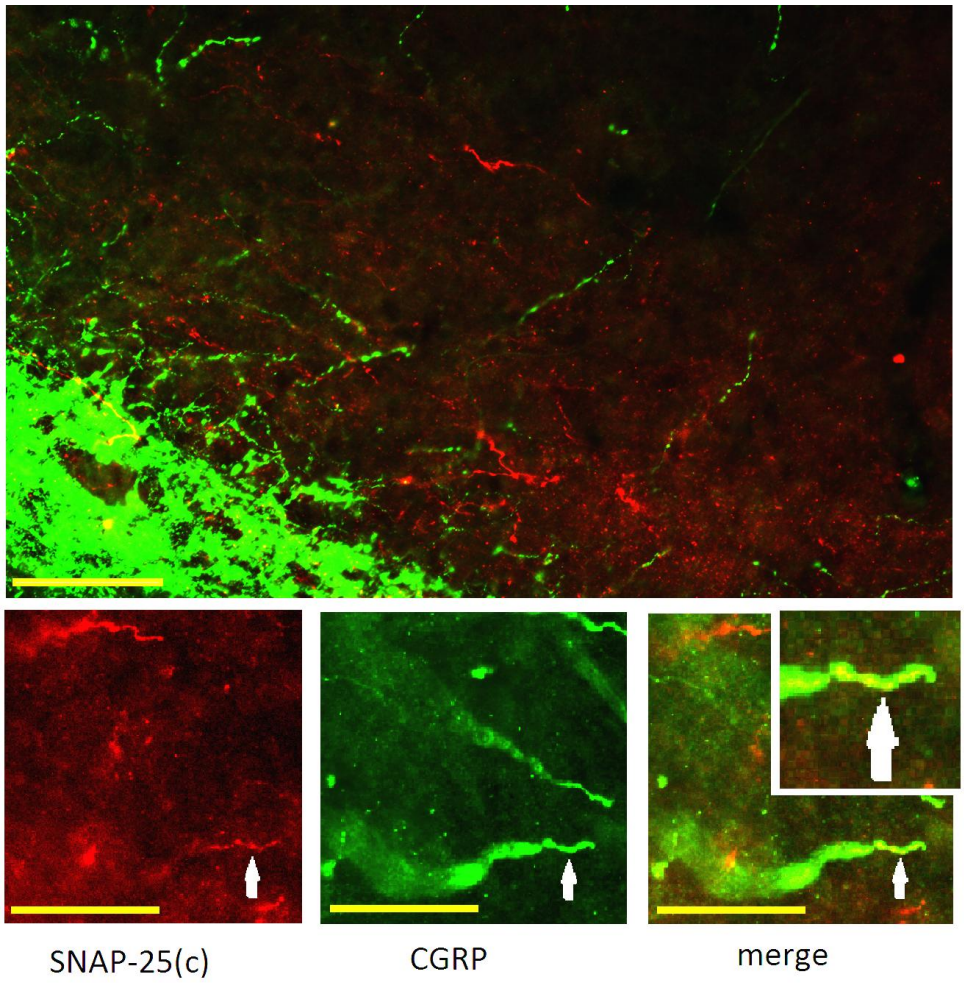

Figure 16 SNAP-25 cleavage occurs outside of CGRP-expressing peptidergic terminals after BoNT/A injection into the whisker pad (*Similar to published in Matak et al., (2014); Appendix V)). Fluorescent microphotographs of ipsilateral TNC 5 days after BoNT/A (15 U/kg) injection into the rat whisker pad. Cleaved SNAP-25 localization (red) was studied in relation to CGRP (green), marker of peptidergic primary afferents. Although the majority of BoNT/A-cleaved SNAP-25 did not colocalize with CGRP (upper panel), occasionally, cleaved SNAP-25 profiles appeared to colocalize with bright fluorescent CGRP fibers (lower panel, arrow). Images are representative of microphotographs obtained from 4 animals (10-15 sections per animals were examined). Scale bar (upper panel = 50 μm, lower panel =25 μm.*

To assess the possible BoNT/A axonal transport from periphery to dura mater, we examined the occurrence of BoNT/A-truncated SNAP-25 in dura mater after BoNT/A 5 U/kg injection into the temporomandibular joint (unpublished data). BoNT/A-truncated SNAP-25 occurred in vascular and non-vascular areas of lateral dura mater. Some cleaved SNAP-25 immunoreactivity was also visible in parietal dura mater. In all observed neuronal terminals, truncated SNAP-25 colocalized with CGRP. These data suggest that BoNT/A may affect the release of neuropeptides in dura mater after its peripheral application (Fig. 17, unpublished data).

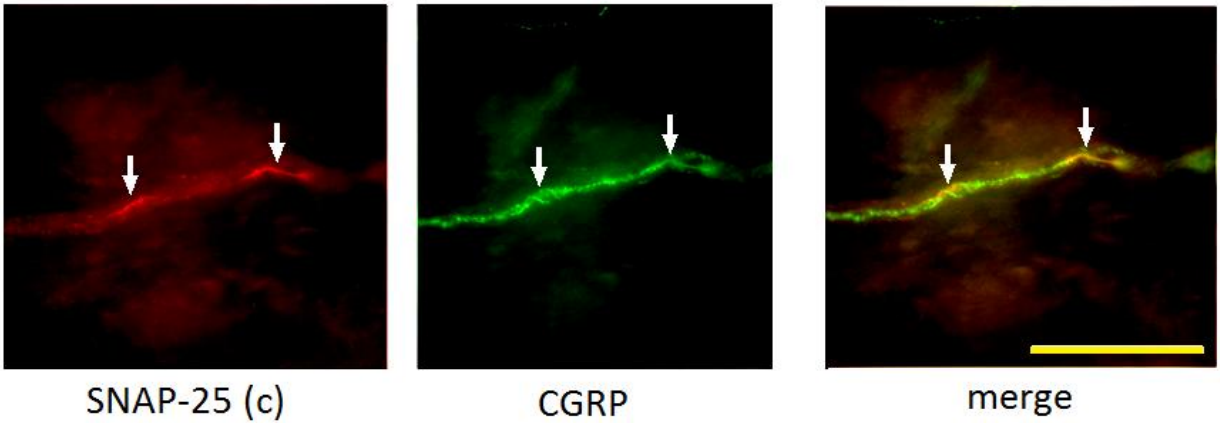

Figure 17 BoNT/A reaches CGRP-expressing dural afferent terminals after peripheral extracranial injection. *Colocalization of BoNT/A-cleaved SNAP-25 (SNAP-25 (c)) and CGRP in ipsilateral dura mater after BoNT/A (5 U/kg) injection into the temporomandibular joint. Scale bar = 50 μm*

4.9 BoNT/A is equally effective after repeated injection and does not induce permanent functional changes

A recent study from Piovesan et al. (2011) reported the lack of effectiveness of a repeated BoNT/A injection in orofacial formalin-induced pain. This study implied the possibility that repeated injections might induce either immunological resistance or putative permanent functional changes leading to BoNT/A inefficiency upon repeated injection. Since BoNT/A efficacy is connected with its axonal transport to CNS, these functional changes might be connected with central synaptic plasticity. We therefore studied BoNT/A effect after single and repeated injections of BoNT/A (Matak et al., 2013; Appendix III), and examined the possible BoNT/A effect on synaptogenesis or neurite outgrowth by measuring the effect on expression of synaptophysin and GAP-43, the markers of synapses and nerve growth (unpublished data).

BoNT/A injection into the whisker pad area evoked a reduced ipsilateral movement of ipsilateral whiskers and their backward direction 1 day after BoNT/A injection. 42 days after the injection there was no visible reduction of whisker pad movement, suggesting that the effects of BoNT/A on the neuromuscular junction has worn off. After second BoNT/A injection, the whisker pad paralysis re-occurred, rulling out a possible immunological resistance to BoNT/A (Matak et al., 2013; Appendix III).

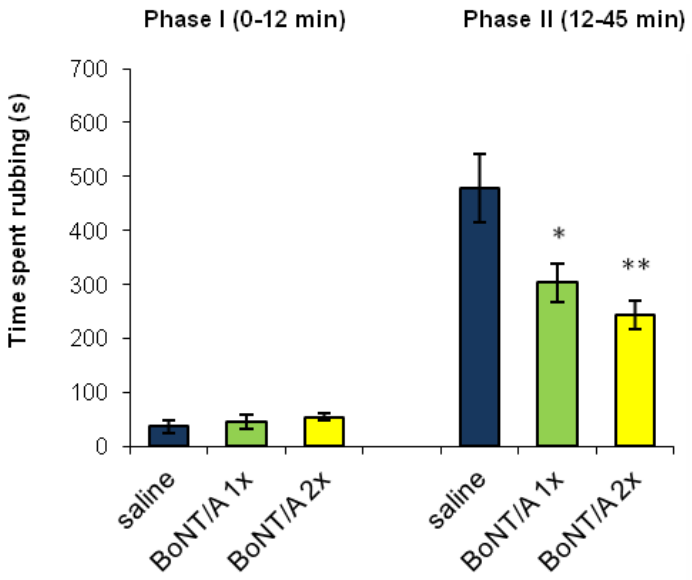

Figure 18 Repeated BoNT/A injections reduce the orofacial formalin (2.5%)-induced pain (Matak et al., 2013). *Animals were pretreated into ipsilateral whisker pad one time or two times with 5 U/kg BTX-A (42 days period between the two injections). Nociceptive testing was performed 6 days following the single or second, repeated injection of BTX-A. Data are represented as mean±SEM, * - p<0.05 ** - p<0.01 in comparison to saline control (one way ANOVA followed by Newman-Keuls post hoc test). N (animals/group)=6.*

Animals injected once or twice with BoNT/A exhibited similar reduction of phase II behavior, demonstrating that the BoNT/A was effective after single and repeated injections (Fig. 17). These data do not confirm possible functional changes or immunological resistance to BoNT/A which might lead to inefficiency of repeated BoNT/A injections (Matak et al., 2013; Appendix III).

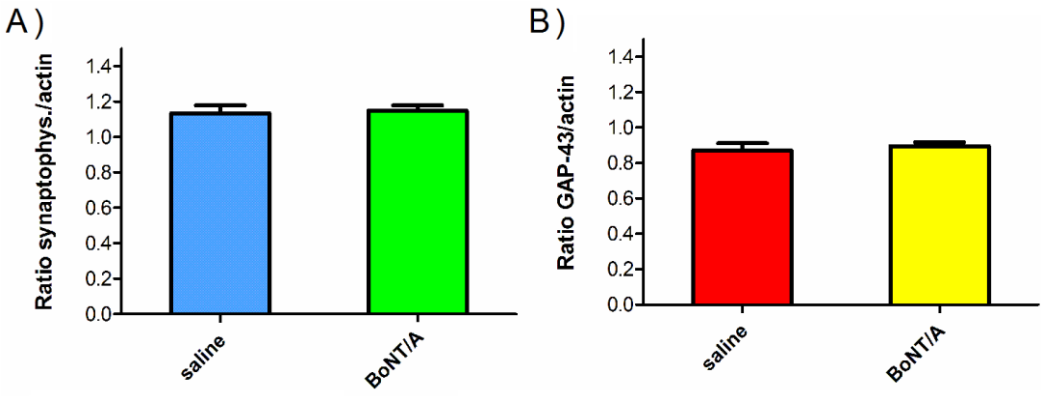

Figure 19 BoNT/A does not induce measurable synaptogenesis or neuritogenesis in the TNC. *Relative expressions (calculated as ratio to actin expression) of A.) synaptophysin (marker of synapses) and B.) GAP-43 (marker of neurite growth) in the ipsilateral TNC were measured by Western blot 5 days after 15 U/ kg BoNT/A injection into the whisker pad. Data are represented as mean±SEM; N (animals/group)=6.*

Analysis of expression of markers of synapses and axonal growth cones (synaptophysin and GAP-43) do not confirm possible nerve growth or synaptic plasticity in the TNC (Fig. 17, unpublished data). These results, however, can be considered preliminary because of low spatial resolution, and a single time point of analysis. In present experiment, tissue of the whole TNC region was analyzed. Changes of expression of synaptic and axonal growth cone markers might have been more localized within the trigeminal dorsal horn. Possibly, changes might be restricted only to central afferent terminals, which comprise a small fraction of total number of neuronal terminals in the dorsal horn. Further studies with higher spatial resolution are needed to confirm or exclude possible BoNT/A-induced synaptic plasticity in the dorsal horn.

5. DISCUSSION

5.1 Toxin's traffic to CNS after its peripheral application

In the present PhD thesis the axonal transport of BoNT/A from periphery to CNS has been characterized in details. Our experiments, enabled by detection of the product of BoNT/A enzymatic activity (cleaved SNAP-25), revealed BoNT/A axonal transport from periphery to CNS within sensory neurons (both trigeminal and spinal), as well as within spinal motoneurons. In addition, we discovered that BoNT/A is axonally transported to CNS via peripheral nerves by a microtubule-dependent mechanism. In the sensory system, BoNT/A enzymatic activity was localized in central afferent terminals (Figs. 6-12).

BoNT/A axonal transport has for a long time been regarded as non-existent, due to the dominant peripheral effects in cholinergic synapses, and the lack of pronounced central effects. However, already 50 years ago subtle central effects have been proposed based on alterations of H-reflex in a man suffering from botulism (Tyler, 1963). In the 1970s, experiments involving intramuscular injection of ^{125}I isotope-radiolabeled BoNT/A have demonstrated the progressive movement of radioactivity within sciatic nerve, ventral roots and ventral horn of corresponding spinal cord segments in cats (Habermann et al., 1974; Wiegand, 1976). Movement of radiolabelled BoNT/A has also been reported at ultramicroscopic level within the axonal compartment (Black and Dolly, 1986), however, it was proposed that BoNT/A molecule is inactivated during the axonal transport. The possibility that some active BoNT/A was transported to CNS has been demonstrated in cat abducens motoneurons. At high dose of toxin applied (3 ng) the spontaneous activity of abducens motoneurons was reduced, which was accompanied by build-up of synaptic vesicles at synapses contacting the motoneuronal cell bodies (Pastor et al., 1997; Moreno Lopez et al., 1997). However, up to recently, there was no firm evidence that the axonally transported BoNT/A represented the enzymatically active toxin molecule. Along with behavioral experiments from our laboratory which suggested BoNT/A axonal transport in sciatic nerve (PhD thesis from Bach-Rojecky, 2006), detection of BoNT/A-truncated SNAP-25 by immunohistochemistry and Western blot in central neurons has provided the proof that enzymatically active BoNT/A is axonally transported within central neurons (limbic system and optic system involving tectum, optic nerve and retina) (Antonucci et al., 2008). In addition, when BoNT/A was applied in high doses into the rat whisker pad, authors

demonstrated the toxin movement and SNAP-25 cleavage in ipsilateral facial nucleus, suggestive of BoNT/A axonal transport in facial motoneurons (Antonucci et al., 2008).

This study was later criticized due to the use of non-commercial BoNT/A, suggested lack of characterization of specificity of the antibody to BoNT/A-cleaved SNAP-25, and the use of high toxin dose not equivalent to the doses used clinically (Alexiades-Armenakas 2008; Aoki and Francis, 2011). In addition, study of BoNT/A and BoNT/E axonal transport within cultured sympathetic neurons suggested passive diffusion as the underlying mechanism of BoNT/A traffic within neuronal processes (Lawrence et al., 2011). However, passive diffusion is a very slow movement of molecules unlikely to happen over large distances *in vivo*. Experiments performed as a part of present PhD thesis have responded to all of the mentioned questions. By comparing the position of Western blot signals for intact and cleaved SNAP-25 in control and toxin-injected hippocampus we demonstrated that the antibody used by Antonucci et al. (2008) is specific for 24 kDa signal belonging to cleaved SNAP-25 (Fig. 4, Matak et al., 2011; Appendix I). By using the same cleaved SNAP-25 antibody, we demonstrated toxin's axonal transport at low 3.5 U/kg and 5 U/kg toxin doses in both trigeminal and spinal sensory neurons, after application of commercially available BoNT/A (INN: Clostridium botulinum type A neurotoxin complex) (Section 4.2; Figures 6 and 7). These doses are comparable to the doses used of therapeutical purposes in humans (Intiso, 2012). In addition, we excluded the possibility that BoNT/A axonal transport was mediated by passive diffusion. By employing intraneural toxin and colchicine injections, we demonstrated that the axonal transport of BoNT/A within peripheral nerve is an active process mediated by microtubules (Figure 8). Moreover, long distance between the sites of toxin application and the CNS, and the time-course of occurrence of BoNT/A enzymatic activity in CNS (3-5 days after peripheral application) rule out the passive diffusion of toxin molecules. Interestingly, cleaved SNAP-25 has been observed in peripheral nerve terminals in the bladder after BoNT/A intrathecal injection (Coelho et al., 2014). These observations suggest that the long-distance axonal traffic of BoNT/A may be directed from CNS to periphery, as well.

In further experiments Caleo and co-workers have demonstrated that BoNT/A is anterogradely transported and transcytosed within optic system (Restani et al., 2011). When BoNT/A was microinjected into the eye vitreous, cleaved SNAP-25 signal was found in second-order neurons in superior colliculus, suggesting the BoNT/A axonal transport through

the retina and optic nerve. The signal of cleaved SNAP-25 was unaltered following the degeneration of retinal terminals induced by optic nerve transection, suggesting the occurrence of BoNT/A in second-order neurons following transcytosis. Moreover, the cleaved SNAP-25, after its degradation by transiently active BoNT/E, re-appeared in superior colliculus, suggesting that the enzymatically active protease remained active for a long period in second order neurons after BoNT/A transcytosis. In addition, impaired neurotransmission was observed in second and third-order synapses in retina after toxin application into superior colliculus (Restani et al., 2012). In rat pups, it was demonstrated that BoNT/A injection into the optic tectum has prevented the cholinergic-driven activity in starbust amacrine cells, retinal interneurons which synapse with retinal ganglion neurons. This experiment suggested that BoNT/A prevented the release of acetylcholine in second order retinal cells after its transcytosis from retinal ganglion neurons. At ultrastructural level, swelling of synapses and build up of synaptic vesicles in second order terminals was observed (Restani et al., 2012a).

Apart from experiments with BoNT/E which induced transient disappearance and re-occurrence of BoNT/A-cleaved SNAP-25 in regions where the toxin was axonally transported, axonal transport of BoNT/A molecule was demonstrated more directly in a compartmentalized culture of motoneurons (Restani et al., 2012b). Fluorescently labeled BoNT/A was shown to be loaded as a cargo for fast axonal transport within non-acidic vesicles. The authors suggested that BoNT/A shares the same mechanism of axonal transport with tetanus toxin, viral pathogens and neurotrophic factors (Restani et al., 2012b).

Axonal transport of BoNT/A was also suggested in patients treated for spasticity (Marchand-Pauvert et al., 2013). The authors observed impairment of recurrent inhibition in muscles distant from injected site. They ruled out possible BoNT/A action on muscle spindles or toxin diffusion away from the injected site as the underlying explanation. The likely mechanism of BoNT/A action is its axonal transport to the cholinergic synapse between recurrent axon collaterals of motoneurons and Renshaw cells in the ventral horn (Marchand-Pauvert et al., 2013).

In addition to facial motoneurons (Antonucci et al., 2008), our study for the first time demonstrated the axonal transport of enzymatically functional BoNT/A within spinal motoneurons (Matak et al., 2012, Appendix II). This was based on occurrence of cleaved SNAP-25 immunoreactivity surrounding the motoneurons after BoNT/A injection into the

hind-limb (Figs. 8 and 9). Additionally, by employing confocal microscopy we found that the immunoreactivity colocalized with axons immunoreactive for ChAT (marker of cholinergic neurons) (Fig., 11B). The exact synapses affected by BoNT/A enzymatic action in ventral horn remain to be elucidated. Apart from possibility that BoNT/A might be active at the level of cholinergic synapses at recurrent collaterals (Marchand-Pauvert et al., 2013), additional sites and consequences of BoNT/A action in ventral horn remain to be investigated. This might lead to the better understanding of BoNT/A clinical efficacy in movement disorders such as dystonia and spasticity.

5.2 BoNT/A actions on central nociceptive transmission

Based on experiments from PhD study by Bach-Rojecky (2006) performed in our laboratory, a strong indication of BoNT/A central action on pain transmission was discovered. In models of mirror (acidic saline-induced muscular hyperalgesia) and inflammatory pain (induced by carrageenan-evoked paw inflammation), prevention of axonal transport within sciatic nerve by colchicine completely prevented the BoNT/A antinociceptive activity. After transection of sciatic nerve, BoNT/A low dose (0.5 U/kg) injection into the proximal stump of sciatic nerve reduced the contralateral mirror pain. These experiments demonstrated the necessity of axonal transport for BoNT/A antinociceptive action, and the lack of involvement of peripheral sensory nerve endings (Bach-Rojecky, 2006). In addition, BoNT/A antinociceptive action developed sooner if BoNT/A was injected intrathecally (Bach-Rojecky, 2006; Bach-Rojecky et al., 2010). The authors proposed that, in order to reduce pain, BoNT/A was axonally transported to CNS (Bach-Rojecky, 2006; Bach-Rojecky and Lacković, 2009). However, based on behavioral experiments it was not possible to respond to following questions: whether the axonal transport represented the enzymatically active BoNT/A molecules (hypothetically, some other molecule or catalytically inactive fragments of BoNT/A might mediate the central antinociceptive effect); whether the neurons involved in BoNT/A axonal transport and action on pain were sensory neurons (other types of neurons might have been involved, as well); in addition, the destination of axonally transported BoNT/A was unknown.

In present PhD thesis we investigated the role of axonal transport within sensory neurons for BoNT/A antinociceptive action by using injections of BoNT/A and/or colchicine into the trigeminal sensory ganglion. Trigeminal craniofacial nociception was suitable for these

experiments due to the sensory character of trigeminal nerve, and the accessibility of trigeminal ganglion for pharmacological treatments via infraorbital foramen. Colchicine injected into the trigeminal ganglion prevented the BoNT/A antinociceptive activity in orofacial formalin-induced pain (Section 4.4, Figure 9). Similarly, we found that colchicine treatment prevented the BoNT/A antinociceptive activity in a model of trigeminal neuropathy induced by infraorbital nerve constriction (Filipović et al., 2012). These experiments suggested that the axonal transport within sensory neurons was responsible for BoNT/A antinociceptive activity, ruling out possible involvement of autonomic or motor neurons.

Kitamura et al., (2009) suggested that prevention of vesicular neurotransmitter release within trigeminal ganglion may be involved in BoNT/A antinociceptive activity in a model of trigeminal neuropathy. We thus accessed the possibility that BoNT/A antinociceptive activity is located within the trigeminal ganglion by employing BoNT/A direct injections into the ganglion. Hypothetically, BoNT/A antinociceptive action, if mediated within the ganglion, should have a fast onset occurring within 24 h from BoNT/A i.g. injection, similarly to the effect visible after intrathecal injection. In addition, the antinociceptive action of i.g.-injected BoNT/A should not be prevented by inhibition of the axonal transport within the ganglion. The antinociceptive activity of BoNT/A was visible after the injection into the ganglion, which confirmed the necessary role of sensory neurons for its antinociceptive action. However, the onset of the effect on pain was delayed: it developed 2 days after the toxin injection (BoNT/A was inefficient if injected 24 prior to orofacial formalin test). Colchicine injection into the ganglion prevented the antinociceptive activity even if BoNT/A was injected into the ganglion, too (Section 4.1, Figure 5). This experiment suggested the possibility that BoNT/A is transported further from the ganglion into the CNS to exert the antinociceptive action.

In parallel, we tried to confirm the supposed presence of BoNT/A molecules in CNS after toxin peripheral application. These experiments were in the beginning unsuccessfully attempted with immunodetection by antibodies to BoNT/A itself (Fig, 5, unpublished data). However, the amount of toxin which may exert a notable effect on pain transmission in the CNS may be well below the detection limit of classical Western blot or immunohistochemical methods. Detection of cleaved SNAP-25 – the product of toxin's enzymatic activity, proved to be a better strategy since a single BoNT/A LC enzyme molecule may cleave many SNAP-25 molecules, which can then be detected by immunohistochemistry. By immunofluorescent

histochemistry we demonstrated the enzymatic cleavage of SNAP-25 in sensory regions of brainstem and spinal cord after toxin peripheral or intraneural injections (Section 4.2, Figs 6-9). In line with the delayed onset of antinociceptive action after toxin peripheral application, the occurrence of cleaved SNAP-25 molecules was not visible in CNS 24 h after BoNT/A facial injection (Matak et al., 2011, Appendix I). BoNT/A was active in the area of termination of central afferent terminals of sensory neurons connected via peripheral afferent endings to injected sites, suggestive of toxin traffic in sensory neurons.

We examined whether supraspinal regions might also be involved in the BoNT/A antinociceptive action. Hypothetically, BoNT/A might be transported to distant supraspinal sensory regions following transcytosis and axonal transport via central projection neurons. Occurrence of cleaved SNAP-25 was examined in thalamus, hypothalamus, sensory cortex, locus coeruleus or periaqueductal gray. No evident cleaved SNAP-25 occurred within t mentioned regions (Fig. 7). However, our observation might be limited by the detection threshold of the antibody to cleaved SNAP-25, thus, direct BoNT/A effect on supraspinal sensory regions cannot be completely ruled out. The pain-evoked neuronal activation (assessed by c-Fos expression) was reduced in the TNC, bilateral locus coeruleus and periaqueductal gray in a model of orofacial formalin-induced pain. BoNT/A, though, did not prevent the neuronal activation in thalamus, hypothalamus or amygdale, the regions involved in stress response and the affective and emotional processing of pain (limbic system). Assuming that BoNT/A in sensory system is not transported further from dorsal horn, the indirect effect on neuronal transmission within central nociceptive regions may be more widespread compared to direct BoNT/A action mediated by central SNAP-25 cleavage.

Occurrence of cleaved SNAP-25 in TNC suggested that BoNT/A may affect the nociceptive transmission from primary afferents to second order neurons, either 1.) by presynaptic activity in central afferent terminals or 2.) in second order synapses following transcytosis. We examined both possibilities by employing a non-selective trigeminal ganglion ablation 5 days after BoNT/A injection into the whisker pad. The BoNT/A-cleaved SNAP-25 in TNC disappeared after trigeminal denervation (Fig. 12), suggesting that the toxin's enzymatic activity was localized within central afferent terminals. Moreover, this experiment does not support the BoNT/A transcytosis to second-order synapses within the TNC. Possible lack of transcytosis in trigeminal sensory neurons is not in line with data from Restani et al. (2011) and (2012) who reported the BoNT/A transcytosis to second order synapses in optic tectum

and retina.

In line with presumed BoNT/A action in synapses, we observed cleaved SNAP-25 partial colocalization with synaptophysin in punctate terminals. However, we discovered possible BoNT/A activity also within non-synaptic sites. Elongated cleaved SNAP-25 immunoreactivity, most likely situated along the axolemma, did not colocalize with synaptophysin. In line with axonal localization, we did not observe colocalization with MAP-2-positive dendrites or NeuN-stained neuronal nuclei. A recent study from Marinelli et al., (2012) reported the occurrence of cleaved SNAP-25 in astrocytes of neuropathic mice. BoNT/A-cleaved SNAP-25 did not colocalize with GFAP-stained processes, suggesting the lack of transcytosis to astroglial cells. The difference between our experiments and data from Marinelli et al. (2012) may be due to different experimental setup, different region examined (lumbar vs trigeminal dorsal horn), or the animal species (rats vs. mice).

By examining the effect of single and repeated injections of BoNT/A, we excluded possible permanent functional changes which might lead to altered antinociceptive efficacy of BoNT/A upon repeated application. Analysis of markers of synapses and axonal growth cones suggested that the BoNT/A effect in trigeminal dorsal horn is not associated with notable synaptogenesis or neurite outgrowth in TNC. However, this study has been performed at a low spatial resolution level (the entire nucleus), thus, more localized action within the dorsal horn, or within central afferent terminals only, should be further evaluated.

In conclusion, these data suggest that the antinociceptive activity of BoNT/A involves prevention of SNARE-mediated neurotransmitter release from sensory afferent terminals in central nociceptive regions. Along with prevention of nociceptive transmission from primary afferents to second order neurons, other sensory regions involved in descending inhibitory control (locus coeruleus and periaqueductal grey) may be indirectly affected.

5.3 Selectivity of BoNT/A action for hyperalgesia and allodynia is mediated by capsaicin-sensitive neurons

Apart from the lasting BoNT/A efficacy a single peripheral application in chronic pain states, another important benefit of BoNT/A is the ability to normalize the pain hypersensitivity without altering normal sensory thresholds. The explanation why BoNT/A targets the pain hypersensitivity and not the normal sensory transmission has not been addressed in detail up

to now. Possibly, selectivity for hyperalgesic responses might be mediated by specific neuronal population of primary sensory neurons targeted by BoNT/A, important for development of central sensitization. We hypothesized that BoNT/A action might be selective for capsaicin-sensitive or TRPV1-expressing sensory neurons, since the deletion of this type of neurons does not alter the acute nociception (Bishnoi et al, 2014). Growing evidence suggests that central afferent terminals expressing the capsaicin receptor (also known as vanilloid-1 receptor or TRPV1), are important mediators of chronic pain and hyperalgesia (Kim et al., 2014). BoNT/A prevents capsaicin-evoked reduction of mechanical and thermal thresholds and nocifensive behavior in animals and pain in humans (Bach-Rojecky and Lacković, 2005; Gazerani et al., 2009; Shimizu et al., 2012). By performing a literature search, we compared the antinociceptive action of BoNT/A *vs* suppressed function of capsaicin-sensitive neurons induced by TRPV1 antagonists or denervation evoked by high-dose agonists (Table 7). In summary, *in vivo* experiments indicate a considerable similarity of BoNT/A analgesic effects and the effect of suppression of capsaicin-sensitive neurons (reduction of allodynia and hyperalgesia, lack of effect on tactile and acute noxious stimuli).

Table 7 Comparison of the antinociceptive activity of BoNT/A and suppression of TRPV1-expressing neurons performed by denervation or TRPV1 antagonists, on different types of experimental pain.

	Peripheral BoNT/A	**Denervation with TRPV1 agonists**	**TRPV1 antagonists**
Acute innocuous or nociceptive mechanical stimuli	No effect (Blersch et al., 2002; Cui et al., 2004; Bach-Rojecky and Lacković., 2005; Blersch et al., 2002), Table 4.	No effect (Neubert et al., 2008; Jeffry et al., 2009; Mishra and Hoon 2010), Table 4.	No effect (Tang et al., 2007)
Acute nociceptive thermal stimuli	No effect (Blersch et al., 2002; Cui et al., 2004; Bach-Rojecky and Lacković., 2005], or reduction in trigeminal area in humans (Gazerani et al., 2009)	Reduction after systemic agonist application (Bishnoi et al., 2011 Mishra and Hoon 2010]), or No effect after intrathecal agonist application (Jeffry et al., 2009; Bishnoi et al., 2011)	Reduction (Tang et al., 2007)
Formalin-induced phase I acute pain	No effect (Cui et al., 2004; Matak et al., 2011; Drinovac et al., 2013), Table 4.	No effect (Shields et al., 2010), Fig. 11	Reduction (Kanai et al., 2006, Tang et al., 2007)

Formalin-induced phase II hyperalgesic behavior	Reduction [Cui et al., 2004; Matak et al., 2011; Drinovac et al., 2013), present study..	No effect (Shields et al., 2010), Fig. 11 or reduction (Yaksh et al., 1979)	Reduction (Kanai et al., 2006; Tang et al., 2007)
Inflammatory thermal hyperalgesia	Reduction (Bach-Rojecky and Lacković 2005; Bach-Rojecky et al., 2008)	Reduction (Jeffry et al., 2009)	Reduction (Sugimoto et al., 2013)
Inflammatory mechanical hyperalgesia and allodynia	Reduction (Bach-Rojecky and Lacković 2005; Bach-Rojecky et al., 2008; Favre-Guilmard 2009]	No reduction (Jeffry et al., 2009, Mishra and Hoon 2010) or reduction (Neubert et al., 2008)	Reduction (Pomonis et al., 2003; Sugimoto et al., 2013)
Capsaicin-induced thermal and mechanical pain, hyperalgesic behavior	Reduction (Bach-Rojecky and Lacković 2005; Bach-Rojecky et al., 2008; Shimizu et al., 2012)	Reduction (Jeffry et al., 2009)	Reduction (Pomonis et al., 2003; Tang et al., 2007)
Neuropathy-induced thermal hyperalgesia	Reduction (Bach-Rojecky et al., 2005)	Reduction (Kissin et al., 2007; Tender et al., 2008)	Reduction (Pomonis et al., 2003; Kanai et al., 2005; Watabiki et al., 2011)
Neuropathy-induced mechanical hyperalgesia and allodynia	Reduction (Park et al., 2006; Luvisetto et al., 2007; Filipović et al., 2012)	Reduction (Kissin et al., 2007; Tender et al., 2008) or no effect (King et al., 2011)	Reduction (Pomonis et al., 2003; Kanai et al., 2005; Watabiki et al., 2011)

In support of presumed role of capsaicin-sensitive neurons, we found that neuronal terminals stained for cleaved SNAP-25 colocalize with TRPV1 receptor (Fig. 14). By employing high dose capsaicin, we found that the occurrence of cleaved SNAP-25 in TNC was prevented by denervation of TRPV1-expressing neurons (Matak et al., 2014; Appendix IV). These experiments suggested that the central terminals stained for cleaved SNAP-25 in the TNC are, indeed, sensitive to capsaicin. Moreover, in a model of formalin-induced pain we found that the denervation of capsaicin-sensitive neurons completely prevented the BoNT/A antinociceptive action on phase II hyperalgesic behavior (Fig. 13). Lack of BoNT/A effect on mechanical thresholds was similar to the effect of capsaicin-evoked TRPV1 denervation (Table 4.). These findings suggest that peripherally administered BoNT/A may modulate the nociceptive transmission of glutamate and other neurotransmitters associated with capsaicin-sensitive neurons at the first sensory synapse in the CNS. Theoretically, since TRPV1

translocation to plasma membrane is mediated by SNAP-25 (Shimizu et al., 2012), BoNT/A might also block the TRPV1 receptor-mediated nociceptive transmission at central afferent terminals (Matak et al., 2014).

As another potential mechanism for BoNT/A selective action on allodynia and hyperalgesia, hypothetically, its actions might have been mediated by direct prevention of central release of pronociceptive neuropeptides, such as CGRP and substance P, which partially colocalize with TRPV1-expresing neurons. Although *in vitro* or *ex vivo* experimental studies suggested that BoNT/A may prevent the neuropeptide release from sensory neurons (Durham et al., 2004; Lucioni et al., 2008), the causal relation between BoNT/A antinociceptive action and prevention of neuropeptide release either in periphery or CNS up to now has not been conclusively demonstrated. In the CNS, we examined possible colocalization of BoNT/A enzymatic activity and CGRP. The terminals stained for cleaved SNAP-25 mainly did not colocalize with CGRP (Fig. 16), suggesting that the direct prevention of central CGRP release is not the dominant mechanism of BoNT/A antinociceptive action in CNS.

Some of BoNT/A's effects cannot be explained only by prevention of neurotransmitter release from central afferent terminals, which at first might appear like a plausible explanation of BoNT antinociceptive mechanism (Marino et al., 2013). In a recent studies from our laboratory, antinociceptive effect of BoNT/A was prevented by systemically or intrathecally applied μ-opioid and GABA-A antagonists, suggesting the involvement of endogenous opioidergic and GABA-ergic system in the antinociceptive activity of BoNT/A (Drinovac et al., 2013; Drinovac et al., 2014). In addition, it was observed that BoNT/A suppresses morphine-induced tolerance and potentiates morphine analgesia (Auguet et al., 2008; Vacca et al., 2012; Vacca et al., 2013). The mechanism of observed involvement of GABA-ergic and opioidergic neurotransmission in the BoNT/A antinociceptive action is presently unknown. Interestingly, denervation of TRPV1-expressing neurons or chronic treatment with TRPV1 antagonists exhibits the effects on morphine tolerance or opioid-induced analgesia similar to BoNT/A (Chen et al., 2006; Chen et al., 2007; Chen et al., 2008). Hypothetically, reduced neurotransmission in capsaicin-sensitive neurons might indirectly trigger the observed effect of BoNT/A on endogenous opioidergic and GABA-ergic systems.

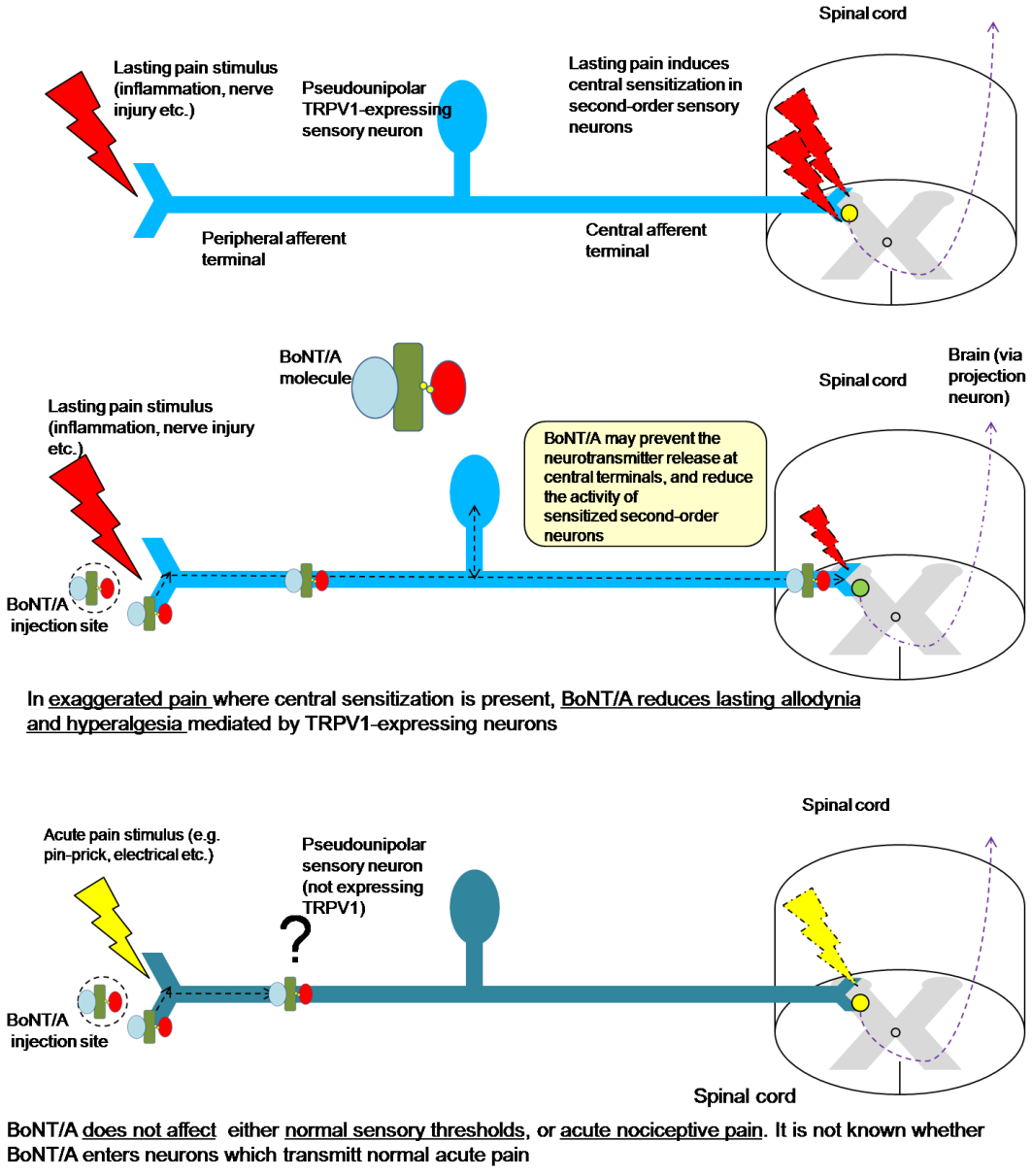

Figure 20 Schematic representation of the possible mechanism of BoNT/A action on central nociceptive transmission. *Selectivity of BoNT/A action for allodynia and hyperalgesia, and the lack of effect on acute pain or other sensory thresholds, involves its selective action on TRPV1-expressing neurons.*

In conclusion, BoNT/A effect on pain is related to its axonal transport to capsaicin-sensitive central afferent terminals. The resulting prevention of pain transmission reduces the central sensitization (facilitated activation of second order sensory neurons leading to hyperalgesic response). This explain the selectivity of toxin's effect to pain hypersensitivity and resulting allodynia and hyperalgesia, which is in line with toxin's efficacy in chronic pain conditions associated with central sensitization (Fig. 20).

5.4 Central vs. peripheral BoNT/A action

Up to now, there is still no consensus about the site and underlying mechanism of antinociceptive action of BoNT/A. Initial opinion, based primarily on the assumption that BoNT/A action is localized to the injection site, suggests that BoNT/A prevents the pain by inhibiting peripheral neurotransmitter/inflammatory mediator release (Aoki, 2005). Additionally, this opinion was modified by possibility that BoNT/A might prevent the local expression of pain receptors such as TRPV1 on peripheral sensory nerve endings by preventing their SNARE-mediated translocation to plasma membrane (Aoki and Francis, 2011).

Strong evidence against the peripheral site and mechanism of BoNT/A action was provided by findings from Bach-Rojecky and Lacković (2005), who discovered that BoNT/A effects on pain are not necessarily connected with local reduction of inflammation and peripheral neurotransmitter release. BoNT/A induces bilateral effects after unilateral injection in mirror or polyneuropathic pain of different origins. In addition, Bach-Rojecky and Lacković (2009) discovered that the antinociceptive effect of peripherally applied BoNT/A is dependent on axonal transport in peripheral nerves. In present PhD thesis, we confirmed the axonal transport of enzymatically active BoNT/A molecules to CNS, and found that the sensory neurons involved in toxin's central action are sensitive to capsaicin (TRPV1-expressing). Enzymatic activity of BoNT/A has been immunohistochemically visualized in the spinal cord or brainstem areas receiving sensory input from toxin's peripheral injection site. Additionally, BoNT/A's antinociceptive activity is shown to be associated with central μ-opioid and GABA-A receptors (Drinovac et al., 2013, 2014). Summary of experimental data in favor of peripheral *vs.* central site of action is given in Table 9. While the experimental data show that BoNT/A may prevent the sensory neurotransmitter release from peripheral afferent terminals, the relation of mentioned effect and BoNT/A antinociceptive action is yet unknown. Based on experimental data obtained in present PhD thesis and the data available in the literature, we suggest that the peripheral site of action cannot explain the antinociceptive activity of BoNT/A.

Table 8 Summary of experimental data supporting peripheral hypothesis of BoNT/A action on pain (left) versus experimental data supporting central antinociceptive activity of BoNT/A (right) (Matak and Lacković, 2014).

Evidence supporting peripheral hypothesis	References	**Evidence supporting central hypothesis**	References
Analogy with the effect on neuromuscular junction and autonomous synapses	*(Aoki, 2005; Aoki and Francis, 2011)*	Bilateral effect of unilateral injection in neuropathic and mirror pain models	*(Favre Guilmard., 2009; Bach-Rojecky and Lacković, 2009; Bach-Rojecky et al., 2010)*
Reduction of formalin-induced increase in hind-paw glutamate peak concentration in rats, reduction of capsaicin-induced glutamate release in human skin	*(Cui et al., 2004; Bittencourt da Silva et al., 2014).*	Prevention of antinociceptive effect of peripheral BoNT/A by intraneural or intraganglionic colchicine	*(Bach-Rojecky and Lacković, 2009, Matak et al., 2011, Filipović et al., 2012)*
Decreased TRPV1 and P2X3 sensory receptor expression in neurogenic bladder	*(Apostolidis et al., 2005)*	Contralateral effect after BoNT/A injection into the distally transected sciatic nerve in a model of bilateral pain	*(Bach-Rojecky and Lacković, 2009)*
Reduction of peripheral neuropeptide release in iris muscle and urinary bladder	*(Ishikawa et al., 2000; Rapp et al., 2006; Lucioni et al., 2008)*	Evidence of SNAP-25 cleavage in caudal medulla and spinal cord sensory regions after low dose peripheral BoNT/A injection	*(Matak et al., 2011; Matak et al., 2012; Marinelli et al., 2012; Matak et al., 2014)*
Decreased glutamate-evoked mechanical sensitivity of craniofacial muscle nociceptors	*(Gazerani et al., 2010)*	Abolishment of trigeminal pain-evoked dural neurogenic inflammation	*(Filipović et al., 2012)*
		Efficacy of intracerebro-ventricular, intrathecal, or intraganglionic BoNT/A injections. Higher potency of intraneuronal and centrally applied BoNT/A in comparison to peripheral application	*(Luvisetto et al., 2006; Bach-Rojecky et al., 2010; Lee et al., 2011; Matak et al., 2011; Coelho et al., 2014)*
		Blockage of neurotransmitter release from distant synapses after retrograde axonal transport	*(Restani et al., 2012)*
		Inhibition of antinociceptive activity of BoNT/A by intrathecally-applied opioid or GABA-A antagonists, prevention of morphine-induced tolerance	*(Drinovac et al., 2013; Drinovac et al., 2014; Vacca et al., 2012; Vacca et al., 2013)*

5.5 Implications of present results for BoNT/A clinical use

5.5.1 Therapeutic BoNT/A application into sensory nerves or ganglia.

Present PhD study has conclusively confirmed the assumption that BoNT/A antinociceptive activity is mediated by toxin's occurrence in CNS, provided by the axonal transport in sensory neurons. Increased potency of BoNT/A after application into the peripheral nerve (Bach-Rojecky and Lacković, 2009), intrathecal space (Bach-Rojecky, 2006) or ganglion (Matak et al., 2011) suggests that only a small portion of peripherally applied BoNT/A is axonally transported towards the CNS in order to exhibit its antinociceptive activity. Possible application of present discoveries is, by employing known anesthetic techniques like peripheral nerve or ganglion blocks, to inject smaller doses of BoNT/A directly into the nerve or ganglia in humans. Selective targeting of nerves or ganglia by BoNT/A may be employed for analgesia in the whole dermatome of injected sensory ganglion or the innervation area of injected nerve. In addition, selective BoNT/A application to sensory nerves may reduce the risk of unwanted autonomic or motor side-effects. Another potential benefit is the lower immunological titer of toxin, which may reduce the risk for development of immunological resistance to BoNT/A upon repeated injections. The major concern of such application of BoNT/A are potential central side effects after toxin axonal transport and transcytosis into distant brain regions. Although some studies suggested that BoNT/A may be transcytosed over two or more synapses in optic and motor system (Restani et al., 2012a; Akaike et al., 2013), our results suggest that there are no detectable signs of toxin's enzymatic activity in second order synapses in TNC or in distant central sensory regions (other than TNC) after toxin's application into the trigeminal area.

5.5.2 Synergism with centrally-acting analgesics

Auguet et al. (2008) and Vacca et al. (2012,13) demonstrated synergistic antinociceptive effect of low, individually ineffective doses of BoNT/A and morphine. In addition, BoNT/A prevented the morphine-induced tolerance (Vacca et al., 2012, 2013). The mechanism of synergistic interaction of BoNT/A and morphine is most likely connected with the activation of endogenous opioid system at spinal cord level via μ-opioid receptors (Drinovac et al., 2013).

As previously discussed (Section 5.3), denervation of TRPV1-expresing neurons or chronic

application of TRPV1 antagonist have similar effect like BoNT/A on morphine-induced tolerance and potentiation of opioid analgesia (Chen et al., 2006; Chen et al., 2007; Chen et al., 2008). Since the synergistic effect of BoNT/A and low-dose morphine may be connected by action on TRPV1-expressing neurons, potential additive or synergistic activity of BoNT/A and low-dose TRPV1 antagonist needs to be examined. As the role of TRPV1-expressing neurons in development of morphine-induced tolerance has been demonstrated to be mediated by increased activity of presynaptic NMDA receptors (Zhao et al., 2012), synergistic effect of BoNT/A and presynaptic glutamatergic receptors like NMDA and AMPA should be evaluated, as well. In addition, potential synergistic or additive effect of BoNT/A and of other types of analgesics acting presynaptically of postsynaptically to central terminals of primary afferents, such as triptans, CGRP antagonists etc. should be assessed. Combination of BoNT/A with other types of analgesics could be a useful therapeutic strategy in humans, potentially reducing the risk of use of high dose classical analgesics associated with development of tolerance or medication overuse.

5.5.3. Prediction of clinical response to BoNT/A treatment

As previously discussed in Section 1.3.1, response to BoNT/A may vary between different patients suffering from the same chronic pain disorder, with some subpopulations more likely to respond to BoNT/A treatment. Theoretically, with better knowledge of the mechanism and types of neurons involved in the BoNT/A antinociceptive efficacy, clinicians might have a better understanding about chronic pain disorders and subpopulations of patients which are more likely to benefit from BoNT/A treatment. In present PhD thesis we found that BoNT/A antinociceptive action is associated with capsaicin-sensitive (TRPV1-expressing) primary sensory neurons, whose central terminals are involved in development of hyperalgesia and allodynia. As a first obvious consequence of present results, it can be predicted that chronic pain disorders with pathological involvement of TRPV1-expressing neurons resulting in allodynia and hyperalgesia could respond better to BoNT/A treatment. In addition, preserved function of TRPV1-expressing neurons may be necessary for response to BoNT/A treatment. We predict that BoNT/A could be less effective in neuropathies resulting in destruction of TRPV1-expressing primary afferents.

5.5.4 Implications for migraine treatment

BoNT/A has been approved for chronic migraine treatment since 2010. However, relatively little is known about the mechanism of BoNT/A action in migraine. Although BoNT/A is injected based on a standard protocol into several extracranial head and neck muscles, peripheral site of action cannot explain the effect of BoNT/A on migraine headache, which involves intracranial activation of trigeminovascular system and dural neurogenic inflammation (Geppetti et al., 2012). Recent research from our laboratory suggests that BoNT/A, after its axonal transport within trigeminal nerve, prevents the neurogenic inflammation (measured as plasma protein extravasation) in dura mater induced by orofacial pain evoked by formalin and IoNC (Filipović et al., 2012). By radioimmunoassay, we have demonstrated that BoNT/A may prevent the increased expression of CGRP in dura mater evoked by CFA-induced inflammatory pain of temporomandibular joint (Table 6). This experiment suggests that, in models of trigeminal pain, BoNT/A reduces the CGRP-mediated activation of trigeminovascular system. In addition, we demonstrated the toxin enzymatic activity in CGRP-expressing dural afferents after low-dose BoNT/A peripheral injection to extracranial area. Present results suggest that BoNT/A effect on migraine headache is mediated by its direct activity on CGRP-expressing afferents in dura mater, thereby, reducing the peripheral sensitization of trigeminal dural afferents perceived as intensive throbbing pain during migraine attack (Mathew, 2011). This is in line with recent clinical studies which reported that higher level of blood plasma CGRP may be a predictive marker for BoNT/A efficacy in chronic migraine (Cernuda-Morollón et al., 2014) as well as that BoNT/A may reduce the CGRP levels in saliva of migraine patients (Cady et al., 2014). In addition, within dural afferents BoNT/A may be axonally transported to central afferent terminals presynaptic to second order sensory neurons, and reduce the development of central sensitization leading to cutaneous allodynia. In support of reduction of central sensitization in the TNC by peripheral BoNT/A, we demonstrated reduced c-Fos expression in a model of orofacial formalin-induced pain (Table 5). BoNT/A, in addition to TNC, reduced the neuronal activation measured as c-Fos expression in some supraspinal regions related to migraine: periaqueductal grey and locus coeruleus (Table 5).

5.6 What is unknown about BoNT/A actions in CNS?

Unlike previously simple idea which assumed the BoNT/A action in periphery localized to the injection site, recent discoveries in both animals and humans revealed a more complex mechanisms of action related to BoNT/A activity in the CNS. These findings lead to novel questions which need to be answered:

1. Possibility that BoNT/A central action on pain, apart from prevention of SNARE-mediated neurotransmitter release from central afferent terminals, involves reduced expression or activation of TRPV1 receptors, as well as of other types of pain receptors or voltage-gated ion channels.

2. The mechanism of BoNT/A interaction with opioidergic and GABA-ergic neurotransmission in the dorsal horn.

3. Possible *in vivo* consequences of BoNT/A action in CNS along axons, away from synaptic zones

4. The mechanism of BoNT/A bilateral action in bilateral mirror or polyneuropathic pain models.

5. The exact basis for apparent lack of dose response in BoNT/A antinociceptive action at low peripheral and intrathecal doses.

6. Transcytosis to distant sensory or motor regions in the CNS, with possible unknown effects *in vivo*.

7. Unknown clinical significance of BoNT/A action in ventral horn, apart from reduced recurrent inhibition (Marchand-Pauvert et al., 2013). Hypothetically, additional actions on neuronal circuitry within ventral horn leading to reduced hyperexcitability of motoneurons may be at least in part responsible for BoNT/A beneficial effects in spasticity and dystonia.

8. Recently, beneficial effects on major depression and impaired processing of emotional language have been reported after BoNT/A injections into facial muscles (Magid et al., 2014; Havas et al., 2012). In addition, BoNT/A-mediated preventive effect on vocal tics and associated premonitory urges in Gilles de la Tourette's syndrome has been observed (Porta et al., 2004). Although authors proposed indirect effects due to neuroparalysis of injected muscles, it remains to be elucidated whether BoNT/A central activity in certain brainstem areas may be involved in observed effects.

9. Potential effects on synaptic sprouting. While at the neuromuscular junction BoNT/A induces axon sprouting, it prevents the outgrowth of dendrites and axons in cultured central neurons. It remains to be examined whether peripherally injected BoNT/A may induce either of these effects *in vivo* in CNS.

10. Possible additional molecular targets of BoNT/A action. Are there any *in vivo* effects of BoNT/A action in the CNS not related to its effects on SNAP-25?

6. CONCLUSIONS

In this PhD thesis we:

1. found that BoNT/A action on pain involves a microtubule-dependent axonal transport within peripheral sensory neurons, and enzymatic activity of active BoNT/A molecules in central sensory nociceptive regions.

2. observed the BoNT/A axonal transport from periphery to CNS in peripheral sensory and motor nerves at low peripheral doses injected, suggesting that it occurs commonly following the peripheral toxin application.

3. found that BoNT/A is axonally transported from periphery to central afferent terminals of TRPV1-expressing sensory neurons. This explains its selective activity in chronic pain states accompanied by allodynia and hyperalgesia, and the lack of its effects on nociceptive acute pain thresholds.

4. found that BoNT/A applied in the trigeminal extracranial area is axonally transported to CGRP-expressing afferents in dura mater, and prevents CGRP neurotransmission in trigeminovascular system. This finding might lead to the explanation of its beneficial effects in chronic migraine and other headache disorders.

In summary, these results, together with experimental data from Bach-Rojecky (2006) have revealed that BoNT/A actions on pain is dominantly a central phenomenon, provided by axonal transport of enzymatically active toxin molecules in sensory neurons. These effects, selective to capsaicin-sensitive neurons, are involved in prevention of central sensitization leading to allodynic and hyperalgesic responses in chronic pain conditions. Although these findings have brought us closer towards the explanation of BoNT/A action on pain, the exact fine details of the mechanism of toxin's antinociceptive activity remain to be further investigated.

7. ABSTRACT

Central antinociceptive activity of botulinum toxin A

Background: Botulinum toxin type A (BoNT/A) is an emerging long-acting therapeutic for chronic pain. In contrast to previously assumed local action, novel evidence point to the CNS as the possible site of BoNT/A action on pain after its axonal transport. The aim of this thesis was to characterize the sites and mechanisms of BoNT/A action on central pain transmission.

Methods: BoNT/A antinociceptive activity was characterized by behavioral nociceptive assessment, immunodetection of BoNT/A enzymatic product (cleaved synaptosomal-associated protein 25 (SNAP-25)) and c-Fos neuronal activation in different rat sensory regions. Peripheral, intraneural and intraganglionic BoNT/A injections, and microtubule-blocker colchicine were employed to assess BoNT/A axonal transport in peripheral sensory nerves. By employing trigeminal nerve ablation we examined possible transcytosis of BoNT/A in sensory regions. Denervation of trigeminal afferents with capsaicin was employed to examine the potential role of capsaicin-sensitive (vanilloid 1-expressing) neurons.

Results: Microtubule-dependent axonal transport of BoNT/A, necessary for its antinociceptive activity, occurred in sensory neurons. Following different toxin peripheral injections, cleaved SNAP-25 has been observed in corresponding sensory nuclei of brainstem (trigeminal nucleus caudalis) and spinal cord dorsal horn, but not in higher level sensory areas. BoNT/A enzyme activity was localized presynaptically in capsaicin-sensitive (vanilloid 1 receptor -expressing) central afferent terminals. BoNT/A reduced the pain-associated neuronal activation in TNC and supramedullary regions involved in descending pain control.

Conclusion: After its axonal transport in sensory neurons, BoNT/A modulates pain transmission at the central synapse of primary afferents. Involvement of capsaicin-sensitive neurons is associated with the selectivity of BoNT/A action for pain hypersensitivity. These findings contribute to the explanation of BoNT/A mechanisms of action in pain and possible refinement of its clinical use.

Key words: botulinum toxin A, antinociceptive activity, axonal transport, synaptosoma-associated protein 25, capsaicin-sensitive neurons, central afferent terminals

8. PROŠIRENI SAŽETAK

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SREDIŠNJE ANTINOCICEPTIVNO DJELOVANJE BOTULINUM TOKSINA A

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UVOD I CILJ ISTRAŽIVANJA: Botulinum toksin tipa A (BoNT/A), neurotoksin iz anaerobne bakterije *Clostridium botulinum*, je jedan od najpotentnijih bioloških toksina. Ulaskom u živčane terminale uzrokuje enzimsko cijepanje sinaptosomalnog proteina molekulske mase od 25 kDa (eng. *synaptosomal-associated protein of 25 kDa*; SNAP-25), što sprječava lučenje neurotransmitora. Intoksikacija organizma preko hrane ili infekcija sporama bakterija pri određenim uvjetima uzrokuje neuroparalitičku bolest botulizam koju karakterizira kljenut mišića. Pročišćeni farmakološki pripravak u malim dozama se koristi kao terapija određenih hiperkinetskih poremećaja pokreta i autonomnih poremećaja, te kao kozmetički pripravak za smanjenje bora. Zbog dugotrajnog djelovanja nakon jednokratne primjene koji traje nekoliko mjeseci BoNT/A se sve više koristi u liječenju određenih tipova kronične boli. BoNT/A je odobren za liječenje kronične migrene, a njegova učinkovitost je pokazana kod niza drugih bolnih poremećaja, poput različitih vrsta neuropatskih boli, artritisa, boli u leđima, temporomandibularnih poremećaja, miofascijalne boli, itd. Zajedničko obilježje svih navedenih poremećaja je postojanje bolne preosjetljivosti i centralne senzitizacije.

Posebne prednosti njegove primjene, osim dugotrajnog djelovanja, su manje nuspojave nego kod konvencionalnih analgetika poput opioida, te manjak utjecaja na transmisiju akutne nociceptivne boli.

Unatoč sve značajnijoj kliničkoj uporabi, malo se zna o mjestu i mehanizmu antinociceptivnog djelovanja BoNT/A. Prevladavajuće hipoteze predložene od strane proizvođača farmakološkog pripravka su da BoNT/A sprječava lokalno lučenje neurotransmitora glutamata i neuropeptidnih posrednika upale s perifernih senzornih završetaka, te na taj način inhibira perifernu transmisiju boli. Novi eksperimenti, poglavito iz našeg laboratorija, upućuju na manjak povezanosti perifernog učinka BoNT/A s njegovim antinociceptivnim djelovanjem. U bilateralnim modelima boli, BoNT/A je nakon jednostrane primjene pokazao bilateralan antinociceptivni učinak, ovisan o aksonalnom transportu kroz periferne neurone. Ti eksperimenti, potvrđeni na nekoliko eksperimentalnih modela, su ukazali na moguće centralno mjesto djelovanja BoNT/A posredovano aksonalnim transportom. Stoga, cilj ovog doktorata je bilo ispitati mogući aksonalni transport molekula BoNT/A u središnji živčani sustav kroz senzorne neurone, okarakterizirati mjesta njegova djelovanja na centralnu transmisiju boli, te ispitati mehanizam selektivnosti djelovanja na bolnu preosjetljivost u odnosu na akutnu senzornu transmisiju.

METODE: Moguće djelovanje enzimski aktivnog BoNT/A u centralnim senzornim regijama štakora je istraženo imunodetekcijom enzimskog produkta djelovanja BoNT/A (pocijepani odn. krnji SNAP-25) nakon različitih načina periferne primjene: subkutanog davanja u područje trigeminusa (područje brkova na licu) ili u stražnju šapicu, te nakon injiciranja u mišić gastrocnemius ili nakon direktne injekcije u izolirani ishijadikus. Paralelno s mikroinjiciranjem u ishijadikus primijenili smo i kolhicin, kako bismo potvrdili mogući aktivni mikrotubularni mehanizam aksonalnog transporta kroz periferne živce. Da bismo potvrdili ulogu aksonalnog transporta BoNT/A kroz senzorne neurone, antinociceptivno djelovanje BoNT/A je ispitano u modelu orofacijalne boli uzrokovane formalinom nakon periferne i(ili) intraganglijske primjene BoNT/A, i intraganglijskog kolhicina.

Nakon davanja toksina u područje lica, imunohistokemijskom lokalizacijom pocijepanog SNAP-25 smo pokušali utvrdili mjesto djelovanja BoNT/A u središnjem živčanom sustavu. Pojava pocijepanog SNAP-25, osim u kaudalnoj jezgri trigeminusa, je ispitana i u supraspinalnim senzornim regijama poput talamusa, moždane kore, hipotalamusa, locus

coeruleus-a itd. Ablacijom trigeminalnog živca smo provjerili mogućnost transcitoze BoNT/A u kaudalnoj jezgri trigeminusa. Pokusima u kojima smo primijenili konfokalnu mikroskopiju smo provjerili kolokalizaciju pocijepanog SNAP-25 s neuronskim i staničnim markerima sinapsi, aksona, dendrita, neuronskih jezgri, astrocita itd. Kako bismo dodatno istražili regionalna mjesta djelovanja BoNT/A na centralnu transmisiju boli, istražili smo neuronsku aktivaciju u trigeminalnoj jezgri i drugim supraspinalnim nociceptivnim regijama imunohistokemijskom analizom ekspresije c-Fos proteina nakon bolnog podražaja uzrokovanog formalinom.

Budući da BoNT/A ne djeluje na akutnu transmisiju boli i ostalih senzornih podražaja, ispitali smo moguću povezanost njegova antinociceptivnog djelovanja s neuronima osjetljivim na kapsaicin. Nakon injekcije BoNT/A u trigeminalnu regiju, istražili smo pojavnost pocijepanog SNAP-25 u kapsaicin-osjetljivim neuronima kolokalizacijom pocijepanog SNAP-25 s vaniloidnim-1 receptorom prolaznog receptorskog potencijala (TRPV1), te denervacijom trigeminalnih aferentnih neurona s intraganglijskom injekcijom visoke doze kapsaicina (2%). Denervacijskim postupkom također smo istražili ulogu kapsaicin-osjetljivih nociceptora pri antinocicepcijskom djelovanju BoNT/A u modelu orofacijalne boli uzrokovane formalinom.

Dalje, ispitali smo moguću povezanost djelovanja BoNT/A i neuropeptida povezanog s genom za kalcitonin (eng. *calcitonin gene-related peptide*, CGRP), koji posreduje središnju senzitizaciju kod boli i migrene. Mogući utjecaj BoNT/A na lučenje i ekspresiju CGRP-a smo istražili imunodetekcijskim mjerenjem koncentracije CGRP-a u cerebrospinalnom likvoru pri različitim bolnim podražajima metodama enzimske imunoadsorpcijske analize (ELISA) i radioimunološkog testa. Nadalje, ispitali smo kolokalizaciju CGRP-a i pocijepanog SNAP-25 u trigeminalnoj jezgri i kranijalnoj duri, te utjecaj perifernog BoNTA na ekspresiju CGRP-a u gangliju, kranijalnoj duri i trigeminalnoj jezgri.

REZULTATI: Pocijepani SNAP-25 se nakon različitih mjesta periferne primjene BoNT/A pojavio u odgovarajućim senzornim nociceptivnim regijama (trigeminalna kaudalna jezgra i stražnji rog leđne moždine. Također, pocijepani SNAP-25 se pojavio i u prednjem rogu leđne moždine. Pojava pocijepanog SNAP-25 u leđnoj moždini nakon primjene BoNT/A u periferni živac je spriječena blokadom aksonalnog transporta pomoću kolhicina. BoNT/A primijenjen periferno i intraganglijski je smanjio bolnu preosjetljivost kod štakora u drugoj fazi formalinskog testa. Kolhicin primijenjen intraganglijski spriječio je antinociceptivno

djelovanje BoNT/A na drugu fazu orofacijalnog formalinskog testa.

Nakon ablacije trigeminalnog živca došlo je do unilateralne osjetne denervacije, ali i nestanka imunoreaktivnosti pocijepanog SNAP-25 u kaudalnoj jezgri trigeminusa, što ukazuje na enzimsku aktivnost BoNT/A u središnjim završecima osjetnih neurona. Kolokalizacija pocijepanog SNAP-25 je pokazala da je enzimski aktivni BoNT/A bio prisutan u središnjim sinapsama i aksonima. Pocijepani SNAP-25 nakon periferne primjene BoNT/A nije bio vidljiv u supraspinalnim regijama. BoNT/A je smanjio nociceptivnu aktivaciju neurona (mjerenu pomoću ekspresije c-Fos proteina) u kaudalnoj jezgri trigeminusa, locusu coeruleusu i periakveduktalnoj sivoj tvari.

Pocijepani SNAP-25 se nakon facijalne primjene pojavio u središnjim živčanim završecima neurona osjetljivih na kapsaicin koji izražavaju TRPV1 receptor. Učinak BoNT/A na orofacijalnu bol uzrokovanu formalinom, te pojava pocijepanog SNAP-25 u CNS-u su spriječeni denervacijom neurona osjetljivih na kapsaicin.

Mjerenjem koncentracije CGRP-a u cerebrospinalnom likvoru nije bilo moguće utvrditi utjecaj BoNT/A na središnje lučenje neuropeptida. Kod eksperimentalne boli uzrokovane injekcijom Freundovog adjuvansa u područje čeljusnog zgloba, BoNT/A je značajno spriječio pojačanu ekspresiju CGRP-a u kranijalnoj duri. U trigeminalnoj jezgri nije bilo značajne kolokalizacije pocijepanog SNAP-25 i CGRP-a. dok se pocijepani SNAP-25 u kranijalnoj duri pojavio u aferentima koji sadrže CGRP.

ZAKLJUČCI: Aksonalni transport enzimski aktivnog BoNT/A kroz senzorne neurone posredovan mikrotubulima odgovoran je za njegovo djelovanje na bol. Antinociceptivni učinak BoNT/A je povezan s njegovom enzimskom aktivnošću na centralnim završecima senzornih neurona osjetljivih na kapsaicin. Djelovanje BoNT/A na bol i centralnu senzitizaciju je praćeno smanjenjem aktivacije neurona u dorzalnom rogu i nekim supramedularnim regijama uključenima u silaznu inhibiciju boli. Selektivna uključenost neurona osjetljivih na kapsaicin objašnjava i selektivnost djelovanja BoNT/A na alodiniju i hiperalgeziju kod određenih kroničnih bolnih stanja, te manjak učinka na normalni prijenos akutne nociceptivne boli. Nalaz da BoNT/A smanjuje neurotransmisiju CGRP-a u kranijalnoj duri mogao bi objasniti njegov učinak na kroničnu migrenu. Ovi nalazi doprinose razumijevanju djelovanja BoNT/A na bolne poremećaje i mogućem poboljšanju njegove kliničke primjene.

Ključne riječi: botulinum toksin tipa A, antinociceptivno djelovanje, aksonalni transport, sinaptosomalni protein od 25 kDa, senzorni neuroni osjetljivi na kapsaicin, središnji završeci osjetnih neurona

Pojmovnik stručnih naziva koji dosad nisu prevedeni u stručnoj literaturi na hrvatskom jeziku

Central afferent terminal – središnji završetak osjetnog neurona

Enzyme-linked immunosorbent assay –enzimski vezana imunoadsorpcijska analiza

Glial fibrillary acidic protein – kiseli vlaknasti protein glije

Intraganglionic application – intraganglijsko davanje

Intraneural application – intraneuralno davanje (u živac)

Radioimmunoassay – radioimunonološki test

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor – receptor koji veže topivi faktor osjetljiv na N-etilmaleimid

Synaptosomal-associated protein of 25 kDa -sinaptosomalni protein od 25 kilodaltona

Transient receptor potential – prolazni receptorski potencijal

Trigeminal nucleus caudalis –kaudalna jezgra trigeminusa

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OnabotulinumtoxinA (marketed as Botox/Botox Cosmetic), AbobotulinumtoxinA (marketed as Dysport) and RimabotulinumtoxinB (marketed as Myobloc) http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/DrugSafetyInformationforHeathcareProfessionals/ucm174949. htm (accessed Sept 2014)

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10. BRIEF CURRICULUM VITAE

I was born in Zadar, Croatia, on 20th of December, 1984. From 1999 I attended Juraj Baraković Grammar School in Zadar. In 2001/2002 I was awarded a full one year scholarship from Open Society/HMC, to study in Bootham School, York, United Kingdom. After finishing Advanced Subsidiary (AS)-levels at Bootham School, I returned to Juraj Baraković Grammar School in Zadar to finish the secondary education in 2002/2003. From 2003 to 2008 I studied Molecular Biology at the Division of Biology, Faculty of Science, University of Zagreb. In 2008 I defended a diplom thesis, titled „G703T polymorphism of tryptophan hydroxylase 2 gene in autistic subjects“, under mentorship of Associate professor Dubravka Hranilović (Department of Animal Physiology, Division of Biology, Faculty of Science). Since 2009 I have been employed as a Junior Researcher-Assistant at the Department of Pharmacology, University of Zagreb School of Medicine, and enrolled in a doctoral study „Biomedicine and Health Sciences“. I had several shorter laboratory research stays abroad at the Department of Psychiatry, University of Würzburg, Germany (4.5 months in total from 2009 to 2012), and at the Department of Pharmacology, University of Pécs, Hungary (2 months in total from 2012 to 2014).

11. PUBLISHED ARTICLES

Articles included in the PhD thesis:

1. Matak, I., Bach-Rojecky, L., Filipović, B., Lacković, Z., 2011 **Behavioral and immunohistochemical evidence for central antinociceptive activity of botulinum toxin A**. Neuroscience 186, 201-207. *Research article*

Web link: http://www.sciencedirect.com/science/article/pii/S0306452211004362

Q2 (Neurosciences)IF (2011)=3.38, total/independent citations=(38/31)

2. Matak I., Riederer P., Lacković Z., 2012 **Botulinum toxin's axonal transport from periphery to the spinal cord**. Neurochem. Int. 61, 236-239. *Research article*

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3. Matak, I., Stracenski, I., Lacković, Z. 2013 **Comparison of analgesic effects of single versus repeated injection of botulinum toxin in orofacial formalin test in rat**s. J. Neural Transm. 120, 141-144. *Research article*

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4. Matak, I., Rossetto, O., Lacković, Z. 2014 **Botulinum toxin type A selectivity for certain types of pain is associated with capsaicin-sensitive neurons**. Pain 155, 1516-1526. *Research article*

Web link: http://www.sciencedirect.com/science/article/pii/S0304395914002061

Q1 (Neurosciences, Pharmacology and pharmacy) IF (2013/2014)=5.836, total/independent citations=(1/1)

5. Matak, I., Lacković, Z. 2014 **Botulinum toxin A, brain and pain**. Prog. Neurobiol. 119-120, 39-59. *Review article*

Web link: http://www.sciencedirect.com/science/article/pii/S0301008214000689

Q1 (Neurosciences) IF (2013/2014)=10.301, total/independent citations=(1/1)

Cummulative IF= 25.047
N (independent citations) = 41

Sources: ISI Web of Knowledge Journal Citation Reports and Scopus (December 2014)

Co-authored articles related to the subject, not included in present PhD thesis:

1. Filipović, B., Matak, I., Bach-Rojecky, L., Lacković Z. 2012 **Central action of peripherally applied botulinum toxin type a on pain and dural protein extravasation in rat model of trigeminal neuropathy**. PLoS One 7: e29803

2. Drinovac, V., Bach-Rojecky, L., Matak, I., Lacković, Z. 2013 **Involvement of μ-opioid receptors in antinociceptive action of botulinum toxin type A**. Neuropharmacology 70, 331-337.

APPENDIX I

Article Matak et al. (2011).

Source web link: http://www.sciencedirect.com/science/article/pii/S0306452211004362

Neuroscience 186 (2011) 201–207

BEHAVIORAL AND IMMUNOHISTOCHEMICAL EVIDENCE FOR CENTRAL ANTINOCICEPTIVE ACTIVITY OF BOTULINUM TOXIN A

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Abstract—Botulinum toxin A (BTX-A) is approved for treatment of different cholinergic hyperactivity disorders, and, recently, migraine headache. Although suggested to act only locally, novel observations demonstrated bilateral reduction of pain after unilateral toxin injection, and proposed retrograde axonal transport, presumably in sensory neurons. However, up to now, axonal transport of BTX-A from periphery to CNS was identified only in motoneurons, but with unknown significance. We assessed the effects of low doses of BTX-A injected into the rat whisker pad (3.5 U/kg) or into the sensory trigeminal ganglion (1 U/kg) on formalin-induced facial pain. Axonal transport was prevented by colchicine injection into the trigeminal ganglion (5 mM, 2 μl). To find the possible site of action of axonally transported BTX-A, we employed immunohistochemical labeling of BTX-A-truncated synaptosomal-associated protein 25 (SNAP-25) in medullary dorsal horn of trigeminal nucleus caudalis after toxin injection into the whisker pad. Both peripheral and intraganglionic BTX-A reduce phase II of formalin-induced pain. Antinociceptive effect of BTX-A was prevented completely by colchicine. BTX-A-truncated SNAP-25 in medullary dorsal horn (spinal trigeminal nucleus) was evident 3 days following the peripheral treatment, even with low dose applied (3.5 U/kg). Presented data provide the first evidence that axonal transport of BTX-A, obligatory for its antinociceptive effects, occurs via sensory neurons and is directed to sensory nociceptive nuclei in the CNS.

Key words: axonal transport, botulinum toxin A, synaptosomal associated protein 25, antinociceptive activity, sensory neurons.

Apart from its well known therapeutic use in muscular hyperactivity and certain autonomic disorders (Ward et al., 2006; Truong et al., 2009), botulinum toxin A (BTX-A) was recently registered for migraine treatment (Dodick et al., 2010). Besides migraine, its beneficial effects not associated with cholinergic neurotransmission were reported in cluster headache, neuropathic pain, joint pain, back pain, etc (Querama et al., 2010). It was suggested that BTX-A, similar to its activity in cholinergic neurons, inhibits the local neurotransmitter release from sensory nerve endings by peripheral SNAP-25 (Synaptosomal Associated Protein of 25 kDa) cleavage (Cui et al., 2004; Aoki, 2005).

Recent studies of rat "mirror pain" (muscular hyperalgesia) and polyneuropathy models (paclitaxel-induced polyneuropathy, diabetic neuropathy) demonstrated bilateral effects following unilateral BTX-A injection (Bach-Rojecky and Lacković, 2009; Favre-Guilmard et al., 2009; Bach-Rojecky et al., 2010). Obviously, such effects cannot be explained only by local action on the sensory nerve endings adjacent to the site of injection. Importantly, BTX-A effects were prevented by colchicine-induced blockage of axonal transport in the sciatic nerve, suggesting that retrograde axonal transport of BTX-A is necessary for its antinociceptive action (Bach-Rojecky and Lacković, 2009). However, neurons involved in axonal transport are unknown as well as the destination of transported BTX-A. So far, axonal transport of BTX-A from periphery to the CNS was demonstrated only in motoneurons (Antonucci et al., 2008), but with unknown functional significance.

Aims of this study were to investigate (a) in which neurons does BTX-A axonal transport, essential for its antinociceptive effects, take place, and (b) to locate the destination of axonally transported BTX-A. Therefore, we examined the effects of low doses of BTX-A injected into the whisker pad or trigeminal ganglion on formalin-induced orofacial pain, and used intraganglionic colchicine to prevent the axonal transport in trigeminal sensory neurons. To investigate the possible site of BTX-A antinociceptive action we employed immunolabeling of BTX-A-truncated SNAP-25 in trigeminal nucleus caudalis.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (University of Zagreb School of Medicine, Croatia), weighing 300–400 g, kept on 12 h/12 h light and dark cycle, were used in all experiments. The experiments were conducted according to the European Communities Council Directive (86/609/EEC) and recommendations of the International Association for the Study of Pain (Zimmerman, 1983). All efforts were made to reduce the number of animals used and to reduce their suffering. Animal procedures were approved by the Ethical Committee of University of Zagreb School of Medicine (permit No. 07-76/2005-43).

BTX-A injections

For peripheral administration, conscious, restrained rats were injected unilaterally with 30 μl of saline-diluted BTX-A 3.5 U/kg (Botox®, Allergan Inc., Irvine, CA, USA) into the whisker pad

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Abbreviations: BTX-A, botulinum toxin A; CGRP, calcitonin gene-related peptide; i.g., intraganglionic (in trigeminal ganglion); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNAP-25, synaptosomal-associated protein 25.

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tissue using a $27^1/_2$-gauge needle. 3.5 U/kg dose was chosen based on previous experiments by Cui et al. (2004) and from our laboratory (Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2005). For intraganglionic (i.g.) injections, animals were anesthetized with chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA; 300 mg/kg, i.p.). Trigeminal ganglion was injected as described by Neubert et al. (2005). In brief, 0–10 μl Hamilton syringe needle (Hamilton Microliter #701, Hamilton, Bonaduz, Switzerland) was inserted through the skin into the infraorbital foramen, which lies in the medial part of zygomatic process, and through the infraorbital canal and foramen rotundum directly into the trigeminal ganglion. Saline-diluted BTX-A (1 U/kg, 2 μl) was slowly injected into the ganglion. Dose of 1 U/kg was chosen based on preliminary experiments and on the dose needed for antinociceptive effect after intrathecal application (Bach-Rojecky et al., 2010). Site of injection was verified by injecting 2 μl of Methylene Blue (Sigma-Aldrich, St. Louis, MO, USA) to five animals. The dye resided only in trigeminal ganglion.

Behavioral testing

Antinociceptive activity of BTX-A was assessed in a model of formalin-induced facial pain. Conscious rats were injected with 50 μl of saline-diluted 2.5% formalin (Kemika, Zagreb, Croatia) (0.92% formaldehyde) into the whisker pad ipsilateral to BTX-A pretreatment and placed in transparent cages for observation (45 min). Observer was blind to the animal treatment (however, experienced observer could see slightly atonic rat whisker movement in BTX-A peripherally treated animals). The number of seconds of formalin-induced ipsilateral facial rubbing/grooming was measured in 3 min periods during phases I and II of formalin-induced pain (Raboisson and Dallel, 2004). Phase I (0–12 min) represents the acute nociceptive pain characterized by direct stimulation of nerve endings with formalin, while phase II (12–45 min) is characterized by the release of inflammatory mediators and sensitization (Cui et al., 2004; Raboisson and Dallel, 2004).

- For testing of peripherally applied BTX-A (3.5 U/kg), rats were divided in three groups (five to six animals per group): (1) saline (i.g.)+saline peripherally, (2) saline (i.g.)+BTX-A peripherally, (3) colchicine (i.g.)+BTX-A peripherally.
- For intraganglionic BTX-A testing (1 U/kg), rats were divided into five groups (four to seven animals per group): (1) saline (i.g.), (2) BTX-A (i.g.) 1 day, (3) BTX-A (i.g.) 2 days, (4) colchicine (i.g.)+BTX-A (i.g.) 2 days, (5) colchicine (i.g.)+saline (i.g.) 2 days.

Colchicine (Sigma-Aldrich, St. Louis, MO, USA) was injected i.g. (5 mM, 2 μl), as described above, 24 h prior to second injection (BTX-A or saline), ipsilaterally. To reduce the number of animals used, effects of intraganglionic colchicine (colchicine+saline group) on formalin-induced pain were tested only in the experiment with intraganglionic BTX-A application. Rats were tested 3 days after peripheral BTX-A injection based on data of Antonucci et al. (2008) and our preliminary experiments, and 1 and 2 days after intraganglionic BTX-A injection.

Characterization of the antibody specificity to BTX-A-cleaved SNAP-25 by Western blot

The antibody used for immunohistochemical detection of BTX-A-cleaved SNAP-25 (a kind gift from Assist. Prof. Ornella Rossetto, University of Padua, Italy) was used previously in study from Antonucci et al. (2008). One of the questions to the authors, regarding the specificity of the antibody to cleaved SNAP-25, is the lack of controls to differentiate between cleaved and non-cleaved SNAP-25. Thus, using the similar protocol as Antonucci et al. (2008), we injected BTX-A (4 U/rat) in rat dorsal hippocampus. One day following the treatment rats were sacrificed and hippocampus excised. Protein isolation, SDS-PAGE and Western

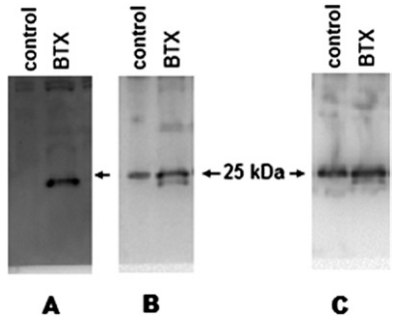

Fig. 1. Characterization of specificity of polyclonal antibody to cleaved SNAP-25 fragment. (A) Western blot membrane processed with primary antibody to cleaved SNAP-25 (1:500 dilution) and appropriate secondary antibody; (B) the same membrane subsequently, before secondary antibody, incubated with antibody to total SNAP-25 (SMI-81, 1:5000 dilution) which recognizes both cleaved and intact SNAP-25; (C) Separate experiment with the membranes incubated only with primary antibody to total SNAP-25 (the same as in experiment B) and appropriate secondary antibody. Control– hippocampus from saline injected animal; BTX– hippocampus injected with BTX-A.

blots were performed as described previously (Antonucci et al., 2008; Constantin et al., 2005). To visualize cleaved SNAP-25, and then the total SNAP-25, two sequential Western blots were performed on the same membrane. Membranes were blocked and incubated firstly with rabbit anti-cleaved SNAP-25 (1:500) in blocking solution overnight at 4 °C, and then with goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (BioSource, Invitrogen, Carlsbad, CA, USA). After development in chemoluminescent (Super Signal West Femto, Pierce, Rockford, IL, USA) and visualization by ECL camera (BioRad, Hercules, CA, USA), membrane was washed and incubated with the mouse monoclonal antibody to total SNAP-25 (1:5000, overnight at 4 °C). Antibody to total SNAP-25 (SMI-81, Sternberger Monoclonals, Baltimore, MD, USA) is well characterized and recognizes both intact and BTX-A-cleaved SNAP-25 (Jurasinski et al., 2001). Then, the membrane was incubated with goat anti-mouse secondary antibody (BD Pharmingen, San Diego, CA, USA) and visualized.

As shown in a Fig. 1, cleaved SNAP-25 (approximately 24 kDa band) was visible only in BTX-A-treated hippocampus and positioned under non-cleaved SNAP-25 (25 kDa band).

Immunohistochemistry

For the time-course experiment rats were injected into the whisker pad with 15 U/kg BTX-A, and sacrificed 1, 3, or 5 days after BTX-A injection. To access the BTX-A effects at different peripheral doses, rats were injected with 3.5 U/kg, 15 U/kg, and 30 U/kg into the whisker pad and sacrificed after 5 days. Doses were chosen based on study from Cui et al. (2004). Rats were deeply anesthetized using chloral hydrate (300 mg/kg, i.p.) and transcardially perfused with 250 ml saline, followed by 250 ml of fixative (4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.01 M phosphate buffer saline (PBS), pH 7.4). Brainstems with upper cervical spinal cords were dissected and cryoprotected at 4 °C overnight in 15% sucrose in fixative, followed by 30% sucrose in PBS the next day, until the tissue sank. Immunohistochemical protocol was similar to that previously described (Antonucci et al., 2008). 40 μm coronal sections (medullas caudal from obex and upper cervical spinal cords) were cut on a cryostat and collected for free floating in PBS. Sections were washed 3×5 min in 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS (PBST), blocked in 10% normal goat serum (Monosan, Uden, Holland) (NGS) for 1 h and incubated overnight at room temperature with 1:400 anti-BTX-A-cleaved SNAP-25 rabbit polyclonal antibody in

1% NGS. The next day, sections were washed, blocked with 1% NGS for 30 min and further incubated with 1:400 goat anti-rabbit Alexa Fluor-555 (Invitrogen, Carlsbad, CA, USA), in the dark for 2 h at room temperature. Sections were then washed with PBST, mounted on glass slides with anti-fading agent (FluoroGel, Electron Microscopy Sciences, Hatfield, PA, USA) and visualized with fluorescent microscope (Olympus BX51, Olympus, Tokyo, Japan) connected to digital camera (Olympus DP-70, Olympus, Tokyo, Japan).

To obtain high-resolution image of the whole section, parts of brainstem sections were photographed using 10× magnification, and subsequently connected using Microsoft Paint software. Images were processed for brightness and contrast using Adobe Photoshop software.

Statistical analysis

The results of orofacial formalin test were presented as mean±SEM. Between-group differences were analyzed by the Newman–Keuls post hoc test. $P<0.05$ was considered significant.

RESULTS

BTX-A reduces formalin-induced orofacial pain: necessity of axonal transport in sensory neurons

BTX-A had no significant antinociceptive effects during phase I of formalin-induced pain. However, peripheral BTX-A pretreatment (3.5 U/kg) significantly reduced the time of facial grooming during phase II of formalin-induced pain (measured 3 days post BTX-A injection). Injection of colchicine (5 mM) into trigeminal ganglion abolished the effect of subsequently applied BTX-A (Fig. 2).

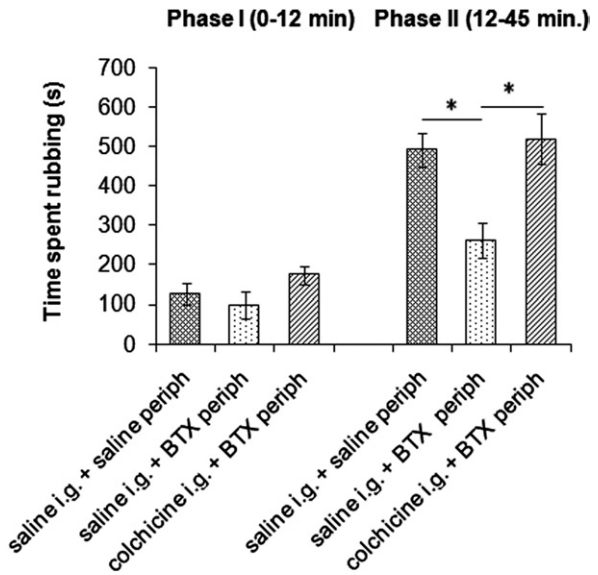

Fig. 2. Effects of peripherally applied BTX-A on pain (facial rubbing) in first and second phase of orofacial formalin test and essential role of axonal transport. Facial pain was produced by formalin injection into the whisker pad (2.5% formalin, 50 μl). BTX-A (3.5 U/kg) was also applied into the whisker pad. Colchicine was injected into the trigeminal ganglion (5 mM, 2 μl) 24 h prior to BTX-A or saline injection into the whisker pad. Measurements were performed 3 d after BTX-A injection. i.g., intraganglionic application into the trigeminal ganglion; periph, peripheral application into the whisker pad. Data are represented as mean±SEM, $n=5–6$, * $P<0.05$ (Newman–Keuls post hoc).

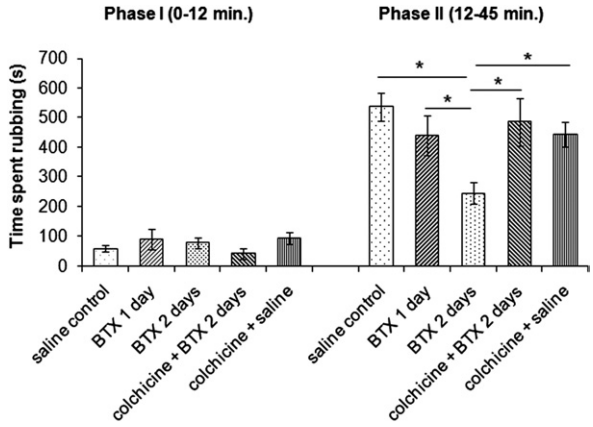

Fig. 3. Efects of intraganglionic BTX-A on pain (facial rubbing) in first and second phase of orofacial formalin test and essential role of axonal transport. Facial pain was produced by formalin injection into the whisker pad (2.5% formalin, 50 μl). BTX-A (1U/kg) was injected into the trigeminal ganglion. Colchicine was also injected into the trigeminal ganglion (5 mM, 2 μl) 24 h before BTX-A or saline. Effect of BTX-A were measured on first and second day after the application. BTX 1 day—BTX-A applied 1 d before the formalin test; BTX 2 days—BTX-A applied 2 d before the formalin test. Data are represented as mean±SEM, $n=4–7$, * $P<0.05$ (Newman–Keuls post hoc).

Intraganglionic BTX-A (1 U/kg) reduced the formalin-induced face grooming 2 days after the injection (Fig. 3). Pain was not significantly reduced when BTX-A was applied i.g. 1 day before the formalin test. Intraganglionic pretreatment with 5 mM colchicine prevented the antinociceptive effect of intraganglionic BTX-A. Intraganglionic injection of colchicine (5 mM) alone did not alter formalin-induced pain (Fig. 3).

Central SNAP-25 cleavage in trigeminal nucleus caudalis (TNC) after BTX-A peripheral application

Following application of BTX-A into the rat whisker pad (15 U/kg), fiber-like cleaved SNAP-25 immunoreactivity, apparently with varicosities, appeared in dorsal horn of ipsilateral TNC (Figs. 4 and 5). Occurrence of truncated SNAP-25 in dorsal horn was evident starting on day 3 after the injection but not on day 1 (Fig. 5A). Truncated SNAP-25 in TNC also appeared at lower and higher peripheral doses (3.5 and 30 U/kg) (Fig. 5B). We did not quantify the immunoreactivity; however, our impression was that 3.5 U/kg dose produces less intensive cleaved SNAP-25 immununofluorescence than higher doses (15 U/kg and 30 U/kg). Cleaved SNAP-25 was present throughout the rostro-caudal length of the TNC and confined to the middle part of the medullar dorsal horn coronal section (TNC according to rat brain atlas (Paxinos and Watson, 2005)). This is in accordance with the dorsoventral somatotopic organization of spinal trigeminal nuclei, whose middle portion belongs to maxillary branch of trigeminal nerve, which innervates the whisker pad (Florence and Lakshman, 1995; Capra and Dessem, 1992). In some sections only few immunoreactive fiber-like structures were visible in contralateral dorsal horn. Some immunoreactivity was also observed inconsistently in C1 upper cervical dorsal horn.

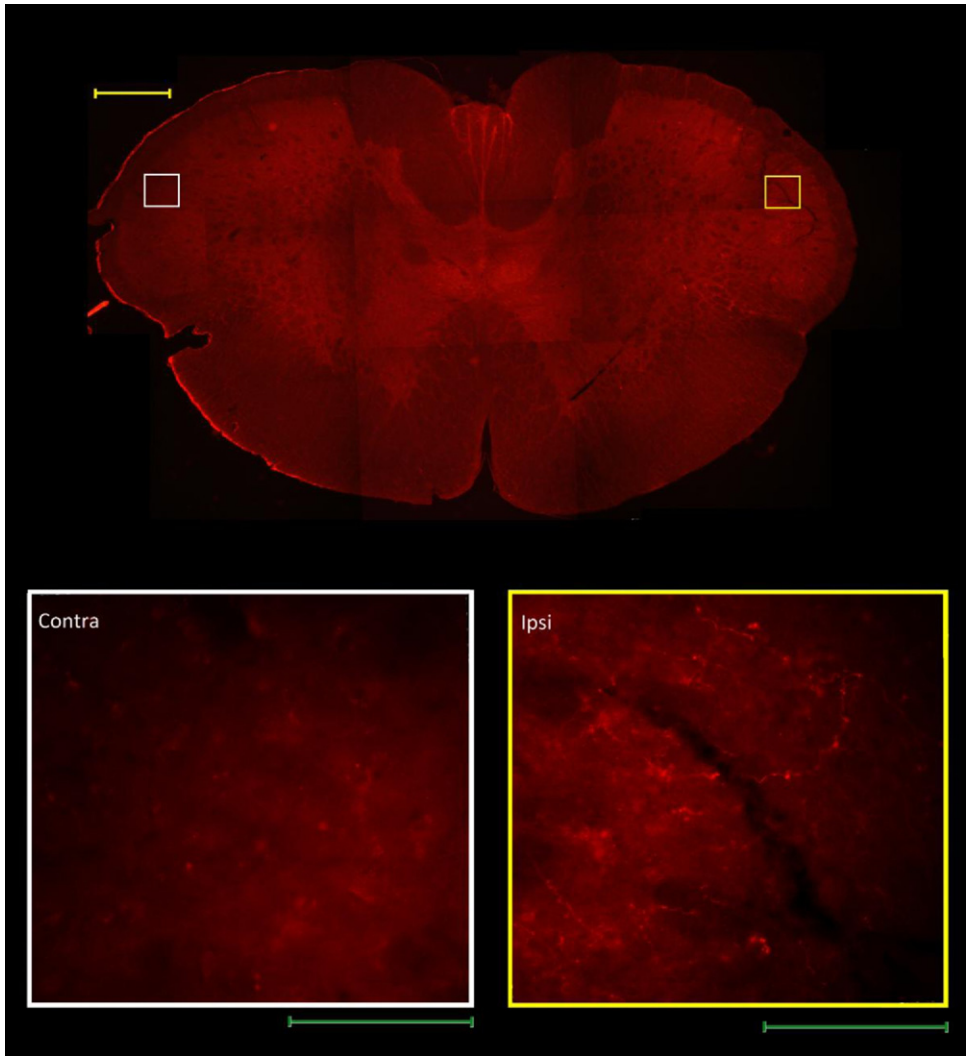

Fig. 4. Immunofluorescently labeled truncated SNAP-25 (light red) in coronal sections of rat caudal medulla 5 d after BTX-A (15 U/kg) injection into the whisker pad. Contra—TNC contralateral to the site of injection. Ipsi—TNC ipsilateral to the site of injection; Section approx. 1.4 mm caudal from obex. Yellow scale bar, 500 μm (10× magnification); green scale bar, 100 μm (40× magnification).

DISCUSSION

Cleavage of SNAP-25 at neuromuscular junctions or autonomic synapses, induced by enzymatic activity of BTX-A light chain, results in blockade of acetylcholine (ACh) release. Analogous mechanism was proposed as an explanation of antinociceptive activity in peripheral sensory neurons (Cui et al., 2004; Aoki, 2005). In formalin-induced inflammatory pain, peripheral BTX-A pretreatment had no effect on the acute nociceptive pain in phase I but it reduced pain during inflammatory phase II, accompanied by lowered peripheral glutamate release and reduction of edema in inflamed paw tissue. The most logical explanation was that both antinociceptive and anti-inflammatory actions of BTX-A are mediated by blockage of neurotransmitter and inflammatory mediator release from sensory nerve endings, as a consequence of peripheral SNAP-25 cleavage (Aoki, 2005).

However, further studies with other inflammatory pain models showed dissociation between anti-inflammatory and antinociceptive activity of BTX-A. When applied in doses that effectively reduced pain, BTX-A had no effect on capsaicin-induced neurogenic inflammation and carrageenan-induced edema (Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2008; Favre-Guilmard et al., 2009). Since inflammation is a peripheral phenomenon, the lack of the BTX-A effect on inflammation while concomitantly reducing pain brought into question the inhibition of peripheral exocytosis as a main mechanism of the antinociceptive action.

Involvement of CNS and importance of axonal transport for BTX-A antinociceptive activity

Evidence of antinociceptive activity distant from the site of peripheral unilateral BTX-A injection was found in paclitaxel-induced polyneuropathy (Favre-Guilmard et al.,

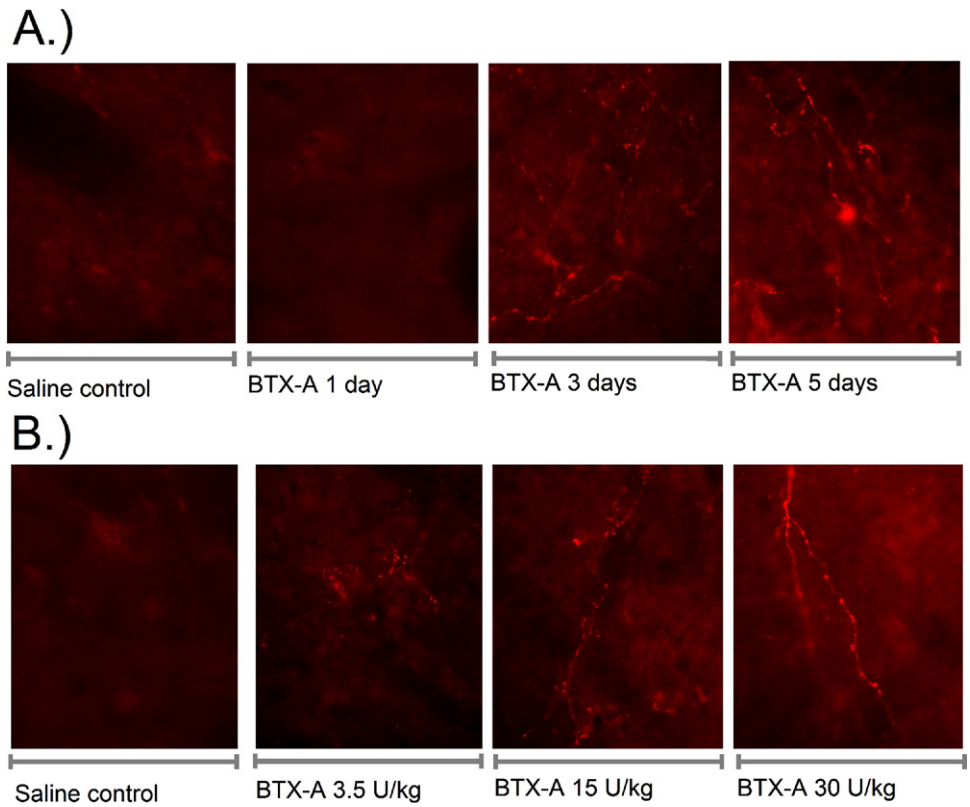

Fig. 5. Evidence of central enzymatic activity of BTX-A in ipsilateral TNC after peripheral application. (A) Time course: occurrence of truncated SNAP-25 1, 3 and 5 d after BTX-A injection into the whisker pad (15 U/kg). (B) Effect of different doses: truncated SNAP-25 in ipsilateral TNC 5 d after peripheral BTX-A application at different doses (3.5, 15 and 30 U/kg). Sections approx. 1–1.5 mm caudal from obex. 40× magnification; scale bar (gray line), 100 μm.

2009), acidic saline-induced muscular hyperalgesia (Bach-Rojecky and Lacković, 2009), and diabetic neuropathy (Bach-Rojecky et al., 2010). In those reports, apart from the injected side, BTX-A reduced the pain on contralateral side, too. Also, BTX-A injection into the distally cut sciatic nerve was still able to reduce contralateral pain in a model of bilateral muscular hyperalgesia, thus excluding the involvement of peripheral nerve endings (Bach-Rojecky and Lacković, 2009). In this model, effect of peripheral BTX-A was prevented by colchicine injection into the sciatic nerve (Bach-Rojecky and Lacković, 2009). These observations demonstrated the necessity of retrograde axonal transport for BTX-A antinociceptive activity, and probably a central site of toxin's action. In line with that, antinociceptive activity of BTX-A is obtained with lower doses and with faster onset after intrathecal than after peripheral injection (Bach-Rojecky et al., 2010). Recently, it was found that increased vesicular release from trigeminal ganglionic cells acutely isolated from rats with experimental trigeminal neuropathy was prevented if animals were pretreated with peripherally applied BTX-A (Kitamura et al., 2009). The authors suggested BTX-A retrograde transport from periphery and transcytosis within ganglionic somata as a possible explanation. Most recent studies of BTX-A effects on regenerative processes in sciatic nerve and neuroimmunological changes in dorsal root ganglia and lumbal dorsal horn of rats with experimental neuropathy also suggest the possible direct BTX-A action distant from the site of injection due to retrograde axonal transport (Marinelli et al., 2010; Mika et al., 2011; Pavone and Luvisetto, 2010).

Observations described up to now suggest that antinociceptive action of BTX-A is centrally mediated and axonal transport-dependent. To verify the hypothesis that BTX-A is axonally transported through sensory neurons, in present experiments we investigated BTX-A effects in trigeminal sensory system. In a model of pain induced by formalin injection into the whisker pad, peripherally applied BTX-A (3.5 U/kg) reduced inflammatory phase II of formalin pain. Effects of peripheral BTX-A were completely abolished by colchicine injection into the trigeminal sensory ganglion (Fig. 2). Intraganglionic BTX-A in a dose of 1 U/kg in our experiment had similar effect like peripheral dose of 3.5 U/kg BTX-A. These observations confirm the assumption that in present experiments, axonal transport of BTX-A occurs via trigeminal sensory neurons and rules out the importance of axonal transport via motor or sympathetic neurons for antinociceptive effects of BTX-A.

Since Kitamura et al. (2009) suggested blockage of vesicular release and BTX-A transcytosis within ganglionic somata, we investigated whether BTX-A antinociceptive activity is caused by its actions inside the ganglion. We speculated that direct i.g. delivery of BTX-A would cause the spread of toxin within the ganglion and should enable fast antinociceptive effect. However, in the present exper-

iment this action was delayed, occurring after 2 days, opening the possibility that the ganglion itself is not the main site of BTX-A antinociceptive activity. Moreover, by blocking axonal transport with colchicine pretreatment, effect of intraganglionic BTX-A was prevented (Fig. 3), suggesting that BTX-A, even when delivered directly to the ganglion, still requires axonal transport to exert its antinociceptive effects. Thus, we hypothesized that BTX-A could be axonally transported, via sensory root, to trigeminal nociceptive projections in central nervous system.

Occurrence of truncated SNAP-25 in the CNS after BTX-A injection in trigeminal innervation area

To verify our assumption about the axonal transport of peripherally applied BTX-A to central trigeminal projections, we employed immunohistochemical labeling of truncated SNAP-25 in TNC, the region that predominantly receives facial nociceptive input. By demonstrating SNAP-25 cleavage in TNC we found that peripherally applied BTX-A, or its catalytically active fragments, most probably reach central projections of primary sensory neurons by axonal transport (Figs. 4 and 5). Time of occurrence of truncated SNAP-25 in our experiment (Fig. 5A) is similar to the time course of BTX-A traffic in central neurons and motoneurons (Antonucci et al., 2008).

Theoretically it might be possible that, instead of BTX-A, the truncated SNAP 25—product of its proteolytic activity, was axonally transported to central trigeminal nuclei. However, previously it was shown that BTX-A injection into the sciatic nerve, which was cut distally to the place of toxin's injection, still reduces pain on contralateral side (Bach-Rojecky and Lacković, 2009). This experiment rules out the possibility that cleaved SNAP-25, transported from peripheral nerve endings, would be involved in antinociceptive effects of BTX-A. Possibility of contribution of SNAP-25 cleavage in trigeminal ganglion cannot be ruled out completely.

Confinement of cleaved SNAP-25 immunoreactivity to medullary dorsal horn excludes possible systemic spreading of BTX-A. Moreover, animals injected into whisker pad with 3.5 and 15 U/kg did not exhibit impaired rotarod performance (results not shown). However, in some sections, few immunoreactive fiber-like structures were also visible in contralateral dorsal horns (data not shown), which could be associated with contralateral crossing of central afferent terminals (Jacquin et al., 1990).

Occurrence of truncated SNAP-25 in TNC suggests that peripherally applied, axonally transported BTX-A can affect second order central sensory neurons, either presynaptically by SNAP-25 cleavage in central terminals of primary afferent neurons, or following transcytosis. Transcytosis of BTX-A to second-order synapses has been suggested in retinal ganglionic cells after axonal transport within visual system (Antonucci et al., 2008). Recently Kitamura et al. (2009) on the basis of *in vitro* experiments proposed existence of BTX-A transcytosis in trigeminal ganglia, too.

SNAP-25 cleavage in medullary dorsal horn (Figs. 4 and 5) suggests that BTX-A can alter central nociceptive transmission of presently unknown neurotransmitters, which requires further investigation. There is evidence that, when applied directly to brainstem slices or intrathecally at the level of spinal cord, BTX-A alters release and expression of CGRP (calcitonin gene-related peptide), a neuropeptide involved in pain transmission (Meng et al., 2009; Lee et al., 2011).

Importantly, we found that BTX-A-truncated SNAP-25 appeared in TNC even at 3.5 U/kg, the lowest peripheral antinociceptive dose (Bach-Rojecky and Lacković, 2005). Therefore, the axonal traffic of BTX-A from periphery to the CNS is not a phenomenon occurring only at high doses, as proposed by Alexiades-Armenakas (2008). Although comparison of doses in rats and humans can always be questioned, dose used in our experiment (3.5 U/kg) corresponds to 245 U dose in 70 kg human. BTX-A doses typically used in migraine treatment range from 100 to 260 U (Aurora et al., 2007), and in recent study which resulted in FDA approval for migraine treatment, from 155 to 195 U (Dodick et al., 2010).

CONCLUSION

Antinociceptive effect of BTX-A requires axonal transport through sensory neurons and it is associated with occurrence of truncated SNAP-25 in central sensory nociceptive nuclei.

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APPENDIX I I

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Rapid communication

Botulinum toxin's axonal transport from periphery to the spinal cord

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ABSTRACT

Axonal transport of enzymatically active botulinum toxin A (BTX-A) from periphery to the CNS has been described in facial and trigeminal nerve, leading to cleavage of synaptosomal-associated protein 25 (SNAP-25) in central nuclei. Aim of present study was to examine the existence of axonal transport of peripherally applied BTX-A to spinal cord via sciatic nerve.

We employed BTX-A-cleaved SNAP-25 immunohistochemistry of lumbar spinal cord after intramuscular and subcutaneous hind limb injections, and intraneural BTX-A sciatic nerve injections. Truncated SNAP-25 in ipsilateral spinal cord ventral horns and dorsal horns appeared after single peripheral BTX-A administrations, even at low intramuscular dose applied (5 U/kg). Cleaved SNAP-25 appearance in the spinal cord after BTX-A injection into the sciatic nerve was prevented by proximal intrasciatic injection of colchicine (5 mM, 2 μl). Cleaved SNAP-25 in ventral horn, using choline-acetyltransferase (ChAT) double labeling, was localized within cholinergic neurons.

These results extend the recent findings on BTX-A retrograde axonal transport in facial and trigeminal nerve. Appearance of truncated SNAP-25 in spinal cord following low-dose peripheral BTX-A suggest that the axonal transport of BTX-A occurs commonly following peripheral application.

1. Introduction

It is a textbook knowledge that botulinum toxin type A (BTX-A) in botulism, as well as in therapeutic applications, exerts the neuromuscular paralysis by enzymatic cleavage of peripheral synaptosomal-associated protein 25 (SNAP-25), involved in neuroexocytosis. Unlike related tetanus toxin, which is known to be retrogradely transported and transcytosed to second-order synapses (Schwab et al., 1979), it was generally accepted that BTX-A acts directly only on peripheral nerve endings. Nevertheless, already in the 70-ties some authors reported axonal transport of radioactively labeled botulinum toxin A (BTX-A) within peripheral nerves to spinal cord (Habermann, 1974; Wiegand et al., 1976). Those observations remained forgotten and questioned in later studies (Tang Liu et al., 2003). Main objections to these early studies were that it was not known if radioactively labeled BTX-A retained the enzymatic activity by the time it reached spinal cord. However, axonal transport of functional BTX-A molecules was recently found in hippocampus, visual system and in facial motoneurons (Antonucci et al., 2008). BTX-A axonal transport followed by enzymatic cleavage in CNS has been demonstrated in trigeminal sensory neurons (Matak et al., 2011). Recent *in vitro* study suggests spread of BTX-A within cell bodies and distal processes of cultured sympathetic neurons (Lawrence et al., 2012).

Retrograde axonal transport of low dose BTX-A in spinal sensory neurons has been suggested by behavioral experiments in models of bilateral muscular hyperalgesia (Bach-Rojecky and Lacković, 2009) and diabetic neuropathy (Bach-Rojecky et al., 2010).

In present study we found the enzymatic activity of BTX-A in rat motor and sensory regions of the spinal cord after intramuscular, subcutaneous, or intraneural toxin application.

2. Materials and methods

2.1. Animals

Eighteen male Wistar rats (University of Zagreb School of Medicine, Croatia), weighing 300–400 g, kept on 12 h/12 h light and dark cycle with unlimited access to food and water, 3 months old, were used. Experiments were conducted according to the European Communities Council Directive (86/609/EEC). Animal

Abbreviations: BTX-A, botulinum toxin A; SNAP-25, synaptosomal-associated protein 25; s.c., subcutaneous; i.m, intramuscular; i.n, intraneural; PBS, phosphate-buffered saline; NGS, normal goat serum; ChAT, choline acetyltransferase; GFAP, glial fibrillary acidic protein; SDS, sodium dodecyl sulfate; BTX-E, botulinum toxin E.

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procedures were approved by the Ethical Committee of University of Zagreb, School of Medicine (Permit No. 07-76/2005-43). All efforts were made to reduce the suffering of animals and the number of animals used.

2.2. Experimental procedure

Animals were injected unilaterally with BTX-A (Botox, Allergan, Irvine, CA, USA) diluted in 0.9% saline. One international unit (1 U) of BTX-A, equal to mouse LD_{50}, contains approximately 48 pg of 900 kDa neurotoxin. Animals were divided into 6 treatment groups (3 animals per group): group 1: BTX-A injected subcutaneously (s.c.) into the plantar side of hind-paw pad (30 U/kg, in volume of 30 μl); groups 2 and 3: BTX-A injected intramuscularly (i.m.) into the gastrocnemius (5 U/kg and 30 U/kg, 30 μl). In groups 4 and 5 BTX-A was injected intraneurally (i.n.) into the sciatic nerve (10 U/kg, 2 μl). 2 μl of 5 mM axonal transport blocker colchicine (Sigma, St. Louis, MO, USA) (group 4) or saline (group 5) were injected into the sciatic nerve 24 h prior to the more distal i.n. injections of BTX-A. To reduce the number of animals in the experiment, single control group (group 6) consisting of 0.9% saline (i.m.) – treated animals was used.

5 U/kg low dose and 30 U/kg BTX-A high dose was chosen based on previous experiments (Cui et al., 2004; Bach-Rojecky and Lacković, 2005), and 10 U/kg dose was chosen based on preliminary data. 5 U/kg dose in humans (350 U for a 70 kg average human) is within the dose-range regularly used for treatment of spasticity (Intiso, 2012).

For i.m. and s.c. injections rats were restrained, while for the i.n. injections animals were deeply anesthetized (chloral hydrate, Sigma, St. Louis, MO, USA; 300 mg/kg intraperitoneally). Sciatic nerve was exposed after skin incision at mid-femoral level and blunt dissection through the thigh muscles. Special care was made to check for possible leakage by placing piece of parafilm under the nerve prior to i.n. injection. 0–10 μl Hamilton needle (Hamilton, Bonaduz, Switzerland) was used to inject saline/colchicine and BTX-A into the nerves. 3 min following the treatment, parafilm was removed, the nerve returned to previous position and the skin sutured. After the operation animals were left to recover from anesthesia under warm bulb light and returned to their cages.

2.3. Immunohistochemistry

Animal preparation and immunohistochemistry was performed similarly as previously described (Matak et al., 2011; Antonucci et al., 2008). In brief, 5 days after BTX-A s.c. and i.m. injections or 3 days following i.n. injections, rats were deeply anesthetized with chloral hydrate and transcardially perfused with 0.9% saline followed by fixative (4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS)). Lumbal spinal cords were removed, cryoprotected in sucrose (15% in fixative for 1 day and 30% in PBS for 2 days), and kept on −80 °C until further use. 40 μm spinal cord coronal sections were cut on a freezing microtome and transferred to PBS-filled wells for free floating. Following blocking in PBS-diluted 10% normal goat serum (NGS), the sections were incubated with 1: 600 anti-BTX-A-cleaved SNAP-25 rabbit polyclonal antibody (a kind gift from prof. Ornella Rossetto, University of Padua, Italy) diluted in PBS with 1% NGS, overnight at room temperature, and the following day with fluorescently-labeled secondary antibody in dark (goat anti-rabbit Alexa-Fluor 555, Invitrogen, Carlsbad, CA, USA). Sections were then blocked again and counterstained with mouse monoclonal antibody for neurons (Anti-NeuN, Millipore, Temecula, CA, USA) (1:500 dilution overnight at 4 °C), and the next day with secondary anti-mouse Alexa-Fluor 488 (Invitrogen, Carlsbad, CA, USA).

Sections were washed with PBS, mounted on glass slides with antifading agent (Fluorogel, Electron Microscopy Sciences, Hatfield, PA, USA), and visualized with fluorescence microscope equipped with appropriate filters (Olympus BX51, Olympus, Tokyo, Japan) and digital camera (Olympus DP-70). Double-label images were composed with Olympus DP Manager software, assembled with Microsoft Paint and then processed for brightness and contrast using Adobe Photoshop. We checked for the appearance of cleaved SNAP-25 immunoreactivity in 20–25 lumbal spinal cord sections from each animal. In the figures, to show the data of one experimental group, representative image from single animal was chosen.

2.4. Colocalization study

Colocalization study of spinal ventral horns was performed with confocal laser scanning microscope (Leica TCS SP2 AOBS, Leica, Wetzlar, Germany), using 488 and 543 nm lasers. L5 spinal cord sections from animals injected with 30 U/kg s.c. were blocked with donkey serum and incubated overnight at room temperature with primary antibodies for cleaved SNAP-25, and for choline acetyltransferase (Millipore, Temecula, CA, USA), produced in goat, 1:100 dilution. The next day sections were incubated with secondaries: donkey anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 546 (Invitrogen, Carlsbad, CA, USA). In second experiment sections were blocked with goat serum and incubated with anti-cleaved SNAP-25 and mouse anti-glial fibrilary acidic protein (Sigma, St. Louis, MO, USA, 1:1000) overnight at room temperature. The next day sections were incubated with secondary goat anti-mouse Alexa Fluor 546 and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA).

3. Results

Slight ipsilateral flaccidity of rat distal hind limb was visible only following the i.m. treatment (5 and 30 U/kg of BTX-A), but not following the i.n. or s.c. injections.

BTX-A-cleaved SNAP-25 immunoreactivity appeared in ipsilateral lumbal ventral horns of s.c., i.m. and i.n.- BTX-A treated animals (Figs. 1 and 2). Cleaved SNAP-25 immunoreactivity was visible around motoneuronal nuclei of lamina 9 in the form of dense, small fibers, and long neuronal processes. Following i.m.

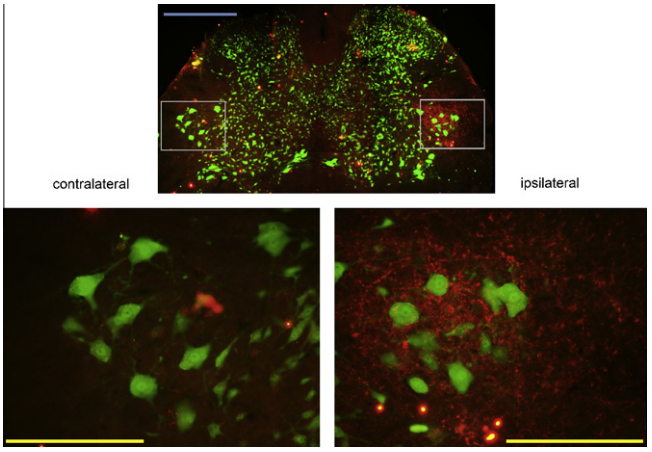

Fig. 1. Immunofluorescently labeled truncated SNAP-25 (red) in ipsilateral ventral horn of rat spinal cord (L5 segment) 5 days after subcutaneous BTX-A (30 U/kg) injection into the hind-paw pad. Green represents NeuN neuronal staining. Contralateral = ventral horn contralateral to the site of injection; ipsilateral = ipsilateral to the site of injection. Blue scale bar, 500 μm; yellow scale bar, 200 μm.

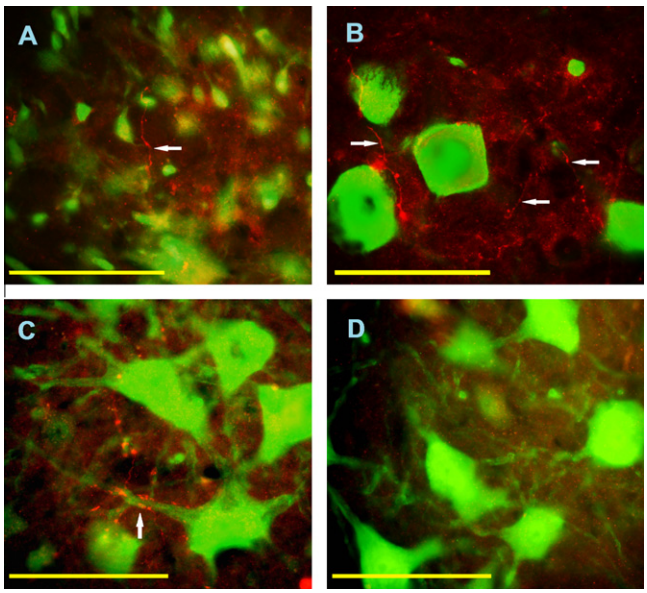

Fig. 2. Truncated SNAP-25 in ipsilateral L4 spinal cord segment. (A) Dorsal horn after intramuscular BTX-A (5 U/kg) injection into the gastrocnemius; (B) ventral horn after intramuscular BTX-A (5 U/kg) injection into the gastrocnemius; (C) ventral horn after saline and BTX-A (10 U/kg) injection into the sciatic nerve and (D) ventral horn after colchicine (5 mM) and BTX-A (10 U/kg) injection into the sciatic nerve. White arrows point to cleaved SNAP-25 fibers (red immunoreactivity). Green represents NeuN neuronal staining. Scale bar, 100 μm.

and i.n. injections, truncated SNAP-25 occurred in L4, mainly in laminas 7 and 9, while following s.c. injection, strongest immunolabeling occurred in L5 segment ventral horn lamina 9 (Fig. 1). Following intrasciatic saline + BTX-A injection at mid-thigh level, truncated SNAP-25 immunoreactivity was more widespread rostro-caudally (L3-L5).

Intramuscularly applied low-dose BTX-A (5 U/kg) also resulted in ventral horn SNAP-25 cleavage (Fig. 2B). Intensity of cleaved SNAP-25 in ventral horn following 5 U/kg i.m. injection, although not quantified, was apparently lower than after higher doses (30 U/kg i.m. and s.c.).

BTX-A enzymatic activity in the form of individual fibers appeared in ipsilateral dorsal horn even at small peripheral i.m. (5 U/kg) dose (Fig. 2A), and following higher dose i.m., i.n. and s.c. injections (not shown), thus, indicating the axonal transport in spinal sensory neurons.

Intrasciatic colchicine pretreatment abolished the cleaved SNAP-25 immunoreactivity in spinal cord of BTX-A (i.n.) treated animals, thus, demonstrating the microtubule-dependent retrograde axonal transport of BTX-A through sciatic nerve (Fig. 2C and D).

To examine the cellular localization of cleaved SNAP-25 in spinal cord motor region, we performed colocalization study with markers of cholinergic neurons and astrocytes. Cleaved SNAP-25 was found to colocalize with choline acetyl transferase (ChAT) - positive fibers (Fig. 3A). Cleaved SNAP-25 did not colocalize with glial fibrillary acidic protein (GFAP), marker of astrocytes (Fig. 3B).

4. Discussion

4.1. Occurrence of cleaved SNAP -25 in the spinal cord after BTX-A peripheral injections

Cleavage of central SNAP-25 in ipsilateral spinal cord segments after single peripheral application of BTX-A suggests the long-distance axonal traffic of enzymatically active BTX-A fragments from periphery to the spinal cord. These results extend recent findings on retrograde axonal transport of functionally active BTX-A in cranial nerves (Antonucci et al., 2008; Matak et al., 2011). Importantly, apart from cranial nerves in facial region with relatively short axons which project to the rat brainstem, this study shows BTX-A axonal transport to CNS over longer distances. Present immunohistochemical evidence of axonal transport to spinal cord is in line with previous behavioral findings, where inhibition of axonal transport in the rat sciatic nerve abolished BTX-A antinociceptive effects in a model of bilateral pain (Bach-Rojecky and Lacković, 2009).

To examine the traffic within peripheral nerves we applied BTX-A directly into the sciatic nerve. Occurrence of truncated SNAP-25 in spinal cord after i.n. injection, preventable by proximally applied

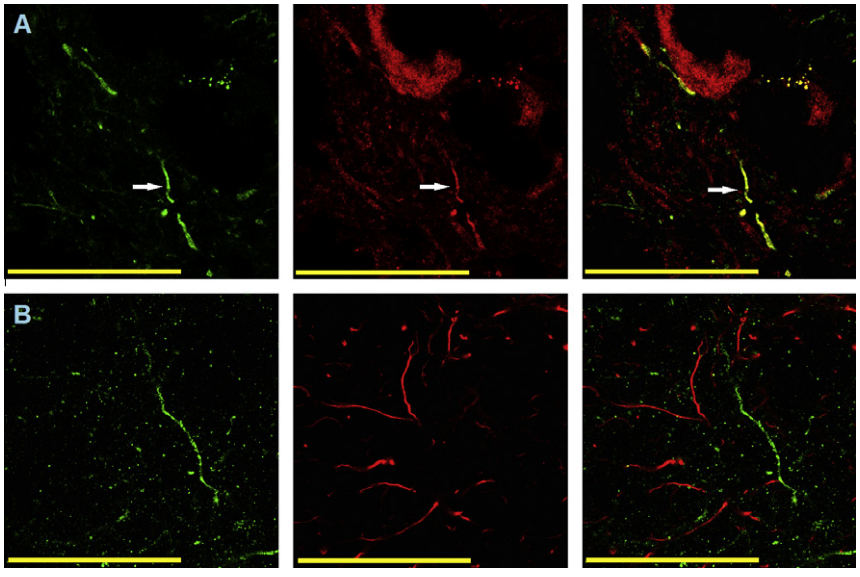

Fig. 3. Truncated SNAP-25 occurs within cholinergic neurons, but not astrocytes. (A) Confocal image of 2 μm optical section of ventral horn ipsilateral to BTX-A treatment (30 U/kg, sc.), showing colocalization of cleaved SNAP-25 (green immunofluorescence) and ChAT (red) fiber. (B) Cleaved SNAP-25 (green) did not colocalize with GFAP (red), marker of astrocytes. Scale bar, 50 μm.

colchicine, shows that BTX-A is retrogradely transported through the peripheral nerve by means of microtubule-dependent axonal transport.

The effect of intrasciatic injection of BTX-A on pain behavior was reported before (Bach-Rojecky and Lacković, 2009). How the BTX-A enters the sciatic axons is unknown. BTX-A uptake into the peripheral nerve endings is mediated by SV2C-receptor mediated endocytosis (Mahrhold et al., 2006). If the similar mechanism exists in axons is not known. Another possibility is that BTX-A diffuses into the axoplasm through damaged axons, caused by i.n. injection itself.

In this study we found that cleaved SNAP-25 was localized in ChAT- positive cholinergic fibers surrounding the motoneuronal cell bodies (Fig. 3), probably belonging to proximal dendrites of motor neurons. This finding indicates that, after axonal transport from periphery, botulinum toxin cleaves SNAP-25 most likely in primary motor neurons. But, the possibility that botulinum toxin, following transcytosis, enters cholinergic nerve terminals of neurons other than motoneurons cannot be ruled out completely. Novel study reported the cleavage of SNAP-25 in the muscles of contralateral forelimb following peripheral BTX-A forelimb injection, thus, indicating the possibility of transcytosis within the motor neurons (Torii et al., 2011).

In this study an indirect method of BTX-A detection (by cleaved SNAP-25 immunolabeling) was used. Antibody specificity to BTX-A-truncated and not to the intact SNAP-25 was previously confirmed by BTX-A injections into the rat hippocampus and SDS polyacrylamide gel electrophoresis followed by Western blot (Matak et al., 2011). Single 24 kDa band corresponding to truncated SNAP-25 was visible only in BTX-A-treated animals, and position of that band was confirmed using antibody which recognizes both cleaved and intact SNAP-25. This experiment excluded possible immunostaining due to the non-specific binding to intact SNAP-25, or due to endogenously cleaved SNAP-25 (Fig. 1 from Matak et al., 2011).

It can be argued that, instead of BTX-A, truncated SNAP-25 was transported from periphery to the spinal cord. Axonal transport of BTX-A was demonstrated previously in the visual system. Antonucci et al. (2008) injected BTX-A into the superior colliculus, and examined the occurrence of truncated SNAP-25 in the rat retina. Then, they cut the optic nerve and depleted the retina from truncated SNAP-25 by transiently active botulinum toxin E (BTX-E), which cleaves both intact and BTX-A-cleaved SNAP-25. After completion of BTX-E effects, BTX-A-cleaved SNAP-25 re-appeared in the retina, demonstrating the presence of BTX-A protease.

Low dose BTX-A (0.5 U/kg) injection into the distally cut rat sciatic nerve reduced contralateral pain in a bilateral pain model (Bach-Rojecky and Lacković, 2009), which, obviously, cannot be associated with peripheral SNAP-25 cleavage.

In sensory system, occurrence of truncated SNAP-25 in CNS seems to be associated with toxin's antinociceptive activity (Matak et al., 2011). On the other hand, significance of SNAP-25 cleavage in central motor regions remains to be investigated.

5. Conclusion

Appearance of truncated SNAP-25 in spinal cord following low dose i.m. BTX-A (5 U/kg) administration suggests that the axonal transport of toxin to CNS commonly occurs following peripheral administration.

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APPENDIX I I I

Article Matak et al. (2013).

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Comparison of analgesic effects of single versus repeated injection of botulinum toxin in orofacial formalin test in rats

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Abstract Long-term effectiveness and repeated administration of botulinum toxin A are the basis for its use in both neuromuscular disorders and certain painful conditions. Botulinum toxin A has been recently approved for migraine treatment, and its off-label use extends to other craniofacial pain disorders. However, recently it was reported that, after repeated injection, botulinum toxin loses its antinociceptive efficacy in rats. In present study with a similar design, we compared the effects of single and repeated injections of botulinum toxin in formalin-induced orofacial pain. No statistically significant differences were found between single or repeatedly treated animal groups. Our results are in line with the clinical experience and suggest that botulinum toxin can be re-administered in orofacial pain treatment.

Keywords Antinociceptive activity · Botulinum toxin A · Orofacial pain · Repeated injection

Introduction

Botulinum toxin A (BTX-A) is used in neuromuscular and autonomous disorders characterized by cholinergic hyperactivity, and in treatment of some forms of pain (Jankovic 2004; Jabbari and Machado 2011). Important for its clinical application is the long-term effectiveness and repeated use (Naumann et al. 2006). Its off-label use extends to various painful conditions, such as peripheral neuropathies, low back pain, various types of headache, etc. (Allam et al. 2005; Dodick et al. 2005; Foster et al. 2001; Jabbari and Machado 2011). BTX-A has been recently approved for chronic migraine treatment (Dodick et al. 2010).

In experimental animals, it has been shown that BTX-A exhibits long-term reduction of inflammatory acute pain, neuropathic pain, visceral and deep somatic pain (Cui et al. 2004; Bach-Rojecky and Lackovic 2005; Bach-Rojecky et al. 2005; Chuang et al. 2008; Krug et al. 2009).

In a recent report its effectiveness was demonstrated in orofacial formalin pain 8 days after the BTX-A pretreatment. However, when toxin was re-administered 42 days after the first treatment, the effect of BTX-A was not reproduced (Piovesan et al. 2011). Mentioned report that BTX-A loses its analgesic effect upon repeated injection in experimental animals is potentially very important for its clinical use in migraine and in some other chronic pain conditions, where repeated use is of high importance.

The aim of this study was to compare the effects of single or repeated injections of BTX-A in formalin-induced orofacial pain.

Materials and methods

Animals

Adult male Wistar rats (University of Zagreb School of Medicine, Croatia) weighing 300 g at the beginning of experiment were used. Animals were kept in 12 h/12 h light and dark cycle, with unlimited access to food and water. Experiment was conducted according to the European Communities Council Directive (86/609/EEC) and recommendations of the International Association for the Study of Pain (Zimmerman 1983). Animal procedures were

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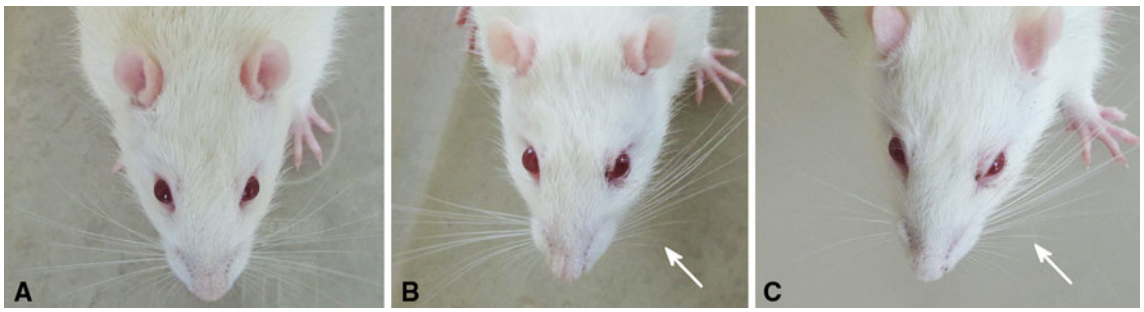

Fig. 1 Whisker appearance 1 day after administration of **a** physiological saline, **b** BTX-A first injection, **c** BTX-A second injection. Saline and BTX-A (5 U/kg) were administered to the left whisker pad

approved by the Ethics Committee of University of Zagreb School of Medicine (Permit No. 07-76/2005-43).

Drugs

100 units of Botulinum toxin type A (Botox®, Allergan Inc., Irvine, CA, USA) were dissolved in 1 ml of 0.9 % saline. 5 U/kg dose was administered unilaterally as 20 µl bolus into the whisker pad tissue using a 27 1/2 gauge needle. 20 µl of 0.9 % saline solution was injected to control rats. Facial formalin injection was carried out using 50 µl of saline-diluted 2.5 % formalin (Kemika, Zagreb, Croatia).

Treatment and behavioral testing

Three months old adult rats were divided into three groups, each consisting of five to six animals. First group of six conscious rats was injected with BTX-A into the left whisker pad tissue, while other two groups were left untreated. After 42 days, first group of rats underwent their second treatment of BTX-A, while the second and third group were injected with BTX-A and saline, respectively. Instead of using two control groups (single and double injection of saline), to reduce the number of animals we used a single control group pretreated only once with saline. In our published experiments, double versus single pretreatment with saline was not found to affect the painful response or BTX-A efficacy (Filipović et al. 2012; Bach-Rojecky and Lacković 2009; Matak et al. 2011). Period of 42 days between the two BTX-A injections was chosen based on the study of Piovesan et al. (2011).

After a period of 6 days, orofacial formalin test was performed to assess the antinociceptive activity of BTX-A. Animals were placed inside transparent chambers for 10-min acclimatization period. Following the acclimatization, conscious, restrained rats were injected with formalin into the whisker pad ipsilateral to BTX-A pretreatment and immediately returned to the transparent chamber for a 45-min observation period. Observers were blind to the animal treatment. Observation period was divided into 15 blocks of 3 min, and the number of seconds the animal spent in ipsilateral face rubbing or grooming was measured during phase I (0–12 min) and phase II (12–45 min) of formalin-induced pain (Raboisson and Dallel 2004).

Statistical analysis

Data were represented as mean ± SEM, and analyzed by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$).

Results

Injection of BTX-A into whisker pad resulted in reduced movement of ipsilateral whiskers and their backward direction (Fig. 1). In group receiving two injections of BTX-A no visible reduction of whisker movement was visible 42 days after the first treatment (prior to second BTX-A injection). Whisker paralysis after second BTX-A injection occurred again, as proof of efficiency of re-administered BTX-A (Fig. 1c).

In orofacial formalin test, phase I of rubbing/grooming behavior was not affected by BTX-A treatment, while phase II in both BTX-A treated groups revealed significant reduction in number of seconds spent rubbing/grooming, in comparison with control (Fig. 2). There was no statistically significant difference in time of facial grooming between the animals administered once or twice with BTX-A (Fig. 2).

Discussion

According to experimental results recently published by Piovesan et al. (2011), BTX-A injections 8 days before formalin test inhibit orofacial pain. However, repeated treatment after 42 days had no effect. This observation could have far reaching clinical consequences because it

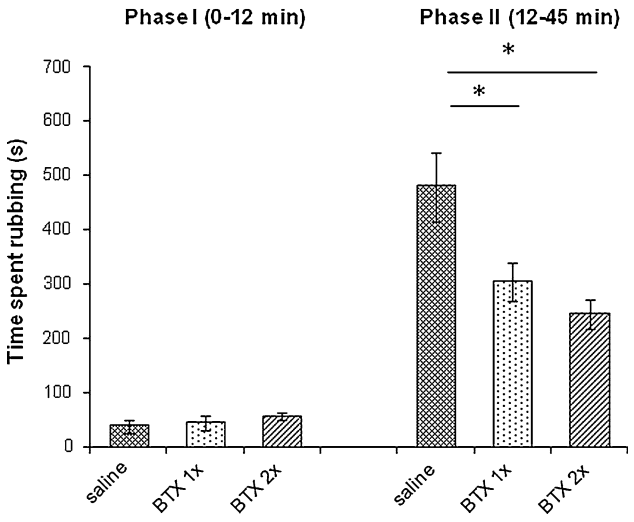

Fig. 2 Repeated BTX-A reduces orofacial pain induced by 2.5 % formalin injection into the whisker pad. Animals were injected into ipsilateral whisker pad one time (BTX 1x) or two times (BTX 2x) with 5 U/kg BTX-A (42-day period between the two injections). Nociceptive testing was performed 6 days following the single or second, repeated injection of BTX-A. Data are represented as mean ± SEM, $*p < 0.05$ (Tukey's HSD test)

questions the usefulness of repeated injections of BTX-A in pain conditions. Basically, it is also intriguing because it might suggest that, after single injection, BTX-A makes permanent functional changes. Such putative changes might be connected either with mechanism of its uptake by peripheral sensory nerves mediated by SV2 proteins (Mahrhold et al. 2006), or with the mechanism of antinociceptive action, which is connected with axonal transport from periphery to central sensory nuclei, and likely with central SNAP-25 cleavage (Bach-Rojecky et al. 2009; Matak et al. 2011; Filipović et al. 2012). It might also suggest immunoresistance to BTX-A or possibly yet unknown long-term effect on synaptic plasticity caused by BTX-A.

However, in contrast to the results from Piovesan et al. (2011), both single and repeated injection of BTX-A were equally effective in our study, which is in line with the clinical experience in pain treatment. It also suggests that BTX-A does not induce permanent functional changes that could lead to ineffectiveness of repeated injection. The presence of ipsilateral whisker paralysis after second injection of BTX-A verifies that no immune resistance towards BTX-A occurred and the second bolus showed equal effectiveness on neuromuscular junction as in animals that were injected only once, which is in agreement with the repeated clinical use in muscular disorders (Naumann et al. 2006).

There are few basic differences between the two studies performed. Adult rats of the same age were used in our experiment, whereas the animals used in study from Piovesan et al. (2011) were juvenile when treated with BTX-A and tested with formalin for the first time. In the aforementioned study, formalin was applied twice to the same experimental animals. Cellular protein precipitation and possible permanent peripheral nerve ending damage in rats treated with formalin upon the first nociceptive testing might have interfered with second nociceptive testing or BTX-A uptake into the sensory neurons after second injection. Therefore, in our study, the formalin test was conducted only once in all experimental groups. As described in previous literature phase I of the formalin test, caused by direct stimulation of nerve endings with formalin, is measured for the first 12 min of the test, while phase II represents central sensitization induced by peripheral nerve activity and is measured for the next 33 min (12–45 min) (Raboisson and Dallel 2004). In our experiments, formalin-induced pain was measured during the whole period of 45 min while Piovesan et al. (2011) measured pain for 30 min only.

Our results did not confirm recent preclinical study from Piovesan et al. (2011), and suggest that BTX-A can be re-administered in orofacial pain and probably in BTX-A treatment of other forms of pain.

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APPENDIX IV

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Botulinum toxin type A selectivity for certain types of pain is associated with capsaicin-sensitive neurons

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ABSTRACT

Unlike most classical analgesics, botulinum toxin type A (BoNT/A) does not alter acute nociceptive thresholds, and shows selectivity primarily for allodynic and hyperalgesic responses in certain pain conditions. We hypothesized that this phenomenon might be explained by characterizing the sensory neurons targeted by BoNT/A in the central nervous system after its axonal transport. BoNT/A's central antinociceptive activity following its application into the rat whisker pad was examined in trigeminal nucleus caudalis (TNC) and higher-level nociceptive brain areas using BoNT/A-cleaved synaptosomal-associated protein 25 (SNAP-25) and c-Fos immunohistochemistry. Occurrence of cleaved SNAP-25 in TNC was examined after nonselective ganglion ablation with formalin or selective denervation of capsaicin-sensitive (vanilloid receptor-1 or TRPV1-expressing) neurons, and in relation to different cellular and neuronal markers. Regional c-Fos activation and effect of TRPV1-expressing afferent denervation on toxin's antinociceptive action were studied in formalin-induced orofacial pain.

BoNT/A-cleaved SNAP-25 was observed in TNC, but not in higher-level nociceptive nuclei. Cleaved SNAP-25 in TNC disappeared after formalin-induced trigeminal ganglion ablation or capsaicin-induced sensory denervation. Occurrence of cleaved SNAP-25 in TNC and BoNT/A antinociceptive activity in formalin-induced orofacial pain were prevented by denervation with capsaicin. Cleaved SNAP-25 localization demonstrated toxin's presynaptic activity in TRPV1-expressing neurons. BoNT/A reduced the c-Fos activation in TNC, locus coeruleus, and periaqueductal gray.

Present experiments suggest that BoNT/A alters the nociceptive transmission at the central synapse of primary afferents. Targeting of TRPV1-expressing neurons might be associated with observed selectivity of BoNT/A action only in certain types of pain.

1. Introduction

Botulinum toxin type A (BoNT/A) proteolytically cleaves synaptosomal-associated protein 25 (SNAP-25), part of the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex involved in vesicular neurotransmitter release [13,31]. Subsequent prevention of SNARE-mediated neurotransmitter release mediates BoNT/A's toxicity in botulism and its therapeutic effects associated with hyperactive neuromuscular and autonomic cholinergic synapses. Small amounts of peripherally applied BoNT/A are used for treatment of different painful disorders (review by Jabbari and Machado [30]). In the craniofacial region, BoNT/A was approved for chronic migraine treatment [17]. Off-label BoNT/A use may be beneficial in other craniofacial painful disorders, such as temporomandibular joint disorders and trigeminal neuralgia [23,67].

Based on the preclinical model of formalin-induced pain [16], it was suggested that BoNT/A reduces both pain and inflammation by preventing local neurotransmitter release from peripheral sensory nerves [2]. However, further studies questioned the association of BoNT/A antinociceptive activity with its antiinflammatory effects. At BoNT/A doses that reduced carrageenan- and capsaicin-induced pain, no significant antiinflammatory effects were observed [4,5,20]. Central antinociceptive activity has been suggested by contralateral BoNT/A effects in experimental bilateral pain after unilateral toxin injection [6,8,20,68,69]. Blockage of axonal

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transport within sciatic and trigeminal nerve with colchicine prevented the antinociceptive activity of peripherally applied toxin [6,21,39]. BoNT/A-induced SNAP-25 cleavage was demonstrated immunohistochemically in trigeminal nucleus caudalis (TNC) and lumbar dorsal horn [37,39,40]. These observations demonstrated that the BoNT/A's antinociceptive effects are dependent on toxin's axonal transport within sensory neurons directed to central nociceptive regions.

BoNT/A is not active in all forms of pain and does not alter normal acute sensory thresholds [5,7,14,16]. We hypothesized that the antinociceptive effects of BoNT/A might be mediated by capsaicin-sensitive transient receptor potential vanilloid 1 (TRPV1)-expressing neurons, since this type of neuron does not convey acute responses to innocuous or noxious stimuli [12,44]. Therefore, we studied formalin-induced hypersensitivity and the occurrence of cleaved SNAP-25 in TNC after peripheral BoNT/A alone or in combination with capsaicin-induced desensitization. We found that the BoNT/A's antinociceptive action and the occurrence of cleaved SNAP-25 in central nociceptive regions are both associated with capsaicin-sensitive primary afferents, which is consistent with the reduction of hyperalgesia and allodynia by BoNT/A, and the lack of its effects on acute mechanical sensitivity.

2. Methods

2.1. Animals

Adult male Wistar rats (Department of Pharmacology, University of Zagreb School of Medicine, Zagreb, Croatia) weighing 300–400 g (12-hour day/night cycle, free access to food and water) were used in all experiments. Experiments were performed according to the 2010/63/EU Directive on the protection of animals used for scientific purposes and recommendations of the International Association for the Study of Pain [71], and approved by the Ethical Committee of University of Zagreb School of Medicine (permit no. 07-76/2005-43).

2.2. BoNT/A injections

Conscious, restrained animals were injected subcutaneously into the whisker pad with 20 μL of 0.9% saline-diluted BoNT/A (Botox; Allergan Inc, Irvine, CA, USA), using a 27 ½-gauge needle. Five- and 15-U/kg doses were chosen based on previous experiments [39,41]. One unit (1 U) of BoNT/A preparation contains 48 pg of purified *Clostridium botulinum* neurotoxin type A complex.

2.3. Intraganglionic denervation of trigeminal nerve with formalin and capsaicin

To study the occurrence of cleaved SNAP-25 in trigeminal central afferent terminals, rats were injected into the whisker pad with 15 U/kg BoNT/A, and formalin was injected intraganglionically (i.g.) 5 days *after* peripheral BoNT/A delivery (sufficient period for cleaved SNAP-25 occurrence in the central nervous system (CNS) [39]). Anesthetized animals (chloral hydrate, 300 mg/kg) were administered slowly (~1 μL/min) with 10 μL formalin (37% aqueous solution of formaldehyde) (Formalin; Kemika, Zagreb, Croatia) into the trigeminal ganglion with a Hamilton syringe, using a percutaneous infraorbital approach [39,45]. Animals were deeply anesthetized and perfused for immunohistochemistry 5 days post formalin-induced denervation (10 days after peripheral BoNT/A).

A procedure similar to the formalin-induced denervation was used to investigate the possible truncated SNAP-25 occurrence in capsaicin-sensitive central afferent terminals. Anesthetized animals (chloral hydrate, 300 mg/kg) were administered percutaneously into the trigeminal ganglion (~1 μL/min) with 2 injections of 10 μL 2% capsaicin (Sigma, St. Louis, MO, USA) or vehicle (0.9% saline + 10% ethanol + 10% Tween-80), separated by 48 hours. First injection of capsaicin was administered i.g. 5 days after BoNT/A (15 U/kg) peripheral treatment. In comparison to 0.5% and 1% doses of capsaicin, which evoked gradual recovery of eye-wipe response within 1 week, 2% capsaicin was chosen for further experiments due to the long-term loss of response (monitored up to 12 days after denervation). Animals were sacrificed 10 days post peripheral BoNT/A (3 days post second capsaicin i.g. injection).

We examined whether the occurrence of BoNT/A enzymatic activity in TNC is dependent on capsaicin-sensitive neurons. In a separate experiment, the denervation of TRPV1-expressing primary sensory neurons was performed *before* the peripheral BoNT/A injection. Animals were subjected to chemical denervation with 2% capsaicin 5 and 3 days prior to BoNT/A (15 U/kg) treatment, and sacrificed by perfusion 5 days post peripheral BoNT/A.

2.4. Behavioral assessment of the effects of trigeminal primary afferent denervation

We assessed the effects of trigeminal denervation procedures on the animal response to mechanical innocuous and noxious stimuli, as well as TRPV1-sensitive sensory function.

Measurements were performed 3–4 days following the trigeminal ganglion ablation with formalin or desensitization of TRPV1-expressing neurons with capsaicin. Prior to behavioral measurements, rats were allowed to accommodate to testing cage environment until normal sniffing/no locomotion posture was assumed. The observer was blinded to the animal treatment.

Whisker pad mechanical or nociceptive sensitivity was first monitored with Von Frey filaments (2 and 8 g bending forces), and then followed by pinprick test (5–10-minute interval between each stimulus). Von Frey filament-bending forces (2 and 8 g) were chosen based on the preliminary experiment with a series of Von Frey filaments (1–15 g) in intact animals. Within the 2 to 8 g range, the filaments elicited a nonpainful response in all control animals (nonaversive behavior, few animals reacted by slow head withdrawal). Von Frey filaments with bending forces higher than 8 g (10 and 15 g) elicited head deflection (filament-bending force was stronger than the rat neck muscles). Pinprick test was employed by using a sterile 27 ½-gauge needle pressed gently against the whisker pad without penetrating the dermis.

Response to innocuous and nociceptive mechanical stimuli in the facial area was quantified by using a semiquantitative behavioral scoring paradigm, originally devised and described in detail by Vos et al. [65]. Aversive behavior was quantified by the following descriptive categories: (1) no response; (2) nonaversive response; (3) mild-aversive response; (4) strong aversive response; (5) prolonged aversive behavior, which consists of a sum of following response elements: (a) detection (exploratory/sniffing behavior directed to stimulating object), (b) withdrawal (animal slowly moves head away from stimulating object), (c) escape/attack (avoids further contact/biting and grabbing movement towards stimulation object), (d) facial grooming (3 or more asymmetric grooming movements).

Each descriptive category, based on sum of present response elements, was assigned a score [65]:

0 = no response (no detection);
1 = nonaversive response (detection);
2 = mild-aversive response (detection + withdrawal);
3 = strong aversive response (detection + withdrawal + escape/attack);
4 = prolonged aversive behavior (detection + withdrawal + escape/attack + facial grooming).

Corneal reflex was employed to check for the normal sensitivity of corneal surface to tactile stimuli prior to capsaicin eye-wipe test. Corneal reflex was examined bilaterally by briefly applying a tipped sterile cotton wisp to the cornea, which elicited a blinking response. To prevent the visual contact-evoked reaction, the rat's head was approached by the experimenter's hand from the posterolateral side, and the cotton tip was gently applied to the cornea across the lateral eye corner. The cotton tip was applied 5 times (>30-second interval between consecutive applications), and the percentage of elicited blinking responses was used as a measure of behavioral response.

Capsaicin eye-wipe test was used to examine the sensory function of TRPV1-expressing trigeminal neurons. A small drop (~10 μL) of saline-diluted 0.01% capsaicin was released on the corneal surface, and the number of ipsilateral eye wipes was counted [15,45]. TRPV1-expressing neurons are considered to be desensitized if the wiping response is greatly reduced or prevented [15,45].

To study the possible role of TRPV1-expressing sensory neurons in BoNT/A antinociceptive activity, the effect of BoNT/A on orofacial formalin test was examined in animals desensitized with i.g. capsaicin. Four days after the completion of capsaicin i.g.-induced desensitization (2 injections within 24 hours), animals were injected into the whisker pad with saline/5 U/kg BoNT/A. Orofacial formalin test was performed 5–6 days after peripheral saline/BoNT/A treatment. Formalin test was employed as described previously [39,41,51]. Animals were injected into the whisker pad with 50 μL of saline-diluted 2.5% formalin and observed for 45 minutes in a transparent cage. Total duration of ipsilateral facial rubbing and grooming evoked by facial formalin was assessed during 3-minute periods divided into phase I (0–12 minutes) and phase II (12–45 minutes). Observer was blinded to the animal treatment.

2.5. Immunohistochemistry of cleaved SNAP-25 in the brain

For the assessment of cleaved SNAP-25 localization, animals were injected with BoNT/A subcutaneously into the whisker pad, and sacrificed after 5–6 days. Apart from TNC, possible occurrence of cleaved SNAP-25 was studied in thalamus, hypothalamus, sensory cortex, locus coeruleus, and periaqueductal gray. Since Marinelli et al. [37] reported the occurrence of cleaved SNAP-25 in lumbar spinal astrocytes of neuropathic mice, we examined the colocalization of cleaved SNAP-25 with marker of astrocytes in animals with trigeminal neuropathy induced by infraorbital nerve constriction, as previously described [21].

Anesthetized animals (chloral hydrate 300 mg/kg intraperitoneal) were perfused transcardially with saline, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brain tissue was excised, cryoprotected with sucrose, and kept at −80 °C as previously described [39,40].

Cryostat-cut 40-μm coronal sections of brainstem and diencephalon were collected for free floating in PBS with 0.25% Triton X-100 (PBST), washed and blocked with 10% normal goat serum (NGS) in PBST. Sections were incubated overnight at room temperature in 1% NGS with 1:1500 rabbit polyclonal antibody to cleaved SNAP-25 (produced by O. Rossetto), which was previously well characterized and recognizes specifically the BoNT/A-truncated SNAP-25 [39]. The following day, the sections were incubated with fluorescent secondary antibody (goat anti-rabbit Alexa Fluor 555; Molecular Probes, Invitrogen, Carlsbad, CA, USA). The tissue was then incubated overnight at 4 °C with mouse monoclonal antibodies to synaptophysin (1:500, Sigma), microtubule-associated protein 2 (MAP-2) (1:1000, Sigma), glial fibrillary acidic protein (GFAP) (1:1000, Sigma), and neuronal nuclear protein (NeuN) (1:500, Millipore, Temecula, CA, USA). The next day, the sections were incubated with goat anti-mouse Alexa fluor 488. Co-staining of cleaved SNAP-25 and TRPV1 was performed with goat anti-vanilloid receptor 1 (TRPV1) polyclonal antibody (1:400, Santa Cruz Biotechnology, Dallas, TX, USA), and donkey anti-rabbit Alexa 488/donkey anti goat Alexa 555 secondary antibodies.

Co-staining of cleaved SNAP-25 with calcitonin gene-related peptide (CGRP) was performed with rabbit polyclonal anti-CGRP (Sigma). To prevent the cross-reactivity of primary antibodies raised in rabbit, a modified antibody elution procedure was used [48]. In brief, after incubation with antibodies to cleaved SNAP-25 and secondary goat anti-rabbit Alexa 555, sections were washed, transferred to Superfrost Plus glass slides (Thermo Fisher Scientific Inc, Waltham, MA, USA), and allowed to adhere and dry. Cleaved SNAP-25 immunoreactivity was photographed in glycerol-coverslipped slides for later comparison. Coverslips were then removed. Slides were washed in PBS and incubated in darkness in preheated acidic elution buffer (50 °C, pH = 2) containing 1% sodium dodecyl sulfate and 25 mM glycine for 30 minutes without shaking. After elution, sections were blocked again and incubated overnight at 4 °C with CGRP antibody (1:5000). Cross-reactivity controls were incubated with 1% NGS. The next day, the sections were incubated with goat anti-rabbit Alexa Fluor 488. In cross-reactivity controls (omitted CGRP antibody), no binding of Alexa fluor 488-labeled secondary antibody was observed. Morphology of cleaved SNAP-25 fibers before elution and after completed immunostaining remained the same.

In studies involving cleaved SNAP-25 immunostaining, sections from 3–4 animals per group (15–25 sections/animal) were examined. Immunostained sections were visualized with an Olympus BX-51 epifluorescent microscope coupled to DP-70 digital camera (Olympus, Tokyo, Japan) or TCS SP2 AOBS confocal microscope (Leica, Wetzlar, Germany). Double-label images were composed using cellSens Dimension software (Olympus). Images were processed for brightness and contrast with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA).

2.6. C-Fos immunohistochemistry after orofacial formalin test

BoNT/A effects on neural activation evoked by orofacial formalin were assessed by quantifying the c-Fos expression in different brain regions of animals injected with 5 U/kg BoNT/A or saline. Immunohistochemical staining for c-Fos was performed on coronal sections from caudal medulla, pons, mesencephalon, and diencephalon, using rabbit anti-c-Fos primary antibody (dilution 1:500, incubation overnight at room temperature; Santa Cruz Biotechnology) and goat anti-rabbit Alexa Fluor 488 fluorescent secondary antibody.

Immunostained sections were visualized with Olympus BX-51 fluorescent microscope coupled to DP-70 digital camera (Olympus). C-Fos-positive neuronal fluorescent profiles were automatically counted using cellSens Dimension software (Olympus). In each region, c-Fos-positive profiles were counted from 4 randomly selected sections per animal. Brain regions were indentified in coronal sections using the rat stereotaxic atlas [47] and appropriate landmarks for each region (central canal, obex, aqueduct, ventricles, etc.).

2.7. Immunohistochemistry of CGRP-expressing central afferent terminals and brainstem neurons after trigeminal ganglion denervation

Denervation of primary afferents in the TNC after formalin-induced ablation of trigeminal ganglion was verified using the immunohistochemistry of CGRP, which is present in central afferent terminals [25]. Since approximately 70% of the CGRP-expressing trigeminal sensory neurons are TRPV1 positive [50], we checked for the reduced CGRP expression after desensitization of capsaicin-sensitive primary afferents.

Ipsilateral and contralateral TNC of each coronal section were visualized with epifluorescent microscope by employing the low-magnification objective (4×) to obtain microphotographs containing the entire TNC region. Images were processed using cellSens Dimension software. Surface area of TNC containing green CGRP immunoreactivity was quantified by using green channel pixel thresholding. To quantify the extent of degeneration, the surface area of the ipsilateral, denervated side was divided by the surface area of the contralateral side, which served as a control.

To assess the possible postsynaptic degeneration of central neurons in the TNC region after i.g. treatment with formalin or capsaicin, NeuN and dendritic (MAP-2) staining was performed.

2.8. Statistical analysis

Parametric data were represented as mean ± SEM, and analyzed by unpaired *t*-test (for comparison between 2 groups) or one-way analysis of variance followed by Newman-Keuls post hoc test (multiple group comparisons). Nonparametric data (response scores of aversive behavior to mechanical stimuli) were represented by scatter plot and median, and analyzed by Kruskal-Wallis test, followed by Dunn's post hoc. $P < 0.05$ was considered significant.

3. Results

3.1. Intraganglionic denervation of trigeminal nerve with formalin and capsaicin

3.1.1. Behavioral effects of trigeminal primary afferent denervation

The animals injected with i.g. formalin showed no response to the ipsilateral whisker pad stimulation with Von Frey filaments, independently of the filament-bending force (2 or 8 g) (Fig. 1A, B). In addition, formalin i.g.-treated animals did not respond to the pinprick test ipsilaterally to formalin-induced ablation (Fig. 1C). Ipsilateral response to capsaicin eye-wipe test in i.g. formalin-treated animals was abolished (Fig. 2). Corneal reflex (blinking response to cotton whip stimulation of cornea) was almost completely prevented (not shown). Contralaterally, the animals responded to whisker pad and corneal mechanical stimulation similarly to control animals (not shown). In addition, capsaicin-evoked eye-wipe response was preserved on the nondenervated side (not shown). Formalin is a chemical fixative that immediately kills the living cells by cross-linking of biological molecules and protein precipitation [57]. In line with that, insensitivity to mechanical and capsaicin-induced stimulation after i.g. formalin suggested a nonselective denervation of trigeminal primary afferents.

Acute mechanical sensitivity was unaltered after i.g. capsaicin-evoked desensitization. Capsaicin i.g.-treated animals showed nonaversive response to whisker pad mechanical stimulation with Von Frey filaments (Fig. 1A, B), responded to noxious pinprick stimulus with strong aversive behavior (Fig. 1C), and exhibited 100% preserved corneal reflex response, similarly to vehicle-treated animals (not shown). Facial BoNT/A pretreatment did not significantly alter the mechanical responses in either vehicle i.g. or capsaicin i.g.-treated animals.

Animals desensitized with 2% capsaicin had a largely reduced response to capsaicin eye-wipe test on the ipsilateral side (Fig. 2), in line with the effects of capsaicin-induced desensitization of TRPV1-expressing neurons [15,45,58]. On the contralateral side, animals responded similarly to vehicle-treated controls (not shown). Present data indicated that the unilateral i.g. capsaicin selectively desensitized TRPV1-expressing neurons only, without altering primary afferents that mediate the acute mechanical sensitivity.

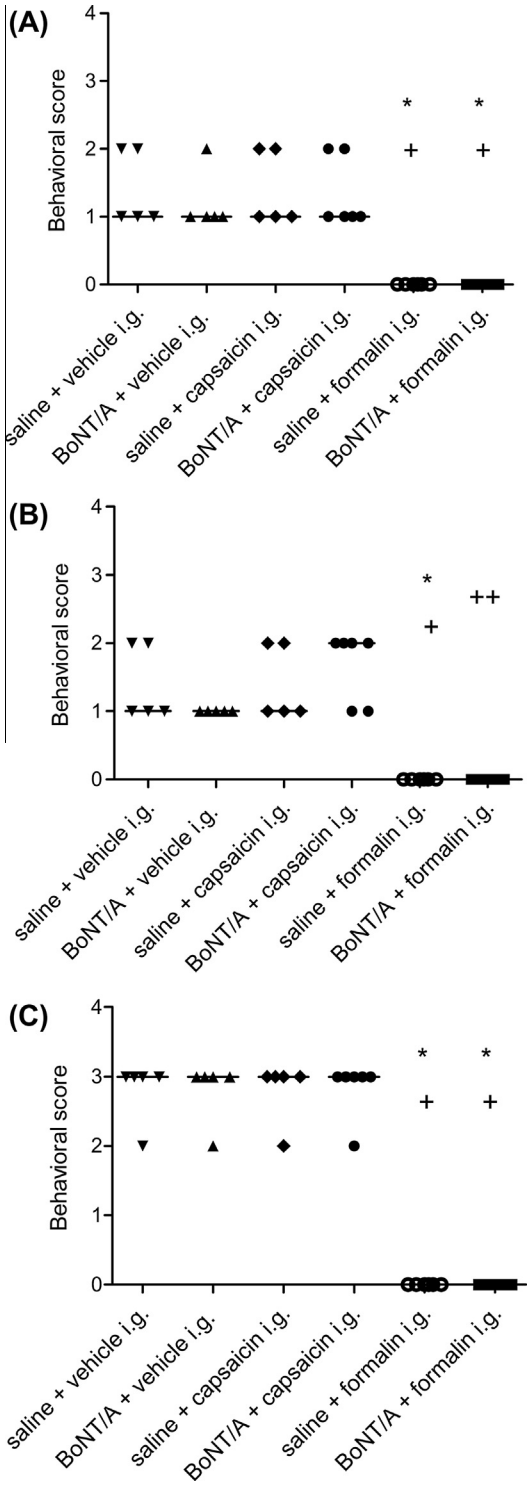

Fig. 1. Mechanical sensitivity of facial area in rats is unaltered by capsaicin-induced desensitization of transient receptor potential vanilloid 1-expressing neurons, and it is abolished after nonselective ablation of trigeminal primary afferents. Five days after the peripheral botulinum toxin type A (BoNT/A; 15 U/kg) or saline injection into the whisker pad, rats were injected intraganglionically (i.g.) with either vehicle, 2% capsaicin (double vehicle or capsaicin treatment separated 24–48 hours), or formalin (single i.g. treatment). Mechanical sensitivity of the facial area was examined 3–4 days after ganglion treatments. (A) Response to ipsilateral whisker pad stimulation with 2-g filament; (B) response to ipsilateral whisker pad stimulation with 8-g filament; (C) response to ipsilateral whisker pad pin-prick stimulation. N (animals per group) = 5–6. Behavioral scores are represented as median (horizontal line), and individual values were represented by scatter plot (dots). $^{*}P < 0.05$ in comparison to vehicle i.g.; $^{+}P < 0.05$ in comparison to capsaicin i.g.; $^{++}P < 0.01$ in comparison to capsaicin i.g. (Kruskal-Wallis test followed by Dunn's post hoc, $P < 0.05$).

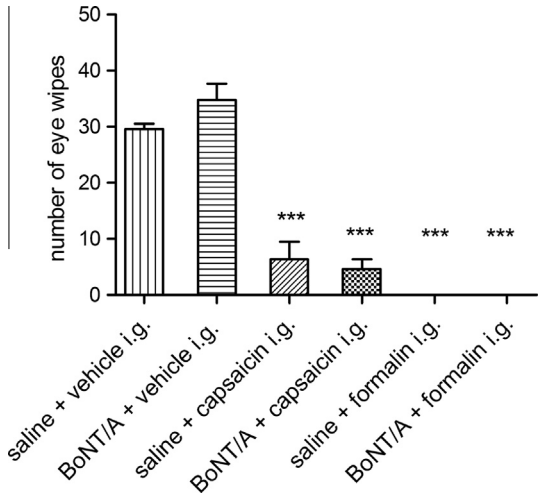

Fig. 2. Capsaicin-induced eye-wipe response after capsaicin or formalin-induced denervation of trigeminal nerve. Five days after peripheral botulinum toxin type A (BoNT/A; 15 U/kg) or saline injection into the whisker pad, rats were injected intraganglionically (i.g.) with vehicle, 2% capsaicin (double vehicle or capsaicin treatment), or formalin (single i.g. treatment). Capsaicin-evoked sensitivity of the eye corneal surface was examined 3–4 days after ganglion treatment. Eye-wipe response (number of eye wipes) was measured after ipsilateral capsaicin application to corneal surface (0,01%, 10 μL). N (animals per group) = 5–6. Results are represented as mean ± SEM. $^{***}P < 0.001$ in comparison to vehicle i.g. (one-way analysis of variance followed by Newman-Keuls post hoc, $P < 0.05$).

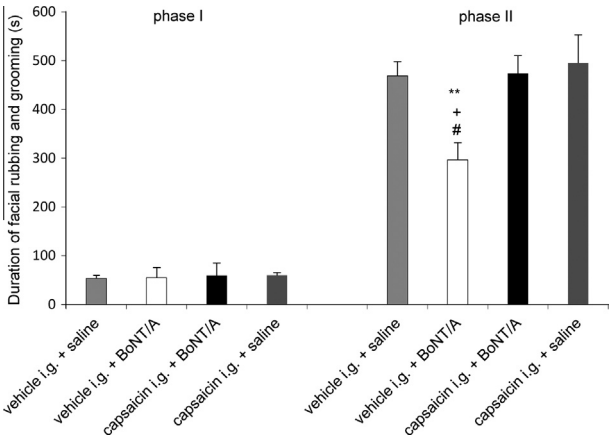

Fig. 3. Antinociceptive activity of botulinum toxin type A (BoNT/A) in orofacial formalin-induced pain is mediated by capsaicin-sensitive sensory neurons. Chemical denervation with 2% i.g. capsaicin prevents BoNT/A's antinociceptive activity in phase II of orofacial formalin-induced pain. Capsaicin/vehicle pretreatment was completed 4 days prior to peripheral saline or BoNT/A (5 U/kg) injection, and formalin test was performed 5–6 days after saline/BoNT/A injection. Number of animals per group = 4–6. Results are represented as mean ± SEM. $^{**}P < 0.01$ in comparison to vehicle control; $^{+}P < 0.05$ in comparison to capsaicin i.g. + BoNT/A; $^{\#}P < 0.05$ in comparison to capsaicin i.g.+ vehicle (one-way analysis of variance followed by Newman-Keuls post hoc, $P < 0.05$).

In animals injected i.g. with vehicle, BoNT/A reduced phase II of formalin-induced orofacial pain, whereas phase I pain was not affected, as previously described [16,39,41]. Capsaicin i.g.-induced denervation prevented the antinociceptive activity of BoNT/A in orofacial formalin-induced pain, while the denervation itself did not influence the duration of nocifensive behavior in formalin test (Fig. 3). These data suggest that the BoNT/A antinociceptive efficacy is dependent on TRPV1-expressing sensory neurons.

3.1.2. Effects of trigeminal ganglion denervation on CGRP-expressing central afferent terminals and brainstem neurons

In line with the abolished unilateral sensory response, trigeminal ganglion ablation with formalin resulted in almost complete unilateral disappearance of CGRP immunoreactivity in the TNC, which is expressed in a subpopulation of central afferent terminals (Fig. 4A).

Capsaicin-evoked denervation induced a large, but in contrast to formalin-induced denervation, incomplete, reduction of CGRP immunoreactivity (Fig. 5B, C).

Decrease of CGRP immunostaining of the ipsilateral TNC in response to i.g. capsaicin is in line with previous studies that reported reduced neuropeptide content in the dorsal horn after desensitization of capsaicin-sensitive central afferent terminals with high-dose TRPV1 agonists [22,32]. Remaining CGRP staining possibly corresponded to the peptidergic afferent population not expressing TRPV1 [50].

Quantification of CGRP immunoreactivity supports the loss of CGRP in i.g. formalin-treated animals (Supplementary Fig. 1A), and CGRP reduction in capsaicin i.g.-treated animals (Supplementary Fig. 1B). Immunostaining of dendrites (MAP-2) and cell bodies (NeuN) of brainstem neurons in the TNC was unaltered by i.g. formalin (Supplementary Fig. 2A, B). Dendritic and somatic staining of central neurons in the TNC was unaffected by i.g. capsaicin (not shown), similarly to i.g. formalin.

3.1.3. Occurrence of BoNT/A enzymatic activity in the TNC after denervation of trigeminal nerve with formalin and capsaicin

Previously, we found the occurrence of BoNT/A-cleaved SNAP-25 in the TNC after toxin injection into the whisker pad [39]. By employing the trigeminal nerve ablation we examined if the BoNT/A's enzymatic activity in TNC was located within primary afferent terminals. Formalin-induced ganglion ablation performed 5 days following BoNT/A peripheral injection induced complete disappearance of cleaved SNAP-25 staining in the TNC (Fig. 4B), indicating that the BoNT/A-cleaved SNAP-25 was located in central afferent terminals.

Double labeling of cleaved SNAP-25 and TRPV1 in TNC demonstrated the occurrence of products of BoNT/A enzymatic activity in TRPV1-expressing neurons (Fig. 5A). Animals subjected to chemical denervation with capsaicin 5 days following peripheral BoNT/A lacked the immunoreactivity for cleaved SNAP-25 in TNC (Fig. 5B), which suggests that BoNT/A enzymatic activity occurs in capsaicin-sensitive central afferent terminals.

Hypothetically, some other types of afferents, which are capsaicin insensitive, might mediate the occurrence of cleaved SNAP-25 in the TNC when the capsaicin-sensitive afferents are desensitized with capsaicin before injection of BoNT/A. However, animals subjected to i.g. capsaicin-induced denervation prior to BoNT/A injection lacked the BoNT/A-cleaved SNAP-25 in TNC (Fig. 5C), suggesting that the occurrence of BoNT/A enzymatic activity in the TNC is dependent solely on capsaicin-sensitive neurons.

3.2. Immunohistochemical localization of cleaved SNAP-25 in the brain

Cleaved SNAP-25 immunoreactivity appeared either as punctate immunoreactivity or fiber-like profiles. Punctate immunoreactivity colocalized with synaptophysin, a presynaptic marker. On the other hand, fiber-like profiles showed no colocalization with synaptophysin (Fig. 6A). Cleaved SNAP-25 was absent from MAP-2-stained dendrites of TNC neurons (Fig. 6B). In BoNT/A-injected naïve (Fig. 6C) and infraorbital nerve constriction-induced neuropathic animals (not shown), cleaved SNAP-25 was detected outside of GFAP-immunoreactive astrocytes.

Cleaved SNAP-25 mainly did not colocalize with neuropeptide CGRP, except in few neuronal terminals (Supplementary Fig. 3). After 5 U/kg peripheral BoNT/A injection, cleaved SNAP-25 was detected in TNC only, but not in other sensory regions (not shown).

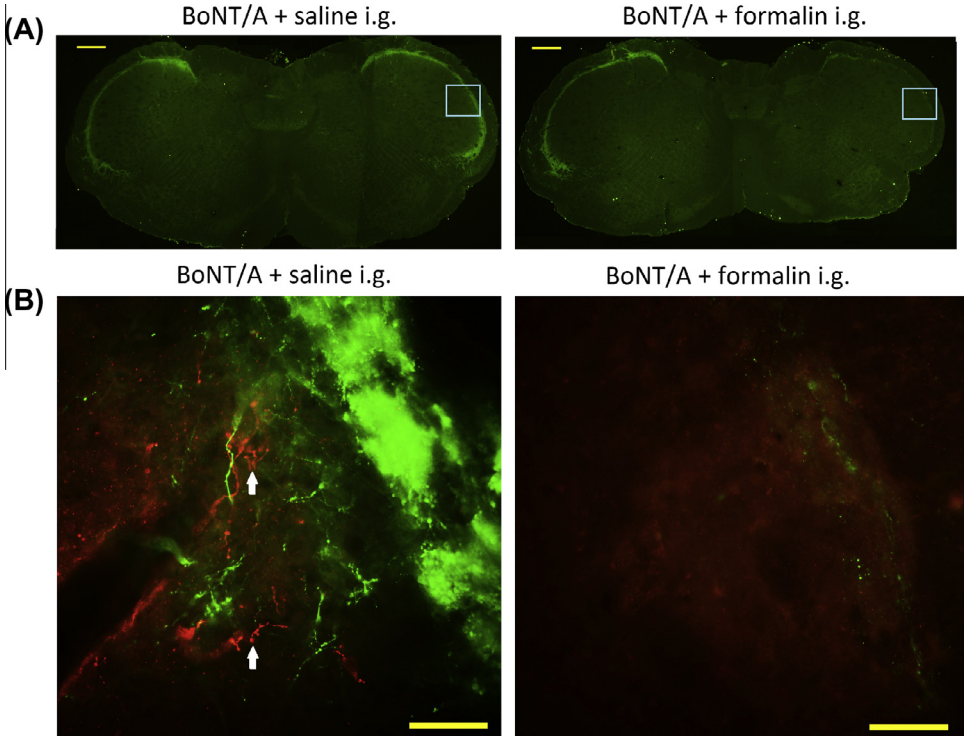

Fig. 4. Proteolytic activity of botulinum toxin type A (BoNT/A) in trigeminal nucleus caudalis (TNC) is located in central afferent terminals of primary sensory neurons. (A) Immunoreactivity for calcitonin gene-related peptide (green), marker of peptidergic primary afferents, is almost completely eliminated from TNC ipsilaterally to formalin intraganglionic (i.g.) treatment, in comparison to i.g. saline treatment (right sides of coronal sections). Scale bar = 200 μm. (B) Formalin i.g. abolishes cleaved synaptosomal-associated protein 25 (SNAP-25) in medullary dorsal horn (red immunofluorescent staining, arrows). Saline or formalin (10 μL) was administered into the trigeminal ganglion 5 days after peripheral BoNT/A injection into the whisker pad (15 U/kg). N (animals per group) = 4 (15–25 sections were examined per each animal). Scale bar = 50 μm.

3.3. BoNT/A effects on regional c-Fos expression in the orofacial formalin test

In the present study, we have examined the effect of BoNT/A on c-Fos expression in the TNC and upstream sensory regions after formalin injection into the orofacial area (Fig. 7, Table 1). Formalin-evoked c-Fos expression in TNC, locus coeruleus, periaqueductal gray, medial thalamus (paraventricular nucleus), amygdala, and hypothalamus was increased 3–9 times compared to saline controls (Table 1, middle column). Increased c-Fos expression in examined regions is in agreement with previous studies involving peripheral formalin test [11].

Similarly to previous findings in spinal cord dorsal horn [2,18,64], in the present experiment, BoNT/A lowered the pain-evoked neural activation (measured by c-Fos expression) in the TNC. Additionally, BoNT/A reduced the formalin-evoked neural activation in locus coeruleus and periaqueductal gray. BoNT/A did not affect the expression of c-Fos in paraventricular nucleus of thalamus, ipsilateral and contralateral hypothalamus, and contralateral central amygdala (Fig. 7, Table 1).

4. Discussion

In contrast to classical analgesics such as opioids, BoNT/A does not alter the acute nociceptive thresholds, but it selectively reduces the allodynic and hyperalgesic responses in certain pain conditions [5,7,14,16]. We previously discovered that the antinociceptive activity of BoNT/A is mediated by its axonal transport to central sensory nociceptive nuclei [6,21,39]. In the present study we investigated the possibility that the selectivity of BoNT/A antinociceptive action is mediated by specific subtypes of sensory neurons targeted by BoNT/A.

4.1. Enzymatic activity of BoNT/A in TNC occurs in central afferent terminals

Occurrence of cleaved SNAP-25 in TNC and lumbar dorsal horn, the regions that receive afferent nociceptive input, suggests that BoNT/A alters central nociceptive transmission [39]. However, the localization of this action in TNC was, up to now, unknown. In the present study, we examined whether BoNT/A's enzymatic activity in the TNC is located in primary sensory neurons. Loss of cleaved SNAP-25 in the TNC after formalin-induced ablation of primary afferents demonstrated that the BoNT/A enzymatic activity occurs in central primary afferent terminals (Fig. 4).

In the present experiments, we did not observe any truncated SNAP-25 remaining after ganglionic denervation, thus, our results do not support possible transcytosis to second-order synapses in the TNC. However, transcytosis of BoNT/A in rats was demonstrated after both anterograde and retrograde axonal transport in the optic system [52,53]. Recently, a decrease of spontaneous and evoked inhibitory glycinergic potentials in isolated rat lumbar substantia gelatinosa neurons following peripheral BoNT/A injection was reported [1]. The authors suggested toxin's transcytosis to glycinergic interneurons.

4.2. BoNT/A's antinociceptive activity is associated with capsaicin-sensitive neurons

After demonstrating that BoNT/A's proteolytic activity in TNC was located within central afferent terminals of trigeminal neurons, we found that the terminals involved are sensitive to capsaicin and express TRPV1 (Fig. 5A, B). Moreover, chemical denervation with i.g. capsaicin prevented the occurrence of cleaved SNAP-25 in the TNC, as well as the antinociceptive activity of

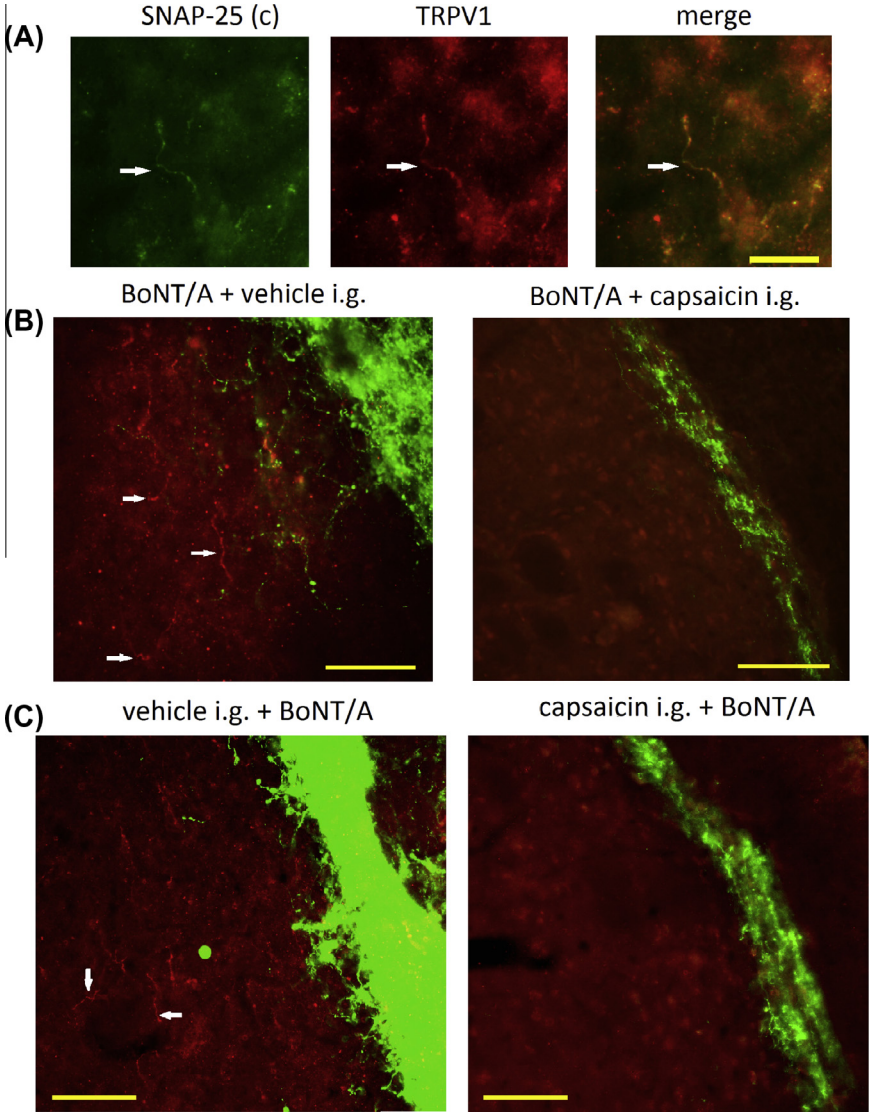

Fig. 5. Botulinum toxin type A's (BoNT/A) proteolytic activity in trigeminal nucleus caudalis (TNC) is associated with transient receptor potential vanilloid 1 (TRPV1)-expressing (capsaicin-sensitive) primary afferents. (A) Fluorescent images of cleaved synaptosomal-associated protein 25 [SNAP-25(c)] and TRPV1-double labeling in ipsilateral TNC 5 days after peripheral injection of BoNT/A (15 U/kg). Cleaved SNAP-25 immunoreactivity (green) in the dorsal horn is localized within TRPV1-expressing neurons (red). Scale bar = 20 μm. (B) Capsaicin 2% i.g. treatments performed 5 and 7 days after administration of peripheral BoNT/A (15 U/kg) eliminates cleaved SNAP-25 (red immunostaining, arrows) in the TNC and reduces calcitonin gene-related peptide (CGRP) immunostaining (green). Animals were sacrificed 10 days post BoNT/A. Scale bar = 50 μm. (C) Chemical denervation with 2% i.g. capsaicin prior to BoNT/A treatment prevents the occurrence of cleaved SNAP-25 in the TNC. Capsaicin 2%/vehicle double pretreatment was completed 3 days before BoNT/A injection (15 U/kg) into the whisker pad, and animals were sacrificed 5 days post peripheral BoNT/A. Red immunostaining represents cleaved SNAP-25 (arrows). CGRP staining (green) was lower in capsaicin i.g.-pretreated animals, in comparison to vehicle control. N (animals per group) = 3–4 (15–25 sections were examined per each animal). Scale bar = 50 μm.

BoNT/A in formalin-induced orofacial pain (Figs. 3, 5C). Mentioned experiments demonstrate that the BoNT/A's antinociceptive activity, mediated by toxin's axonal transport to CNS [6,21,39], involves capsaicin-sensitive (TRPV1-expressing) central afferent terminals.

Enzymatic activity of BoNT/A in capsaicin-sensitive neurons supports the reduction of capsaicin-evoked pain [5,24,55]. It was reported that BoNT/A reduces TRPV1 expression in peripheral sensory neurons, possibly by preventing SNARE-mediated receptor translocation to the cell membrane [3,56,69,70]. Similar effect may occur in central afferent terminals, where BoNT/A might regulate the TRPV1 receptor-mediated central nociceptive transmission.

TRPV1-expressing neurons are primarily glutamatergic [26], but might contain other transmitters such as Substance P or CGRP [9,25,32,50]. Thus, BoNT/A might prevent glutamate as well as other co-transmitters' release from a distinct set of nerve endings [19,21,29]. Recently, it was proposed that BoNT serotype B reduces spinal substance P release from TRPV1-expressing neurons in mice [38].

4.3. BoNT/A selectivity for hyperalgesia and allodynia is associated with capsaicin-sensitive neurons

Since only 16–20% of trigeminal neurons express TRPV1 [9,26,28,50], our observations might suggest a preferential targeting of BoNT/A to TRPV1-expressing central terminals in the TNC. Selective targeting of TRPV1-expressing nerve endings might explain the activity of BoNT/A in only certain types of pain. Comparison between the antinociceptive effects of BoNT/A and suppressed function of TRPV1-expressing neurons in different types of experimental acute nociceptive, inflammatory, and neuropathic pain indicates a considerable agreement of the effects of BoNT/A and TRPV1-mediated antinociceptive effects:

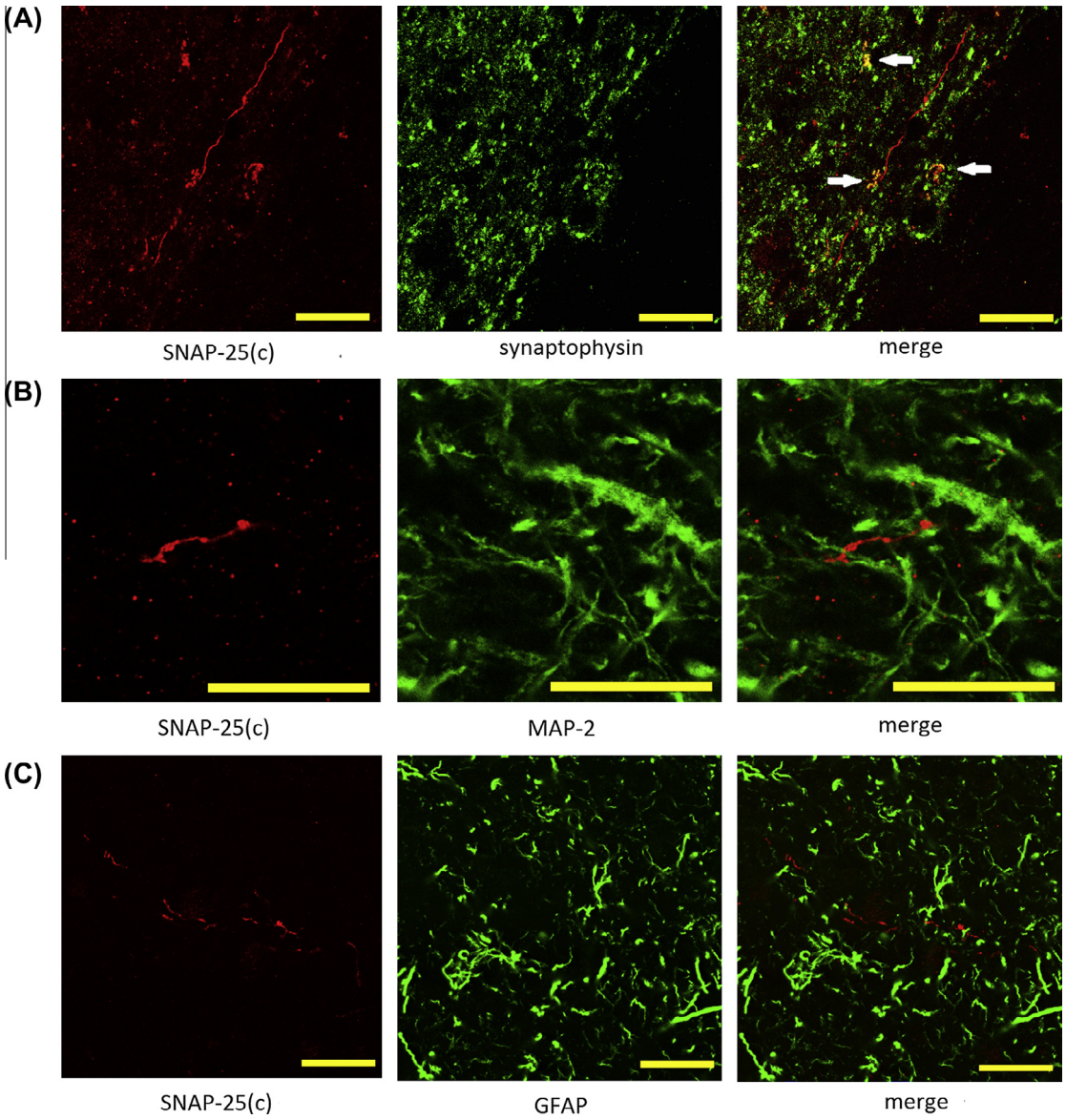

Fig. 6. Cleaved synaptosomal-associated protein 25 (SNAP-25) localization in relation to presynaptic terminals, dendrites, and astrocytes. Confocal images of ipsilateral trigeminal nucleus caudalis (TNC) 5 days after botulinum toxin type A (BoNT/A; 15 U/kg) injection into the rat whisker pad. Cleaved SNAP-25 (SNAP-25(c)-red immunofluorescence) partially colocalizes with synaptophysin (arrows), a presynaptic marker (A). Cleaved SNAP-25 did not colocalize with MAP-2, marker of dendrites (B), and glial fibrillary acidic protein (GFAP), marker of astrocytes (C). Images are representative of confocal microphotographs obtained from 4 animals. Scale bars = 20 μm.

- BoNT/A and suppression of TRPV1-expressing neurons (evoked by denervation of TRPV1-expressing neurons, or TRPV1 antagonists) do not affect acute mechanical thresholds [5,7,14,16,32,43,45,59]. In the present study, we observed preserved acute mechanical sensitivity upon either BoNT/A treatment or denervation of capsaicin-sensitive primary afferents (Fig. 1). Transmission of acute mechanical stimuli by neurons that are not capsaicin-sensitive might explain the lack of effect of BoNT/A on acute innocuous or nociceptive mechanical thresholds.
- BoNT/A, denervation of TRPV1-expressing neurons, and TRPV1 agonists, reduce the nocifensive behavior and mechanical hyperalgesia evoked by capsaicin [4,5,24,32,49,55,59], and thermal hyperalgesia evoked by inflammatory or neuropathic pain [4,5,7,15,34,36,49,56,59,60,66].
- BoNT/A and TRPV1 antagonists reduce the inflammatory and neuropathic mechanical allodynia and hyperalgesia [7,18,21, 34,46,49,66]. The results are ambiguous after denervation with high-dose TRPV1 agonists: some studies report the reduction of mechanical allodynia [36,60], while others do not [35].
- BoNT/A and TRPV1 receptor antagonists reduce formalin-induced pain [16,18,33,39,41,59,63]. However, in present experiments, 2.5% formalin-induced nocifensive response was unaltered by i.g. capsaicin (Fig. 5). This is in accordance with a recent similar study employing i.g. resiniferatoxin (a more potent capsaicin analog) and 2.5% orofacial formalin [15]. Effect of desensitization of TRPV1-expressing neurons on the duration of formalin-evoked nociceptive behavior in mice was shown to be dependent on formalin concentration [54]. While intrathecal capsaicin reduced the 0.5% formalin-evoked behavior, it did not reduce the behavior evoked by higher formalin dose (2%) [54]. Unaltered response to formalin test might be associated with central plastic changes occurring after denervation of TRPV1-expressing afferents, such as the abnormally increased receptive fields of dorsal horn neurons [42]. Another theoretical possibility is that the denervation of TRPV1-expressing neurons might result in compensatory nociceptive activation of other primary afferent types in the formalin test.

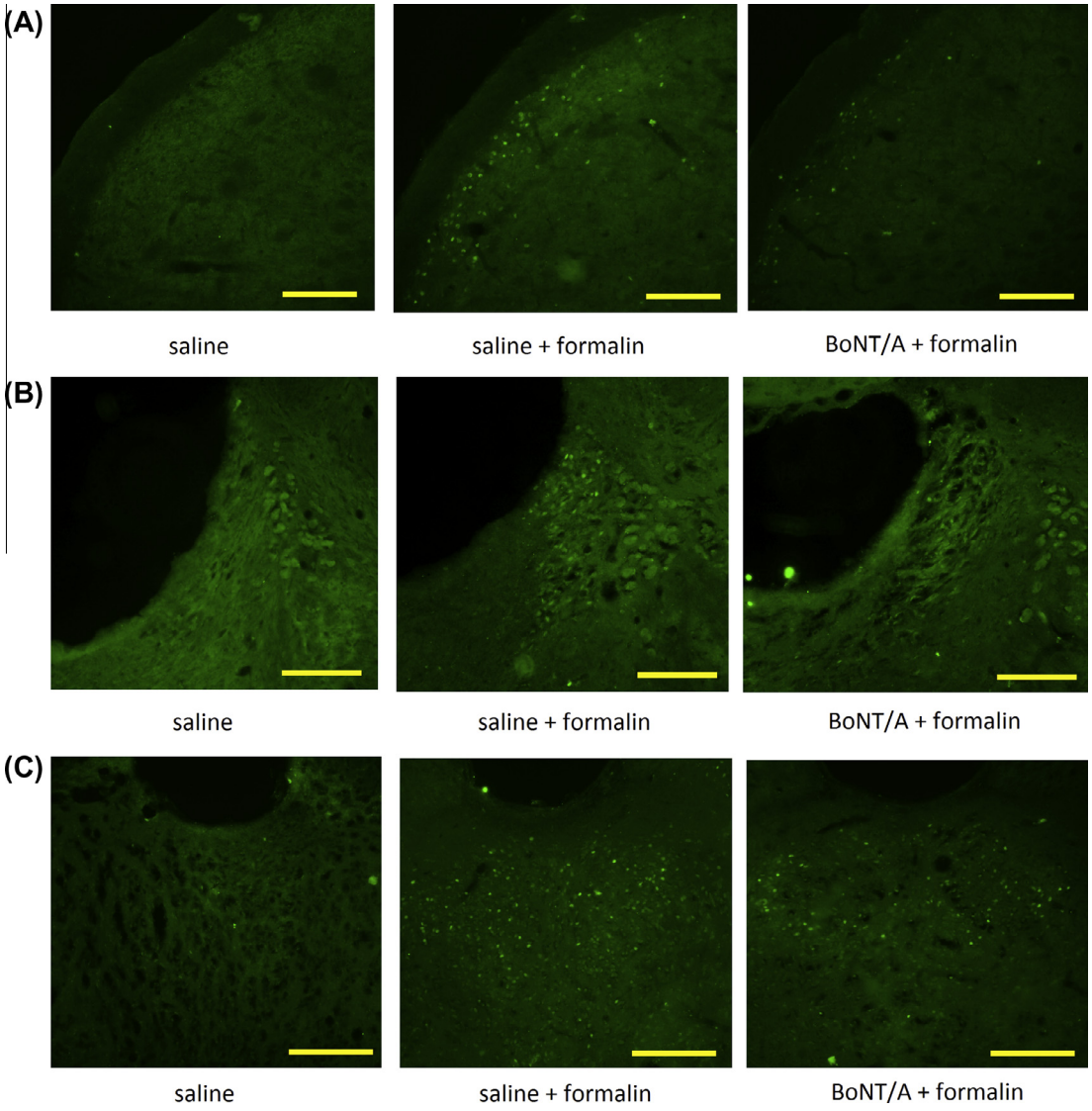

Fig. 7. Botulinum toxin type A (BoNT/A) reduces pain-evoked neural activity in trigeminal nucleus caudalis and locus coeruleus, but not in thalamus. Fluorescent images of orofacial formalin-induced neural activity (assessed with c-Fos expression [green]) in (A) ipsilateral trigeminal nucleus caudalis; (B) ipsilateral locus coeruleus and (C) paraventricular thalamic nucleus. Five U/kg BoNT/A or saline was applied into the whisker pad 5–6 days prior to formalin injection into the whisker pad. *N* (animals per group) = 3–4. Scale bar = 200 μm.

Table 1
Botulinum toxin type A (BoNT/A) differentially alters regional c-Fos activation in orofacial formalin test. Orofacial formalin test was performed 5 days following the saline or 5 U/kg BoNT/A injection into the whisker pad, and animals were perfused 2 hours after formalin injection. Number of immunofluorescently stained c-Fos-positive neuronal profiles in examined regions was automatically quantified in 4 randomly selected sections per animal.

	Saline (n = 3)	Saline + formalin (n = 4)	BoNT/A + formalin (n = 4)
Trigeminal nucleus caudalis (ipsilateral)	14.7 ± 0.7	138.5 ± 14.0	75.7 ± 9.3 (P = 0.003)
Locus coeruleus (ipsilateral)	4.7 ± 2.8	21.2 ± 2.4	13.7 ± 1.7 (P = 0.045)
Locus coeruleus (contralateral)	3.0 ± 1.5	24.6 ± 3.3	15.3 ± 1.5 (P = 0.023)
Periaqueductal gray	90.7 ± 26.4	290.9 ± 20.4	149.7 ± 8.9 (P = 0.001)
Hypothalamus (ipsilateral)	40.7 ± 5.4	342 ± 15.6	338.2 ± 24.3 (n.s.)
Hypothalamus (contralateral)	44.7 ± 16.1	341.8 ± 27.3	294.9 ± 20.7 (n.s.)
Paraventricular thalamic nucleus	19.2 ± 2.5	132.5 ± 17.7	110.1 ± 11.8 (n.s.)
Central amygdaloid nucleus (contralateral)	7.4 ± 2.3	36.0 ± 6.3	45.9 ± 3.9 (n.s.)

Data are represented as mean ± SEM. n = number of animals per group. For BoNT/A + formalin group, P values are shown in comparison to saline + formalin group (one-way analysis of variance followed by Newman-Keuls post hoc, $P < 0.05$ was considered significant); n.s. = nonsignificant.

4.4. Cleaved SNAP-25 cellular and regional localization

Herein we examined the localization of truncated SNAP-25 in relation to cellular markers in the TNC. Cleaved SNAP-25 punctate immunoreactivity colocalized with presynaptic terminals immunolabeled with synaptophysin, consistent with well-known BoNT/A activity in synapses [10]. Cleaved SNAP-25 fiber-like profiles, most likely corresponding to axons, were not immunoreactive

to synaptophysin (Fig. 6A). This is in line with extrasynaptic occurrence of SNAP-25 along the axons [61]. Cleaved SNAP-25 did not colocalize with either MAP-2-positive dendrites of secondary neurons or GFAP, marker of astrocytes (Fig. 6B, C). A recent study by Marinelli et al. [37] reported BoNT/A-truncated SNAP-25 occurrence in spinal astrocytes of neuropathic mice. Differences between the studies might be associated with experimental setup, animal species (mice vs rats), and sensory region examined (lumbar spinal dorsal horn vs TNC).

Following BoNT/A subcutaneous injection into the whisker pad area, we did not observe convincing cleaved SNAP-25 colocalization with CGRP-containing peptidergic afferents (Fig. 4, Supplementary Fig. 3). In rats, a significant portion of TRPV1-expressing trigeminal neurons (~30–56%) does not express CGRP [9,50]. Lack of colocalization could be associated with the site of toxin administration, since TRPV1-expressing afferents that innervate cutaneous structures are primarily nonpeptidergic [9,27,62]. Our results suggest that BoNT/A's antinociceptive action, at least in the present experimental setup, is not mediated primarily by direct prevention of central CGRP release.

Cleaved SNAP-25 in sensory regions examined above the level of TNC (locus coeruleus, periaqueductal gray, thalamus, hypothalamus, sensory cortex) was not observed. However, pain-evoked neural activity (assessed with c-Fos expression) was decreased by BoNT/A in locus coeruleus and periaqueductal gray (but not in thalamus, hypothalamus, and amygdala) (Fig. 7; Table 1). Reduction of pain-evoked neural activity in regions where BoNT/A enzymatic activity was not observed suggests that the toxin's indirect effects in CNS may be more widespread compared to its direct effects mediated by central SNAP-25 cleavage.

4.5. Conclusion

Present results suggest the association of BoNT/A's antinociceptive activity with capsaicin-sensitive central afferent terminals. This could explain the selective action of BoNT/A on only some forms of pain.

Conflict of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2014.04.027.

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APPENDIX V

Supplementary data from Matak et al. (2014).

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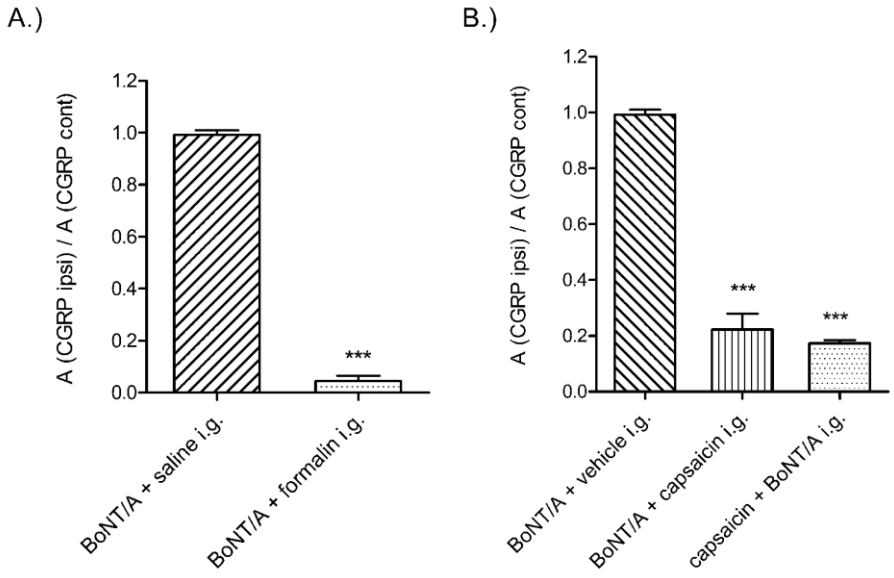
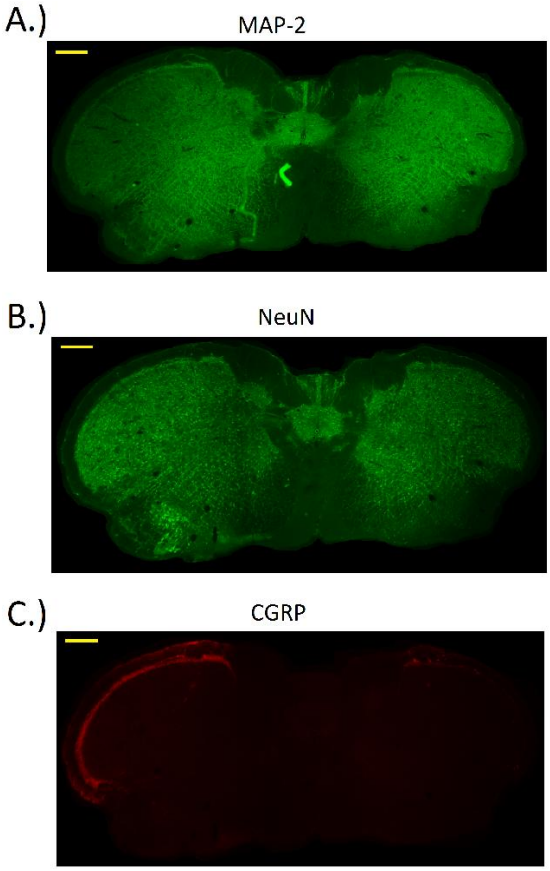
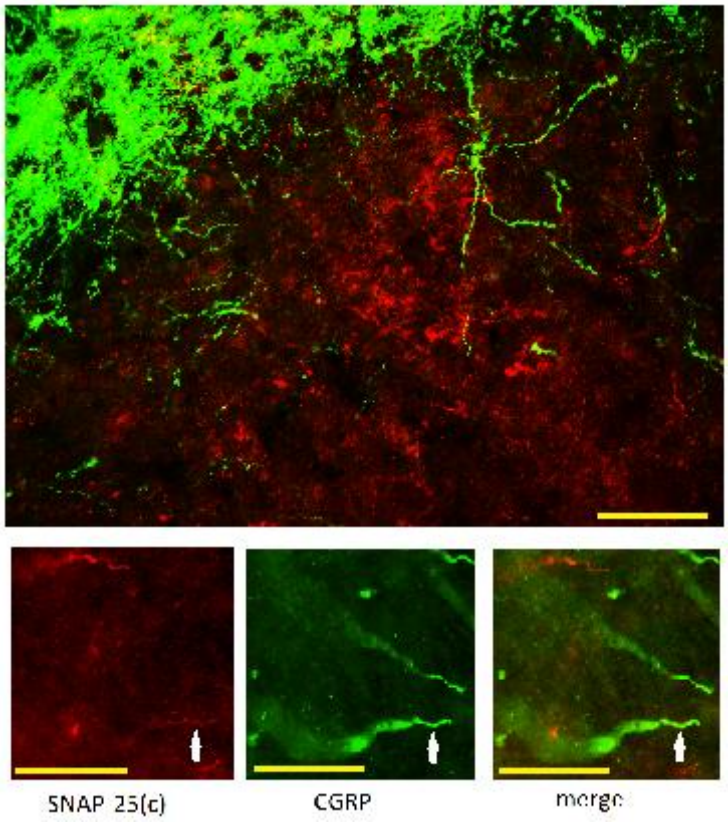


Supplementary figure 1- ***Quantification of reduction of CGRP immunoreactivity after unilateral trigeminal ganglion ablation with formalin (A.) or desensitization with capsaicin B.).*** *Surface areas of ipsilateral and contralateral trigeminal nucleus caudalis covered by CGRP were calculated using pixel thresholding. Surface area of ipsilateral CGRP immunoreactivity was divided by the surface area of CGRP immunoreactivity from contralateral side of the same coronal section. CGRP immunoreactivity was almost completely eliminated by formalin .g. treatment, and largely reduced by i.g. capsaicin. N(animals per group)=3-4, 4-6 coronal sections per animal were analyzed. Data are represented as mean ± SEM; ***- p<0.001 in comparison to saline or vehicle i.g. treatment (A. t-test or B. one-way ANOVA followed by Newman-Keuls post hoc, p<0.05).*



Supplementary figure 2 ***Ablation of primary afferents does not alter secondary brainstem neurons.***
A.) and B.) Formalin i.g. does not alter the immunoreactivities of dendrites (MAP-2) or neuronal bodies (NeuN) of secondary neurons in the TNC (green). C. Immunoreactivity for CGRP (red) is almost completely eliminated from TNC ipsilaterally to formalin i.g. treatment (right). N(animals)=3, 10-15 coronal sections per animal were examined. Scale bar=200 µm.

Supplementary figure 3 ***SNAP-25 cleavage occurs outside of CGRP-expressing peptidergic terminals after BoNT/A injection into the whisker pad****. Fluorescent microphotographs of ipsilateral TNC 5 days after BoNT/A (15 U/kg) injection into the rat whisker pad. Cleaved SNAP-25 localization (red) was studied in relation to CGRP (green), marker of peptidergic primary afferents. Although the majority of BoNT/A-cleaved SNAP-25 did not colocalize with CGRP (upper panel), occasionally, cleaved SNAP-25 profiles appeared to colocalize with bright fluorescent CGRP fibers (lower panel, arrow). Images are representative of microphotographs obtained from 4 animals (10-15 sections per animals were examined). Scale bar (upper panel = 50 µm, lower panel =25 µm*



APPENDIX VI

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Botulinum toxin A, brain and pain

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ABSTRACT

Botulinum neurotoxin type A (BoNT/A) is one of the most potent toxins known and a potential biological threat. At the same time, it is among the most widely used therapeutic proteins used yearly by millions of people, especially for cosmetic purposes. Currently, its clinical use in certain types of pain is increasing, and its long-term duration of effects represents a special clinical value. Efficacy of BoNT/A in different types of pain has been found in numerous clinical trials and case reports, as well as in animal pain models. However, sites and mechanisms of BoNT/A actions involved in nociception are a matter of controversy. In analogy with well known neuroparalytic effects in peripheral cholinergic synapses, presently dominant opinion is that BoNT/A exerts pain reduction by inhibiting peripheral neurotransmitter/inflammatory mediator release from sensory nerves. On the other hand, growing number of behavioral and immunohistochemical studies demonstrated the requirement of axonal transport for BoNT/A's antinociceptive action. In addition, toxin's enzymatic activity in central sensory regions was clearly identified after its peripheral application. Apart from general pharmacology, this review summarizes the clinical and experimental evidence for BoNT/A antinociceptive activity and compares the data in favor of peripheral *vs.* central site and mechanism of action. Based on literature review and published results from our laboratory we propose that the hypothesis of peripheral site of BoNT/A action is not sufficient to explain the experimental data collected up to now.

Contents

Abbreviations: BoNT/A, botulinum toxin type A; LC, light chain; SV2, synaptic vesicle protein 2; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle-associated membrane protein; TeNT, tetanus toxin; GABA, γ-aminobutyric acid; CGRP, calcitonin gene-related peptide; TRPV1, transient receptor potential vanilloid 1; PC-12, pheochromocytoma-12.

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1. Introduction

In the last 15–20 years, therapeutic use of botulinum toxin type A (BoNT/A) has expanded to cover different painful disorders. Initially it was reported that BoNT/A relieves pain associated with spasticity and cervical dystonia. Based on the discovery that BoNT/A may reduce the frequency of chronic migraine attacks and associated pain, its efficacy has been clinically investigated in chronic migraine treatment (Dodick et al., 2010) and approved in USA in 2010. Additionally, in off-label studies BoNT/A beneficial effect has been reported in many clinical disorders, such as lower back pain, myofascial pain, trigeminal neuropathy, temporomandibular joint disorders, osteoarthritis, *etc.* (Section 4.1.2). In different clinical conditions, reduction of pain hypersensitivity lasting for several months after single application makes BoNT/A a unique antinociceptive drug. Although the effect of BoNT/A on peripheral cholinergic synapses in different muscular and autonomous disorders has been well characterized, the mechanism of BoNT/A action on pain is still unknown. In this review we will focus on the most relevant findings and current hypotheses on the mechanism of BoNT/A antinociceptive actions. Though the BoNT/A action on pain is still dominantly believed to be of peripheral origin (Aoki and Francis, 2011; Francisco et al., 2012; Wheeler and Smith, 2013), novel experiments demonstrated that BoNT/A is axonally transported to central sensory regions, and proposed that its antinociceptive action is centrally mediated (Bach-Rojecky et al., 2008; Drinovac et al., 2013; Marinelli et al., 2012; Matak et al., 2011). These new findings raise many additional questions on the mechanism of BoNT/A antinociceptive action, and possibly some other CNS effects, which need to be answered.

2. Botulinum toxin: from the most potent poison to clinical use

Botulinum toxin (BoNT) is produced by a Gram-positive rod-shaped anaerobic bacterium *Clostridium botulinum* and few similar *Clostridia* (*C. butyricum*, *C. baratii* and *C. argentinense*) (Popoff and Bouvet, 2013). BoNT is a protein complex consisting of neurotoxic part (which proteolytically targets synaptic proteins involved in vesicular neurotransmitter release) and auxiliary proteins (in details explained in Section 3). There are seven well known antigenically distinct BoNT serotypes (A–G), with the most recent serotype H being reported and currently characterized (Dover et al., 2014; Barash and Arnon, 2014). Additionally, six serotypes may have additional subtypes (Dover et al., 2009; Kalb et al., 2011).

BoNT/A is one of the most potent toxins known. Estimated intravenous (i.v.) median lethal dose in humans is only 1 ng/kg or 70 ng/70 kg person (Gill, 1982). One gram of toxin could kill more than one million people via inhalational route, making BoNT a potential biological threat (Arnon et al., 2001; Bigalke and Rummel, 2005; Franz et al., 1997; Gill, 1982).

2.1. Botulism

Systemic BoNT intoxication induces botulism, a neuroparalytic disease with a low incidence, but fatal outcome in 5–10% of cases (WHO, 2013). Main features of botulism, caused primarily by inhibition of peripheral cholinergic transmission, are the long-term flaccid paralysis of skeletal muscles, and the impairment of gastrointestinal and autonomic nervous system functions. Effects on sensory system have also been reported occasionally (Goode and Shearn, 1982; Kuruoğlu et al., 1996; Martínez-Castrillo et al., 1991). Symptoms of botulism usually appear within 12–36 h

following the exposure to toxin, but in some cases may occur with a delay of up to 8 days. First symptoms are relatively mild (weakness and vertigo, dry mouth and difficulty in swallowing and speaking), leading to progressive paralysis of skeletal muscles, and ending in respiratory failure in more severe cases. Immediate treatment consists of early administration of antitoxin and intensive respiratory support (WHO, 2013). In humans, botulism is mostly caused by BoNT serotypes A, B, E, and rarely F (Sobel, 2005). Known types of botulism are:

1. *Food-borne botulism* (intoxication with toxin-contaminated food), characterized accurately for the first time by the early 19th century German physician Justinus Kerner, who described symptoms of food poisonings caused by ingestion of contaminated smoked sausages (*botulus* – lat. sausage) (Erbguth, 2008). It is the most common form of BoNT poisoning associated with different types of mainly home-made food preserved in anaerobic conditions. Spores of *Clostridium botulinum* are heat-resistant, while the toxin itself is destroyed by boiling (WHO, 2013).
2. *Inhalational botulism* is very rare, and it can occur due to exposure during industrial production of toxin. Theoretically, it might become a very serious threat if the toxin is used as a biological weapon (Arnon et al., 2001).
3. *Iatrogenic botulism* has been reported after cosmetic use of high-dose of illegal BoNT/A preparation, and in pediatric patients treated with high dose of BoNT/A for spasticity (Chertow et al., 2006; Crowner et al., 2007).

   In addition to poisoning with BoNT, botulism can also be caused by anaerobic toxo-infection with *C. botulinum* endospores, which germinate and produce the toxin inside the body.
4. "*Infant botulism*" is associated with anaerobic conditions adequate for ingested spore germination in the intestinal tract of infants under 6 months of age (Brook, 2007). Up to 20% of the honey specimens from different countries may contain spores of botulinum toxin (Nakano et al., 1990). Parents and caregivers are warned not to feed infants before the age of 1 year with honey (WHO, 2013).
5. *Adult intestinal toxemia botulism* can occur if the normal gut flora has been altered as a result of surgical procedures or antibiotic therapy (WHO, 2013).
6. *Wound botulism* occurs due to wound infection with *C. botulinum*. It has been reported in intravenous drug abusers (Sobel, 2005; Wenham, 2008).

2.2. BoNT/A as a useful drug

Ability to purify botulinum toxins (serotypes A and B) and use them locally in very low doses, as well as their long term activity (lasting up to several months), have been the basis of their clinical use in various neuromuscular and autonomous disorders. The idea of using small doses of BoNT for therapeutic purposes was proposed for the first time by Kerner in 1822 (Kerner, 1822; reviewed by Erbguth, 2008). *C. botulinum* was characterized in 1897, and different BoNT serotypes were identified and purified in the 20th century (Erbguth, 2008). In the late 1960s and 1970s, based on preclinical experiments with monkeys, injections of small doses of purified BoNT/A into the lateral or medial rectus muscle have been initially used in the treatment of strabismus (Scott et al., 1973; Scott, 1980). BoNT/A has been approved for the use in strabismus in 1989, and later in other types of muscular hyperactivity disorders like blepharospasm, hemifacial spasm, focal dystonia and upper limb spasticity (reviewed by Barnes, 2003; Thenganatt and Fahn, 2012). Apart from movement disorders, BoNT/A has been used for treatment of autonomic system disorders (approved in primary axillar hyperhidrosis and urinary incontinence caused by neurogenic detrusor overactivity) (Dressler, 2013; Naumann et al., 2013; Seth et al., 2013), and in non-muscular pain conditions (reviewed in detail in Section 4). Cosmetic use of BoNT/A for wrinkle correction was approved in USA in 2002. Today, due to its applications for medical and cosmetic purposes, BoNT/A is one of the most commonly used therapeutic proteins. Botulinum toxin's growing market is estimated to reach the profit/the sales of $2.9 by 2018 (Chapman, 2012). Presently, BoNT/A is produced by over 20 manufacturers in US, Europe, and other parts of the world (Truong et al., 2009). Although they contain the same active molecule, potency units for each of the BoNT products are specific, and cannot be compared or converted between different products. Therefore, Food and Drug Administration (FDA) approved new names for different BoNT products registered in USA (FDA, 2009, 2013):

- Botox (botulinum toxin type A); new name: onabotulinumtoxinA
- Botox cosmetic (botulinum toxin type A); new name: onabotulinumtoxinA
- Dysport (botulinum toxin type A); new name: abobotulinumtoxinA
- Xeomin (botulinum toxin type A); new name: incobotulinumtoxinA
- Myobloc (botulinum toxin type B); new name: rimabotulinumtoxinB

3. Mechanism and molecular targets of BoNT/A action

3.1. Pharmacokinetics

3.1.1. Structure of BoNT/A complex

BoNT/A molecular complex of 900 kDa consists of toxic part (150 kDa) and auxiliary proteins (750 kDa). The toxic part consists of two polypeptide chains connected with disulphide bridge (Fig. 1). The larger, heavy chain (100 kDa) contains a carboxy

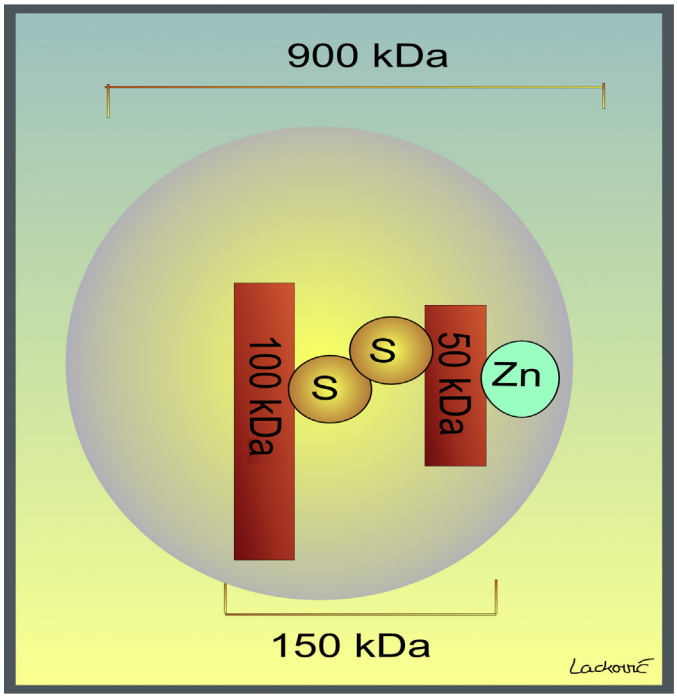

Fig. 1. Schematic representation of 150 kDa BoNT/A neurotoxin molecule consisting of light chain (50 kDa, Zn-endopeptidase, dark red) coupled with heavy chain (100 kDa, light red) by sulphur bridge. S, sulphur; Zn, zinc. Accessory proteins of 750 kDa comprising the rest of 900 kDa complex are not shown.

terminal membrane acceptor-binding domain (H_C) and a translocation domain at the N terminal (H_N), which mediate the toxin binding to nerve terminals and translocation of light chain into the cytosol (Gu and Jin, 2013; Lee et al., 2013).

50 kDa light chain (LC) enters the cytosol and prevents neurotransmitter release by enzymatic cleavage of synaptosomal-associated protein of 25 kDa (SNAP-25) (Section 3.2). Auxiliary proteins containing hemaglutinins and non-hemaglutinins participate in the stabilization of the BoNT/A complex and preservation in extracellular space and throughout the gastrointestinal tract (Chen et al., 1998; Gu and Jin, 2013; Lee et al., 2013).

3.1.2. Absorption and distribution

In the gut, BoNT/A 150 kDa neurotoxin passes from the lumen of small intestine across epithelial lining into the bloodstream by transcytosis. Similarly, inhaled BoNT/A may enter the bloodstream across lung alveolar epithelium. It then reaches extracellular fluid in various tissues and targets peripheral nerve endings (Simpson, 2013). Differences in estimated human LD50 doses of BoNT/A delivered orally and intravenously (70 μg *vs.* 0.09–0.15 μg in 70 kg human, respectively) (Arnon et al., 2001) suggest that only a small fraction of orally ingested active BoNT/A can cross from the gastrointestinal tract into the systemic bloodstream. This is due to the inactivation of a large portion of orally ingested BoNT/A by the low pH HCl in the stomach or degradation by digestion enzymes (Sugii et al., 1977). Dose necessary for inhalational poisoning (0.8–0.9 μg) (Arnon et al., 2001) suggests higher rate of bloodstream penetration than after oral administration. Auxiliary proteins are not necessary for 150 kDa toxin absorption from small intestine and lungs (Maksymowych et al., 1999; Al-Saleem et al., 2012).

Experimental assessment of systemic pharmacokinetic of i.v.-administered BoNT/A was performed in rodents (Ravichandran et al., 2006). The bloodstream elimination half-life of active, non-metabolized toxin was around 4 h. Majority of the toxin was unbound to plasma proteins (85–95%).

The time course of BoNT/A poisoning was studied using i.v. administration of polyclonal neutralizing antibody (BoNT/A antitoxin) at different time points following high-dose toxin i.v. delivery. Antitoxin administered 10 min after BoNT/A only partially prolonged the animal survival. After 20 min it was almost completely unable to prevent the symptoms of BoNT/A poisoning in experimental animals (Ravichandran et al., 2006). This signifies that the process of BoNT/A distribution and entrance into the tissue target peripheral nerve endings occurred within minutes.

Dependently on the volume and dose, therapeutically used BoNT/A may spread from the injection site and induce local side effects (Brodsky et al., 2012; Majlesi, 2008). Peripheral spread away from the site of toxin injection is not dependent on auxiliary proteins (Brodsky et al., 2012; Carli et al., 2009). BoNT/A traffic from periphery to the CNS *via* axonal route is discussed in Section 6.

3.1.3. BoNT/A internalization into nerve terminals

BoNT/A makes the first contact with neuronal terminals by binding to polysialogangliosides in the outer side of plasma membrane, which anchor the toxin from extracellular fluid (Simpson, 2013). Subsequently, the toxin heavy chain binds its high affinity membrane protein acceptors, synaptic vesicle protein 2 (SV2) and fibroblast growth factor receptor 3, which govern BoNT/A binding and endocytotic entry into neurons (Dong et al., 2006; Jacky et al., 2013; Mahrhold et al., 2006). BoNT/A internalization into neurons may be mediated by all three isoforms of SV2 (SV2A–C), with the strongest affinity for SV2C (Dong et al., 2006). After binding to protein acceptors on neuronal membrane, BoNT/A undergoes dynamin-dependent endocytosis into the acidic compartment of small synaptic vesicles (Colasante et al., 2013; Harper et al., 2011). The process of BoNT/A endocytosis is augmented by neuronal activity, which promotes synaptic vesicle recycling (Harper et al., 2011).

50 kDa BoNT/A light chain (LC) is translocated from small synaptic vesicle into the cytosol by a pH-dependent active process mediated by N-terminus translocation domain of heavy chain (H_N) (Kalandakanond and Coffield, 2001; Fischer and Montal, 2007). H_N domain is inserted into the vesicle membrane and acts as a chaperone channel, which unfolds the three-dimensional structure of LC into a polypeptide chain and translocates it from the inside of small synaptic vesicle into the cytosol. Under the influence of acidic pH in vesicles, disulphide bridge between the heavy and light chain is reduced during the translocation process (Fischer and Montal, 2007).

3.2. Pharmacodynamics

3.2.1. Proteolytic activity

After its translocation into the cytosol, LC polypeptide is refolded into a soluble Zn^{2+}-dependent metalloprotease (Kalandakanond and Coffield, 2001). At the cytosolic side of presynaptic plasma membrane, LC protease hydrolyses a distinct peptide bond on membrane-associated protein SNAP-25 (Blasi et al., 1993; Sudhof, 2013). SNAP-25 is a part of the Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) heterotrimeric complex, which has a pivotal role in fusion of vesicular and plasma membrane lipid bilayers during Ca^{2+}-dependent exocytosis. By cleaving unique peptide bonds on SNARE-proteins, different BoNT serotypes and tetanus toxin (TeNT) prevent the fusion of neurotransmitter-containing vesicles with presynaptic plasma membrane (Fig. 2). Along with SNAP-25 (targeted by BoNT serotypes A, E and C1), SNARE complex is comprised of two additional proteins: membrane-associated syntaxin (targeted by BoNT/C1) and vesicle-associated membrane protein (VAMP)/synaptobrevin (targeted by BoNT serotypes B, D, F, G, and TeNT) (Binz et al., 1994; Foran et al., 1996; Pellizzari et al., 1999; Schiavo et al., 1992).

3.2.2. Duration and extent of BoNT/A-induced synaptic paralysis

BoNT/A-mediated cleavage of SNAP-25 in cholinergic synapses leads to reversible long-term prevention of acetylcholine release which, in case of neuromuscular paralysis, can last up to 6 months in humans.

Based on experiments involving introduction of anti-BoNT/A LC antibodies into the cytosol (Bartels et al., 1994), it was found that, after inhibition of BoNT/A catalytic activity, the paralysis may persist for up to 4 days. The delay in recovery was attributed to the time course of synaptic turnover of cleaved SNAP-25 (Keller and Neale, 2001). Thus, this period is not sufficient to explain the long lasting effect of BoNT/A (4 days *vs.* several months). The dominant mechanism for the long-term duration of BoNT/A activity is the unusual stability of its proteolytic light chain in the cytosol. It may persist for at least 180 days *in vivo*, and 80 days *in vitro* in rodents (Antonucci et al., 2008; Keller et al., 1999). Persistence of BoNT/A protease in the cytosol is presumably mediated by toxin's light chain resistance to proteasomal degradation (Tsai et al., 2010). BoNT/E LC is more susceptible to proteasomal degradation which mediates the shorter duration of its effects compared to BoNT/A (Tsai et al., 2010).

It is estimated that BoNT/A-mediated cleavage of less than 10% of SNAP-25 is able to cause an almost complete muscular paralysis (>90%) (Kalandakanond and Coffield, 2001). Additionally, *in vitro* spontaneous cholinergic neurotransmission in cultured autonomic neurons is blocked >80% by 1 pM BoNT/A despite cleaving only <20% of the SNAP-25 (Lawrence et al., 2013). These observations suggest that only a portion of functional SNAP-25 needs to be cleaved to induce a near-complete synaptic paralysis. Truncated SNAP-25 lacks only 9 C-terminal amino acids, which does not impair the forming of heterotrimeric complex with other two SNAREs

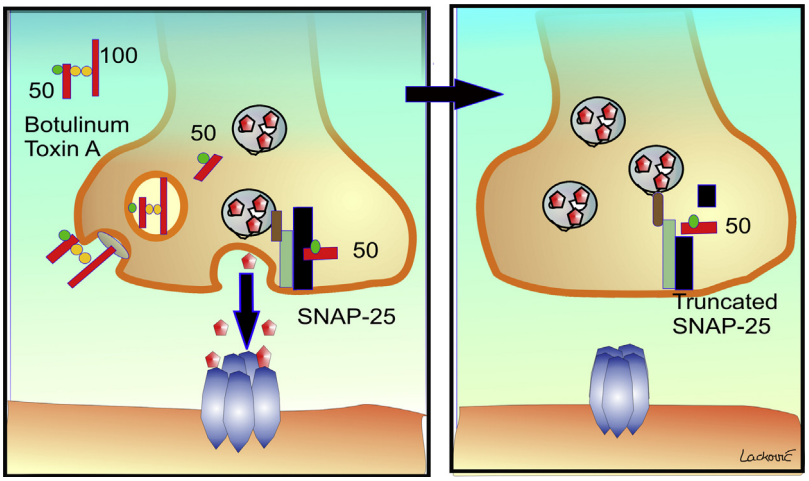

Fig. 2. Mechanism of botulinum toxin type A (BoNT/A)-mediated prevention of neurotransmitter release. *Left*: Schematic representation of 150 kDa BoNT/A neurotoxin (red) consisting of heavy chain and light chain coupled with disulphide bonds (yellow). Light chain contains zinc ion (green). BoNT/A heavy chain recognizes the dual ganglioside-protein acceptors (gray oval shape) on the outer side of plasma membrane and it is internalized into synaptic vesicles. BoNT/A LC is then translocated into the cytosol. *Right*: Light-chain cleaves membrane-associated synaptosomal-associated protein of 25 kDa (SNAP-25) which prevents vesicle fusion with membrane. This induces prevention of neurotransmitter release and build-up of synaptic vesicles in the synapse. 100, 100 kDa BoNT/A heavy chain; 50, 50 kDa BoNT/A light chain; SNAP-25; synaptosomal-associated protein of 25 kDa (black rectangle); light green rectangle represents syntaxin, brown rectangle represents vesicle associated membrane protein/synaptobrevin, blue pentamer represents postsynaptic neurotransmitter receptor; red pentagon represents neurotransmitter. Sizes of schematic representations of proteins, atoms and neurotransmitter are not drawn to scale compared to synaptic structures and vesicles.

(Lawrence et al., 2002; Meunier et al., 2003). Inactive SNARE complex by itself may inhibit the neurotransmitter release (Keller and Neale, 2001). This is augmented by finding that cell transfection with BoNT/A-truncated SNAP-25 induces the inhibition of vesicular release similar to BoNT/A (Huang et al., 1998). It is estimated that several SNARE complexes forming a star-shaped oligomer (SNARE supercomplex) are necessary for the fusion of a single synaptic vesicle with plasma membrane (Megighian et al., 2010).

3.2.3. Toxicity and clinical potency of BoNT/A

BoNT/A dose required to produce clinical symptoms of botulism are minute – required quantities are in order of nanograms (i.v. LD_{50} is 70 ng/70 kg person). Effective therapeutic doses are much lower – in some clinical conditions like spasmodic dysphonia in order of picograms (Upile et al., 2009). Main pharmacokinetic and pharmacodynamic factors which summate and result in high BoNT/A potency are summarized in Sections 3.1.2–3.2.2. These are:

- although it is a large protein, fraction of the toxin penetrates into the bloodstream through epithelial barriers (Section 3.1.2);
- long elimination half-life of BoNT/A in the systemic circulation allows distribution to peripheral tissues and entry into peripheral nerve terminals (Section 3.1.2);
- high affinity of BoNT/A heavy chain to dual ganglioside-protein acceptors at the peripheral nerve terminals (Section 3.1.3);
- BoNT/A light chain is an enzyme which may target many SNAP-25 molecules (Section 3.2.1);
- in the cytosol, BoNT/A light chain is very stable and resistant to proteasomal degradation, which mediates the long-term effect of BoNTA (Section 3.2.2);
- cleavage of only a small portion of SNAP-25 is required for synaptic paralysis (Section 3.2.2).

3.3. BoNT/A activity is not restricted to inhibition of SNAP-25-mediated acetylcholine release

3.3.1. Effect on neurotransmitters other than acetylcholine

Classically, it was assumed that BoNT/A enters cholinergic motor and autonomic neurons only. However, the toxin enters many different neuronal types and blocks the neurotransmitter release from non-cholinergic synapses, too. Mostly *in vitro* experiments demonstrated that BoNT/A prevents the release of serotonin, dopamine, noradrenaline, glutamate, gamma-aminobutyric acid (GABA), enkephalin, glycine, substance P, ATP and calcitonin gene-related peptide (CGRP) (Durham and Cady, 2004; McMahon et al., 1992; Morris et al., 2002; Nakov et al., 1989; Thyssen et al., 2010; Verderio et al., 2007; Welch et al., 2000).

3.3.2. Preferential effect on excitatory vs. inhibitory neurons

BoNT/A is more efficient in impairing the release of excitatory neurotransmitters like acetylcholine and glutamate, in contrast to GABA (Verderio et al., 2007). This was proposed to result from higher transient increase in calcium concentration upon depolarization in GABA-ergic neurons, in comparison to excitatory neurons (Grumelli et al., 2010). High concentrations of intracellular Ca^{2+}, in turn, have the ability to overcome the cleaved SNAP-25-mediated paralysis (Gerona et al., 2000; Grumelli et al., 2010; Lawrence et al., 2002). Loss of SNAP-25 C-terminus affects the Ca^{2+}-dependent interaction of SNARE complex with synaptotagmin I, a protein involved in triggering of Ca^{2+}-mediated neurotransmitter release. This interaction can be restored by increasing the Ca^{2+}-concentration (Gerona et al., 2000).

Since SNAP-25 is also a negative regulator of calcium channels (Pozzi et al., 2008), higher level of SNAP-25 in excitatory neurons makes them more BoNT/A-sensitive in comparison to inhibitory neurons. Reducing the calcium levels by chelators induces a higher sensitivity of GABA-ergic neurons to BoNT/A action (Grumelli et al., 2010; Verderio et al., 2004).

3.3.3. Activity outside of synaptic active zone

Thyssen et al. (2010) demonstrated that BoNT/A activity on neurotransmitter release in neuronal compartments is not confined only to synapses. Authors showed that BoNT/A inhibits the ectopic vesicular release of glutamate and ATP from axons of olfactory receptor neurons (Thyssen et al., 2010). BoNT/A effect in axons suggests its activity on neurotransmitter release outside of active synaptic zones.

3.3.4. Effects on cell types other than neurons

BoNT/A blocks the vesicular release from non-neuronal cell types containing SNAP-25-dependent exocytotic machinery, such as pancreatic beta cell lines (blockage of insulin release), chromaffin cells (acetylcholine) and astrocytes (glutamate) (He et al., 2008; Lawrence et al., 2002; Kanno and Nishizaki, 2012). BoNT/A effect on acetylcholine release from sciatic Schwann cells has also been reported (Marinelli et al., 2012).

3.3.5. Additional actions of BoNT/A mediated by SNAP-25

Besides its role on neurotransmitter release, SNAP-25 modulates the activity of Ca^{2+} channels and possibly other voltage gated ion channels (He et al., 2008; Ji et al., 2002; Pozzi et al., 2008; Zamponi, 2003). By targeting SNAP-25, BoNT/A may prevent the SNARE-mediated translocation of receptors to plasma membrane, such as N-methy-D-aspartate receptor and transient receptor potential vanilloid 1 (TRPV1) (Cheng et al., 2013; Morenilla-Palao et al., 2004; Shimizu et al., 2012). In addition, it may prevent the G protein interaction with SNARE-dependent exocytotic machinery (Gerachshenko et al., 2005).

Process of neurite extension through the axonal growth cone was shown to be dependent on SNAP-25 and sensitive to BoNT/A action. BoNT/A inhibits axonal cone growth in cultured hippocampal, dorsal root ganglion cells, or differentiated pheochromocytoma-12 (PC-12) cells. In addition, BoNT/A prevented the dendritic growth in hippocampal neurons (Grosse et al., 1999; Morihara et al., 1999). The fact that SNAP-25 is not localized at presynaptic sites only, but also along the axons and dendrites (Galli et al., 1995; Duc and Catsicas, 1995) suggests that SNAP-25 might be a multifunctional protein. It was proposed that SNAP-25 (or the whole SNARE complex) may mediate various types of membrane fusion events in the entire axonal compartment (Duc and Catsicas, 1995). Consequently, BoNT/A-cleaved SNAP-25 presence along neuronal processes (Fig. 3) suggests that BoNT/A might also have some additional *in vivo* effects which remain to be investigated.

3.3.6. Possible additional targets of BoNT/A action other than SNAP-25

Up to now, SNAP-25 is the only definitively accepted molecular target of BoNT/A action. However, according to several *in vitro* studies there is a defined effect of BoNT-A on cellular processes like neuroexocytosis, apoptosis and neurite sprouting which do not necessarily depend on enzymatic cleavage of SNAP-25 (Coffield and Yan, 2009; Ishida et al., 2004; Proietti et al., 2012; Ray et al., 1993, 1999; Zhang et al., 2013).

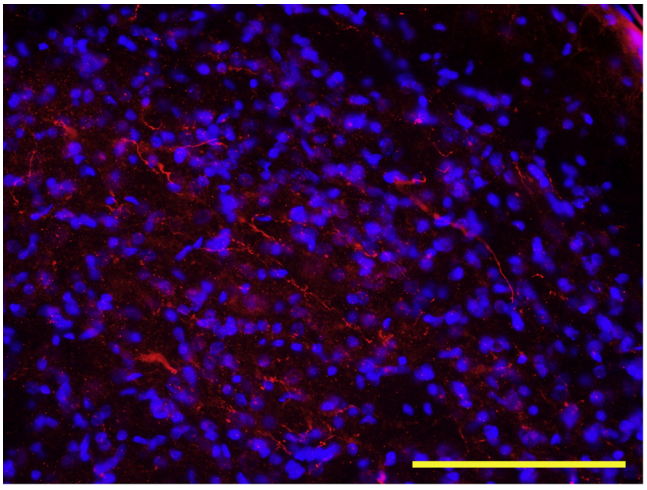

Fig. 3. Fluorescent microphotograph of cleaved SNAP-25 in the ipsilateral trigeminal nucleus caudalis 5 days after BoNT/A (15 U/kg) injection into the rat whisker pad. Immunohistochemical procedure is previously published (Matak et al., 2011). Red fluorescent signal represents cleaved SNAP-25, while blue fluorescence represents nuclear counterstaining with diamidino-2-phenylindole (DAPI). Lateral edge of brainstem section is visible in the upper right corner. Scale bar = 100 μm.

Several studies have shown that the BoNT/A effect on acetylcholine release mediated by arachidonic acid pathway may be independent of SNAP-25. In PC12 cell line, arachidonic acid or phospholipase A2 activation-induced exocytotic release of acetylcholine was found to be prevented by BoNT/A (Ray et al., 1993). Acetylcholine release promoted by arachydonic acid or by phospholipase A2 activation was found to be present even when the expression of SNAP-25 was prevented by antisense oligonucleotides (Ray et al., 1993, 1999). The authors proposed the existence of additional anti-exocytotic mechanisms of BoNT/A action not dependent on SNAP-25 (Ray et al., 1999). Recently, it was shown that phospholipase A2 activator mastoparan-7 partially reverses the BoNT/A-mediated impairment of cholinergic transmission in cultured spinal cord cells (Zhang et al., 2013). The effect was suggested to be unrelated to the activity of BoNT/A proteolytic effect on SNAP-25 (Zhang et al., 2013).

It was suggested that BoNT/A blocks the lysophosphatidic acid-promoted acetylcholine release from differentiated PC12 cells by inhibiting RhoB-dependent signaling pathway (Ishida et al., 2004). RhoB protein is a member of Rho kinases/GTP-ases involved in intracellular signaling pathways leading to actin reorganization. The authors demonstrated that BoNT/A, by an unknown mechanism, promotes the proteasomal degradation of RhoB (Ishida et al., 2004).

BoNT/A induces apoptosis in cell cultures derived from prostate cancer and breast cancer (Bandala et al., 2013; Karsenty et al., 2009; Proietti et al., 2012). Lack of neural elements in the cell culture suggests that BoNT/A effect are mediated through mechanisms independent from neurotransmitter release. In prostate cancer cell lines, BoNT/A increases the concentration of phosphorylated phospholipase A2, which is proposed to be associated with BoNT/A-mediated apoptosis and inhibition of proliferation (Proietti et al., 2012).

In another *in vitro* experiment, neuritogenic sprouting of motor nerve terminals, previously believed to be associated with toxin's paralytic activity on neuromuscular junctions, was proposed to be independent of toxin's light chain (Coffield and Yan, 2009). BoNT/A heavy chain subunit was shown to promote the neurite sprouting in cultured motor neurons similarly to the native 150 kDa toxin. Authors proposed that the binding activity of BoNT/A heavy chain alone is sufficient to promote neuritogenesis (Coffield and Yan, 2009).

Bossowska and Majewski (2012) reported that, at the level of sensory ganglia, BoNT/A bladder injection reduces the expression of substance P, CGRP, calbindin, somatostatin, and neuronal nitric oxide synthase in sensory neurons innervating the bladder in pigs. The mechanism of these changes might be connected with altered gene expression. Up-regulation of CGRP and enkephalin m-RNA expression in motoneurons after intramuscular BoNT/A has been interpreted as an indirect consenquence of peripheral chemical denervation (Humm et al., 2000; Jung et al., 1997; Palomar and Mir, 2012; Zhang et al., 1993).

4. Evidence of the antinociceptive action of BoNT/A

4.1. Clinical evidence of BoNT/A's antinociceptive activity

4.1.1. First clinical observations

As previously mentioned in Section 2.2, small doses of purified BoNT/A are clinically used for treatment of neuromuscular disorders characterized by increased tonicity or overactivity of certain muscles (Barnes, 2003; Thenganatt and Fahn, 2012). Along with the neuroparalytic effect, it was observed that BoNT/A

reduces the pain associated with the neuromuscular hyperactivity disorders, such as dystonic torticollis (Tsui et al., 1986; Brin et al., 1987; Tarsy and First, 1999). BoNT/A-mediated pain relief was initially believed to be associated with decreased contraction of affected muscles (Arezzo, 2002; Cohen et al., 1989; Mense, 2004). It was proposed that BoNT/A, by inhibiting the release of acetylcholine in hyperfunctional muscular end plates, may indirectly prevent the painful ischemia caused by muscle contractures in spasticity and dystonia (Mense, 2004). However, it was reported that the observed pain relief in patients treated for spasmodic torticollis was not concurrent with neuromuscular effects, since it was present in some patients even when dystonic posture of the neck was not improved (Stell et al., 1988). Additionally, BoNT/A-mediated pain relief does not always occur simultaneously with the observed paralytic effect in focal dystonias, and it sometimes even persists after the neuromuscular benefit is no longer visible (Aoki, 2003; Freund and Schwartz, 2003; Relja and Klepac, 2002). In a dose response double blind study of Relja and Klepac (2002), it was observed that BoNT/A effect on pain in spasmodic torticollis occurs after one week, while the beneficial effect on motor function started after 2 weeks. Moreover, it was discovered that the dose necessary to induce beneficial effect on pain (50 U) was lower than the doses needed for motor improvement (100 and 150 U). In patients treated with BoNT/A for temporomandibular disorders it was observed that the beneficial effects on pain persisted longer than the decreased voluntary bite force (Freund and Schwartz, 2003). Additionally, based on the effectiveness of BoNT/A injections into the trigger points in treatment of myofascial pain, BoNT/A effect was proposed to be linked to either indirect or direct effects on pain fibers rather than muscles themselves (Giladi, 1997). Since the onset of BoNT/A action on pain did not correspond to the onset of paralytic muscular effects or lasted longer than the paralysis, toxin's action on sensory or vegetative nerves was suggested (Giladi, 1997; Mense, 2004).

Independently from pain which might be related to increased muscle contraction, antinociceptive effect of BoNT/A was reported in different types of chronic pain not associated primarily with muscular hyperactivity, such as migraine and different types of neuropathic pain (Argoff, 2002; Silberstein et al., 2000). Along with experimental knowledge obtained from pre-clinical *in vitro* and *in vivo* studies (Section 5.2), it is now accepted that BoNT/A effects on pain may be mediated by its direct effects on sensory system.

4.1.2. Current clinical experience

Migraine. Based on results of large (1384 patients) multicentric Phase III Research Evaluating Migraine Prophylaxis Therapy (PREEMPT) studies (Dodick et al., 2010), BoNT/A was approved by the FDA for the treatment of chronic migraine (migraine having >15 headache days per month) in 2010. Therapeutic outcome was a significantly reduced mean number of migraine attacks per month and headache severity, suggesting that BoNT/A may be used as a prophylactic treatment of chronic migraine. In mentioned studies BoNT/A was injected into fixed sites over several cranial and neck muscles and in fixed doses (total dose of 155–195 onabotulinum toxin A preparation units – 1 unit (U) corresponds to intraperitoneal mouse LD_{50} dose, which equals 48 pg of 900 kDa toxin complex).

The value of PREEMPT study, supported by onabotulinumtoxinA manufacturer, was highly prized (reviewed by Láinez-Andrés, 2012) and questioned at the same time because the outcome of some other randomized controlled experiments was mild or not found at all (reviewed by Gady and Ferneini, 2013). As pointed out by Frampton (2012) "Debate surrounding the PREEMPT studies has centered on the small treatment effect of BoNTA relative to placebo, the possibility that blinding was inadequate and relevance of the evaluated population". It is also possible that some patient subpopulations exhibit a larger benefit from BoNT/A use in comparison to others. The directionality of pain may be a marker for predictability of BoNT/A responsiveness in the treatment of migraine headache (Jakubowski et al., 2006; Burstein et al., 2009). In the subpopulation of responders, it was reported that most of them (84%) described their headache as a pressure build-up from outside (imploding headache), while in the subpopulation of non-responders, 83% of patients described their pain as a pressure from inside (exploding headache) (Burstein et al., 2009). It was also suggested that pericranial allodynia, unilaterality of migraine pain and pericranial muscle tenderness in chronic migraine may be the predictive markers for BoNT/A responsiveness (Mathew et al., 2008).

Other types of pain. In the past decade, several hundreds of reports of pain relief due to BoNT/A off-label use in various clinical conditions have been published. Beneficial effects in pain have been reported in interstitial cystitis (Kuo, 2013; Russell et al., 2013), chronic arthritis (Chou et al., 2010), residual limb pain (Wu et al., 2012), different types of peripheral neuropathic pain (Ranoux et al., 2008; Zúñiga et al., 2008), diabetic neuropathy (Chen et al., 2013; Relja and Miletić, 2006; Yuan et al., 2009), masticatory pain (Freund and Schwartz, 2003), *etc.* Although several double-blind, placebo controlled studies have been performed (review by Jabbari and Machado, 2011), clinical reports on BoNT/A efficacy are dominated by studies based on case series or individual case reports. In the literature there is an increasing number of rare conditions with reported BoNT/A efficacy such as Parry Romberg syndrome (Borodic et al., 2014), Morton neuroma (Climent et al., 2013), painful legs and moving toes syndrome (Rodriguez and Fernandez, 2013), post-thoracotomy pain (Fabregat et al., 2013) post-amputation limb pain (Wu et al., 2012), *etc.*

Although BoNT/A seems to be a promising candidate for treatment of chronic pain, the results of clinical studies are contradictory, such as the effects on myofascial pain (Cheshire et al., 1994; Göbel et al., 2006; Wheeler et al., 1998) and tension type headache (Relja, 1997; Relja and Telarović, 2004; Schulte-Mattler and Krack, 2004).

Results of systemic reviews and meta-analyses (Cochrane data base systemic reviews and other) are also mostly inconclusive:

- Winocour et al. (2014) published a systemic review of 7 clinical trials on 427 women with subpectoral breast implants. The results suggest that BoNT/A may alleviate *postoperative pain* but this outcome is inconsistent and lacks methodological rigor.
- Soares et al. (2012) and Gerwin (2012) analyzed the efficacy of BoNT/A in *myofascial pain syndromes in adults*. Based on data from four studies with a total of 233 participants, authors concluded that "there is inconclusive evidence to support the use of botulinum toxin in the treatment of myofascial pain syndromes .... Meta-analyses were not possible due to the heterogeneity between studies".
- *Subacute/chronic neck pain.* Nine randomized and quasi-randomized controlled trials (503 participants) were included in the systemic review performed by Langevin et al. (2011). However "evidence fails to confirm either a clinically important or a statistically significant benefit of BoNT-A injection for chronic neck pain associated with or without associated cervicogenic headache. Likewise, there was no benefit seen for disability and quality of life at four week and six months".
- In systemic review of *low-back pain and sciatica* (Waseem et al., 2010) included three randomized trials ($N = 123$ patients). Only one of the three trials had a low risk of bias and demonstrated that BoNT injections reduced pain at three and eight weeks and improved function at eight weeks compared to saline injections. The second trial showed that BoNT/A injections were better than injections of corticosteroid plus lidocaine or placebo in patients

with sciatica attributed to piriformis syndrome. The third trial concluded that BoNT/A injections were better than traditional acupuncture in patients with third lumbar transverse process syndrome. Heterogeneity of studies prevented the meta-analysis.

- Singh and Fitzgerald (2010) analyzed BoNT/A efficacy in *shoulder pain*. They analyzed six randomized controlled trials with 164 patients receiving either BoNT/A or placebo. BoNT/A decreased pain and improved shoulder function in patients with chronic shoulder pain due to spastic hemiplegia or arthritis, but the sample size was small, and authors concluded that more studies with safety data are needed.
- Hu et al. (2013) published a systemic review analyzing the outcome of BoNT/A treatment of 101 patients with *trigeminal neuralgia* (6 studies: five prospective studies and one double-blind, randomized, placebo-controlled). Beneficial effect was observed in "approximately 70–100% of patients". However, randomized, controlled, double-blinded studies are still lacking.
- In an extensive meta-analysis involving 23 studies and more than 5000 patients, Jackson et al. (2012) analyzed the BoNT/A efficacy in episodic migraine, chronic migraine, chronic daily headache and tension type headache. They concluded that BoNT/A may reduce the mean number of headache days per month in chronic migraine and chronic daily headache, in comparison to placebo. BoNT/A was not beneficial in the treatment of episodic migraine and tension-type headache.

In addition to the small sample size and limited number of randomized controlled clinical trials, the reason for contradictory or negative findings can be the lack of standardized guidelines for BoNT/A application and dosage, and the appropriate definition of study primary outcomes (Jabbari and Machado, 2011).

In spite of controversies, clinical reports on the use of BoNT/A suggest a unique long-lasting pain reduction after a single peripheral application, lasting 3–6 months. This represents the most obvious advantage over classical analgesic drugs, which have a shorter duration of action and need to be taken regularly. Prolonged activity in comparison to other analgesics drives the need for further development of the BoNT/A use in treatment of chronic pain. Another advantage of BoNT/A use may be the lack of serious side effects often associated with certain classic analgesic drugs, such as the development of tolerance and medication overuse. Potential important use of BoNT/A is its reported efficacy in some types of chronic pain refractory to other treatments.

4.2. Preclinical studies

Up to now, the clinical use of BoNT/A in pain has occurred largely empirically. Hundreds of clinical reports on the BoNT/A action on pain (including migraine) can be found In the PubMed database. In contrast to that, if we subtract review articles and commentaries, there are only few dozens of preclinical publications related to BoNT/A and pain in total.

4.2.1. Ex vivo *and* in vitro *studies*

Ex vivo and *in vitro* studies showed that BoNT/A inhibits the evoked release of neuropeptides which modulate the inflammation and pain. In the rabbit iris sphincter muscle, BoNT/A inhibited substance P-ergic component of contraction evoked by electric pulse field (Ishikawa et al., 2000), suggesting that BoNT/A may reduce the peripheral SP release from trigeminal afferents. In rat bladder preparation from rats with cyclophosphamide-induced chronic cystitis, or in bladder preparations treated with HCl, incubation with BoNT/A prevented the *ex vivo* release of SP and CGRP (Rapp et al., 2006; Lucioni et al., 2008). BoNT/A inhibited the release of SP evoked by capsaicin and K^+ in cultured dorsal root ganglion cells (Purkiss et al., 2000; Welch et al., 2000). The toxin's effects were dependent on the presence of extracellular Ca^{2+} (Purkiss et al., 2000). BoNT/A was similarly effective in reducing K^+ or capsaicin-stimulated CGRP release from sensory neurons isolated from trigeminal ganglion, while the basal CGRP release was unaffected (Durham and Cady, 2004). In another study, BoNT/A inhibited the CGRP release from trigeminal sensory neurons evoked by K^+, bradykinin, and to a lesser degree by capsaicin (Meng et al., 2007). In brainstem slices BoNT/A altered the electrophysiologically measured basal CGRP drive on secondary trigeminal neurons, but was unable to alter capsaicin-evoked CGRP drive (Meng et al., 2009). In the same study it was shown that the increase of Ca^{2+} concentration can overcome the BoNT/A-mediated inhibition of K^+-stimulated CGRP release from trigeminal ganglion neurons (Meng et al., 2009). In cultured sensory ganglia BoNT/A prevented the SNARE-mediated TRPV1 translocation to the plasma membrane, which might contribute to its analgesic activity *in vivo* (Morenilla-Palao et al., 2004; Shimizu et al., 2012; Yiangou et al., 2011).

In vitro effect on evoked CGRP release has been screened for different BoNT serotypes: BoNT/A, BoNT/B, BoNT/C1, BoNT/D and BoNT/E (Meng et al., 2007, 2009). It was shown that BoNT/E cannot inhibit the evoked CGRP release in sensory neurons, since SV2A and SV2B protein isoforms, the ectoacceptors for BoNT/E endocytosis, are not present in sensory neurons which express SV2C (2009). In contrast to BoNT/E, BoNT/A binds all three SV2 isoforms, with highest affinity for SV2C. The effect of BoNT/E protease on capsaicin-evoked CGRP release in cultured sensory neurons was established after coupling BoNT/E light chain to BoNT/A heavy chain receptor binding domain (H_C(A)), which yielded EA recombinant chimeric protein (Meng et al., 2009).

4.2.2. In vivo *models*

Chemically induced pain. First preclinical observation of *in vivo* antinociceptive effect of BoNT/A was reported in a model of formalin-induced inflammatory pain. Subcutaneous BoNT/A was found to reduce the inflammatory hyperalgesia during the second phase of formalin test (Cui et al., 2004; Drinovac et al., 2013; Luvisetto et al., 2006; Vacca et al., 2012). Intracerebroventricular injection of BoNT/A, in comparison to peripheral injection, had similar efficacy in reducing formalin-induced pain (Luvisetto et al., 2006). The effect of intrathecal BoNT/A injection on formalin-induced pain has also been reported (Lee et al., 2011). Efficacy of BoNT/A in reducing thermal and mechanical hyperalgesia was also reported in models of acute inflammatory somatic pain evoked by carrageenan and capsaicin (Bach-Rojecky and Lacković, 2005; Favre-Guilmard et al., 2009; Shin et al., 2013). A recent study reported that BoNT/A is equally effective after single and repeated injections in a model of formalin-induced orofacial pain (Matak et al., 2013).

Neuropathic pain. BoNT/A effectiveness in nerve injury-evoked neuropathic pain was found in the partial sciatic nerve transection model (Bach-Rojecky et al., 2005a,b), wherein it reduced the thermal and mechanical hyperalgesia. In the same model BoNT/A was also shown to reduce the mechanical and cold allodynia (Drinovac et al., 2013). Further studies of neuropathic pain reported that BoNT/A reduced the mechanical and cold allodynia in spinal nerve ligation model (Park et al., 2006). Later, its efficacy in reducing mechanical allodynia was reported in sciatic nerve constriction injury-evoked neuropathy (Luvisetto et al., 2007; Marinelli et al., 2010; Mika et al., 2011). It was observed that BoNT/A accelerates the functional recovery of injured sciatic nerve in a model of sciatic constriction injury-induced peripheral neuropathy, assessed by using sciatic static index and regeneration-associated markers (Marinelli et al., 2010; Mika et al., 2011; Pavone and Luvisetto, 2010). In addition, BoNT/A prevented the sciatic

nerve injury-induced upregulation of pronociceptive opioid neuropeptides and SNAP-25 in the sensory ganglia, and markers of glial activation in the spinal cord (Mika et al., 2011; Vacca et al., 2013). These effects were proposed to contribute to the analgesic activity of BoNT/A in neuropathic pain. In a model of chronic constriction injury of the infraorbital nerve, BoNT/A reduced the mechanical allodynia and thermal hyperalgesia (Filipović et al., 2012; Kitamura et al., 2009; Kumada et al., 2012). Along with its analgesic activity, it also reduced the accompanying neurogenic inflammation of cranial dura mater (Filipović et al., 2012).

Bilateral or polyneuropathic pain. BoNT/A efficacy in bilateral pain induced by repeated intramuscular acidic saline injection and diabetic pain evoked by streptozootocin was reported after both peripheral and intrathecal injections (Bach-Rojecky and Lacković, 2009; Bach-Rojecky et al., 2010). BoNT/A was effective in reducing polyneuropathic pain evoked by chemotherapeutic drug paclitaxel (Favre-Guilmard et al., 2009). BoNT/A reduced the bilateral pain evoked by ventral root transection (Xiao et al., 2011, 2013). In these models, unilateral peripheral BoNT/A injection exerted a bilateral antinociceptive effect. (For details on these studies see Section 5.3.)

*Other types of pain. A*part from somatic pain, its efficacy was reported in animal models of acute and chronic visceral pain. BoNT/A prevented the acetic acid-induced bladder pain and capsaicin-evoked prostatic pain, as well as chronic cystitis evoked by cyclophosphamide (Chuang et al., 2004, 2008, 2009). Additionally, BoNT/A efficacy was reported in postsurgical pain (Filipović et al., 2010) and CFA-induced chronic knee arthritis (Krug et al., 2009).

In vivo efficacy of other BoNT serotypes and their recombinantly engineered combinations. BoNT/B efficacy was investigated for the first time in a model of formalin-induced pain in mice (Luvisetto et al., 2006). Peripherally administered BoNT/B, in contrast to BoNT/A, reduced the phase I of formalin-induced pain, while it had no effect on phase II hyperalgesia. Intracerebroventricularly administered BoNT/B had a pro-hyperalgesic activity on the interphase between phase I and phase II of formalin-induced pain (Luvisetto et al., 2006). In a mouse model of chronic knee arthritis induced by collagenase IV intraarticular injection, BoNT/B reduced the joint tenderness and significantly improved the gait score (Anderson et al., 2010). In mice, intrathecally applied BoNT/B reduced the phase II of formalin induced pain and neurokinin-1 receptor internalization in the dorsal horn evoked by central substance P release (Huang et al., 2011). In the *in vivo* model of capsaicin-evoked pain it was shown that a recombinant protein chimera consisting of BoNT/E LC coupled to proteolytically inactive full length BoNT/A may induce a prolonged analgesic activity (Dolly et al., 2011). It seems that proteolytically inactive BoNT/A facilitated the BoNT/E LC entrance into sensory neurons. Additionally, BoNT/A part of the molecule protected the BoNT/E LC from proteasomal degradation, enabling its prolonged activity (Dolly et al., 2011). Recently, analgesic efficacy of chimeric BoNT/A LC coupled to TeNT Hc was demonstrated in complete Freund's adjuvant-induced inflammatory pain (Ferrari et al., 2013).

4.3. Peculiar properties of BoNT/A antinociceptive activity

Unlike most analgesics which affect acute nociceptive pain thresholds, BoNT/A shows a considerable selectivity only in lasting types of pain associated with central sensitization. BoNT/A primarily seems to lower hyperalgesic or allodynic responses in acute inflammatory or chronic pain. It does not alter normal nociceptive thresholds or acute nociceptive pain in both humans and animals, as well as phase I of formalin-induced experimental pain (Blersch et al., 2002; Cui et al., 2004; Bach-Rojecky et al., 2005a,b).

Together with the observed lack of effect on acute nociceptive pain, another important difference between conventional analgesics and BoNT/A is the lack of defined dose-response effects on pain, *i.e.* the correlation between the dose of peripherally delivered toxin and observed antinociceptive effect (Bach-Rojecky et al., 2005a,b, 2010; Bach-Rojecky and Lacković, 2005). At lower toxin doses which do not impair the motor performance, it seems that the full analgesic effect is exerted already at the lowest effective dose (Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2010). For example, at 3 U/kg BoNT/A seems not to affect the carrageenan and capsaicin-evoked pain, but at slightly higher dose (3.5 U/kg), and further increased doses (5 and 7 U/kg) BoNT/A exerts similar and maximal analgesic activity (Bach-Rojecky and Lacković, 2005). Similar antinociceptive effect of 3.5, 7 and 15 U/kg BoNT/A doses was reported in formalin test (Cui et al., 2004). In few studies the authors described the increased analgesic effects occurring at high doses of toxin applied (20–40 U/kg) (Cui et al., 2004; Park et al., 2006). However, systemic spread of BoNT/A impaired the animal motor performance, which most likely interfered with the ability to produce a nocifensive reaction (Cui et al., 2004). Up to now, clinical trials also did not address the dose response of BoNT/A, and the doses employed were defined only empirically.

5. Peripheral or central mechanism of BoNT/A's antinociceptive activity

5.1. Peripheral theory of BoNT/A's antinociceptive effects

As an explanation of its antinociceptive effect it was suggested that BoNT/A, similarly to well-known prevention of acetylcholine release from neuromuscular junction, inhibits the local neurotransmitter release from sensory nerve endings by peripheral SNAP-25 cleavage (Cui et al., 2004; Aoki, 2005; Aoki and Francis, 2011; Wheeler and Smith, 2013). This suggestion was based primarily on the observation of Cui et al. (2004), who found that subcutaneous injection of BoNT/A reduces the licking activity during the second (inflammatory) phase of pain induced by subcutaneous injection of formalin. BoNT/A did not reduce the initial phase of licking behavior caused by direct chemical stimulation of peripheral nerve endings with formalin. Selective reduction of inflammatory pain was accompanied by inhibition of formalin-induced increase of peak glutamate concentration in the rat hind-paw, and reduction of paw edema, assumed to be a consequence of neurogenic inflammation. Antinociceptive activity of BoNT/A was therefore suggested to be linked with its peripheral anti-inflammatory effects (Aoki, 2005; Cui et al., 2004). It was proposed that cleavage of SNAP-25 at peripheral endings of sensory nerves results in consecutive prevention of release of neurotransmitters which mediate pain and inflammation (Aoki, 2005; Aoki and Francis, 2011; Wheeler and Smith, 2013). The authors hypothesized that BoNT/A may reduce the peripheral release of not only glutamate, but also of neuropeptides involved in neurogenic inflammation such as substance P (SP) and calcitonin gene-related peptide (CGRP) (in the report of Cui et al. (2004), peptides were not directly measured) (Aoki, 2005; Cui et al., 2004). The effect of BoNT/A on the release of proinflammatory mediators was demonstrated mostly in *in vitro* and *ex vivo* experiments (Section 4.2.1). In addition, the suppressive effect of BoNT/A on neuronal activation, measured as formalin-induced c-Fos expression or electrophysiologically measured activation of wide dynamic range neurons in lumbar dorsal horn was reported (Aoki, 2005). The authors proposed that BoNT/A, by preventing the peripheral release of neurotransmitters, indirectly reduces the central sensitization (Aoki, 2005; Aoki and Francis, 2011).

Antinociceptive and anti-inflammatory effects of BoNT/A were reported in human models of capsaicin-induced pain (Gazerani et al., 2006, 2009; Tugnoli et al., 2007). Gazerani et al. (2009) reported a reduction of pain and capsaicin-induced vasomotor

reactions in the human skin within the trigeminal area. Similar anti-inflammatory effects were observed by Tugnoli et al. (2007), who reported that BoNT/A reduced pain and capsaicin-induced neurogenic effects in human skin in the area pre-treated by BoNT/A. Contrary to these reports, studies of Schulte-Mattler et al. (2007) and Voller et al. (2003) did not report significant anti-inflammatory or antinociceptive effects of BoNT/A on capsaicin-evoked pain in humans. The differences between the described studies might be related to the experimental setup regarding the mode of toxin injection (subcutaneous, intramuscular or intradermal) or different BoNT/A doses (Gazerani et al., 2006, 2009; Schulte-Mattler et al., 2007; Tugnoli et al., 2007; Voller et al., 2003). Additionally, BoNT/A does not reduce the experimental inflammatory pain evoked by UV light in humans (Sycha et al., 2006).

5.2. Dissociation of BoNT/A antinociceptive activity and peripheral anti-inflammatory effects

As mentioned in the previous section, BoNT/A was found to reduce the formalin-induced inflammation, increased glutamate content in hind paw tissue, and inflammatory phase hyperalgesia (Aoki, 2005; Cui et al., 2004). At the time, it was logical to assume that the relation between BoNT/A effect on inflammation and pain was causal – BoNT/A reduces the pain and indirectly the central sensitization by reducing peripheral neurotransmitter/inflammatory mediator release. However, the link between the effects of BoNT/A on peripheral inflammation and pain could not be confirmed in subsequent reports (Bach-Rojecky et al., 2005a,b, 2008; Favre-Guilmard et al., 2009; Shin et al., 2013).

In experimental carrageenan or capsaicin-induced inflammatory pain, BoNT/A normalized the mechanical and thermal hyperalgesia, but neither local tissue inflammatory edema and proinflamatory cell infiltration evoked by carrageenan, nor plasma protein extravasation induced by capsaicin were reduced (Bach-Rojecky et al., 2005a,b, 2008; Favre-Guilmard et al., 2009; Shin et al., 2013). These experiments demonstrated that the antinociceptive and anti-inflammatory effects of BoNT/A are not necessarily connected as it should be expected if both were mediated by reduced peripheral release of proinflammatory neuropeptides (Bach-Rojecky et al., 2008). In the study of Cui et al. (2004), there seems to be a difference between the minimal BoNT/A doses needed to produce anti-inflammatory and antinociceptive effects. Reduction of formalin-evoked paw edema was significant at a 7 U/kg dose, and was not different from control at 3.5 U/kg. Both doses produced the antinociceptive effect without significant difference.

Dissociation between anti-inflammatory and antinociceptive effects of BoNT/A was also reported by a human experimental studies. Tugnoli et al. (2007) observed pain reduction only when capsaicin was administered to toxin-pretreated area, but not when injected in the area adjacent to toxin treatment. On the contrary, neurogenic flare and vasodilatation was reduced even if capsaicin was administered adjacently to the toxin-pretreated area, but the pain was not affected. Based on this observation the authors proposed that the toxin's anti-inflammatory effect on neurogenic flare and vasodilatation do not significantly contribute to BoNT/A-mediated pain reduction (Tugnoli et al., 2007). Similar conclusion was proposed in the human study of Krämer et al. (2003) which reported that BoNT/A reduced the neurogenic flare evoked by cutaneous electrical stimulation, however, with very limited analgesic effect. The authors suggested that the reduction of neuropeptide-mediated peripheral neurogenic inflammation does not contribute significantly to BoNT/A analgesic effects observed in clinical pain syndromes (Krämer et al., 2003).

Independently of the potential connection between its anti-inflammatory and antinociceptive effects, it was observed that peripherally delivered BoNT/A has significant antinociceptive effect in types of pain where neither peripheral neurotransmitter release nor peripheral inflammation have important role, such as bilateral hyperalgesia induced by intramuscular acidic saline (described in Section 5.3).

5.3. Effects on bilateral pain: indication of central action of BoNT/A

Bilateral pain model studies reported bilateral effects of unilaterally administered BoNT/A (Bach-Rojecky et al., 2005a,b, 2010; Lacković et al., 2006; Bach-Rojecky and Lacković, 2009; Favre-Guilmard et al., 2009; Xiao et al., 2011; Filipović et al., 2012; Xiao et al., 2013). BoNT/A's distant contralateral effects, obviously, cannot be explained only by BoNT/A action on peripheral nerve endings of injected side.

Bilateral hyperalgesia induced by intramuscular acidic saline. Mirror pain is defined as the occurrence of mechanical sensitivity in the uninjured contralateral "mirror image" body structures after unilateral injury, possibly mediated by diffusible signaling molecules or bilateral neural pathways at the spinal cord level (Koltzenburg et al., 1999). Mirror pain might also involve bilateral supraspinal structures and descendent facilitatory pathways (Da Silva et al., 2010).

In our laboratory, the effect of BoNT/A on centrally mediated mirror pain was examined in a model of bilateral muscular hyperalgesia (Bach-Rojecky et al., 2005a,b; Bach-Rojecky and Lacković, 2009). In this model, two injections of acidic saline (pH = 4) into the gastrocnemius lead to development of bilateral mechanical hyperalgesia on both hind-limbs (Sluka et al., 2001). Bilateral hyperalgesia induced by intramuscular acidic saline injections was proposed to be mediated by central sensitization and supraspinal bilateral pathways (Da Silva et al., 2010; Sluka et al., 2001). Hypothetically, if the BoNT/A antinociceptive action is mediated by the prevention of peripheral neurotransmitter release, then it should not reduce the contralateral acidic saline-induced bilateral hyperalgesia. Toxin's effect at the level of CNS seems the only convincing explanation. The puzzling fact is that the toxin injected into the contralateral limb reduced the pain on that side only, ruling out a possible systemic diffusion of BoNT/A.

Paclitaxel-induced polyneuropathy. Bilateral effects of unilateral toxin injection were also observed in a model of peripheral polyneuropathy induced by chemotherapeutic drug paclitaxel (Favre-Guilmard et al., 2009). Paclitaxel-induced polyneuropathy in rats develops after intraperitoneal high-dose injection of chemotherapeutic, resulting in bilateral decrease of hind-paw mechanical withdrawal thresholds. After BoNT/A injection into one hind-paw, decrease in mechanical withdrawal thresholds was reversed on both hind-paws. Since BoNT/A injected contralaterally failed to reduce pain evoked by unilateral carrageenan, the authors ruled out possible systemic spread of BoNT/A as the mechanism of bilateral effect (Favre-Guilmard et al., 2009).

Experimental diabetic pain. In a model of beta-cytotoxic drug streptozotocin-induced type I diabetes accompanied by neuropathy, BoNT/A injected unilaterally induced the bilateral effect on mechanical pain. The effect of BoNT/A was delayed: it was evident on 5th day after BoNT/A injection, but not after 24 h. However, after intrathecal injection of peripherally ineffective low dose (1 U/kg), BoNT/A effect occurred within 24 h.

Due to systemic polyneuropathic effect of paclitaxel, and widespread neuropathy occurring in experimental diabetes type I, pathological neural changes are bilaterally symmetric regarding the side of BoNT/A injection. Bilateral effect of unilaterally delivered BoNT/A is therefore difficult to explain without considering a central site of action. Bilateral effects after unilateral toxin injections were also reported in models of neuropathic pain induced by ventral root transection (Xiao et al., 2011, 2013), and

infraorbital nerve constriction (Filipović et al., 2012). It seems that BoNT/A bilateral effect on bilateral and mirror pain models is a general rule, *i.e.* not dependent on the type of experimental pain.

In some studies, the onset of the antinociceptive effect in formalin-induced pain and experimental model of inflammatory pain (Cui et al., 2004; Mika et al., 2011) started within 24 h. However, most of other studies reported the delay of antinociceptive activity of up to 5–7 days, while peripheral neuromuscular paralysis in animals usually occurs within 24 h (Bach-Rojecky et al., 2005a,b, 2010; Bach-Rojecky and Lacković, 2009; Chuang et al., 2004; Filipović et al., 2012). In addition, BoNT/A's antinociceptive activity occurred within 24 h when applied intrathecally at peripherally ineffective doses (Bach-Rojecky et al., 2010). The observed faster onset of antinociceptive activity of BoNT/A after central application is difficult to explain by presumed peripheral site of BoNT/A action (Bach-Rojecky et al., 2010). Based on the bilateral effect and faster onset of action of intrathecally applied small BoNT/A dose, the authors proposed that BoNT/A effect was centrally mediated (Bach-Rojecky et al., 2010).

6. Axonal transport of BoNT/A

Behavioral data obtained from bilateral pain models suggested that BoNT/A effect on pain might involve the toxin movement and its direct activity in the CNS. Hematogenous route as the underlying pathway for penetration into the CNS is unlikely since experimentally used low doses of BoNT/A do not induce systemic poisoning. In addition, BoNT/A is a large protein (150 kDa) which cannot cross the blood-brain barrier. Other possible explanation is the axonal transport of BoNT/A from periphery to CNS. However, the axonal transport of BoNT/A, up to recently, was classically believed to be non-existent or very limited, and its activity in the brain following peripheral delivery was questionable (Section 6.1).

6.1. Early studies of BoNT/A axonal transport to CNS

It is widely known that BoNT/A induces local paralysis when injected into the muscles treated for neuromuscular disorders or cosmetic use. In the literature, due to the prevailing opinion that BoNT/A axonal transport is either too slow or non-existent, and the lack of clinically observable central side-effects, the possibility of direct central action of BoNT/A has been largely neglected. Although the possibility of retrograde axonal traffic to the spinal cord was hypothesized during the early preclinical research of BoNT/A, prevailing opinion remained that BoNT/A effects are exclusively locally mediated (Aoki and Francis, 2011; Tang-Liu et al., 2003).

As suggested by some earlier studies, the toxin might penetrate into the central nervous system by axonal transport (Habermann, 1974; Wiegand et al., 1976; Wiegand and Wellhöner, 1977). To trace the possible spread of BoNT/A into the CNS, the authors employed ^{125}I-radiolabeled BoNT/A injections into the cat gastrocnemius muscles. Following injection of ^{125}I-labeled BoNT/A, progressive movement of radioactivity was detected along the neuronal pathway directed to CNS: firstly in the sciatic nerve, followed by ipsilateral spinal ventral roots. In the end, radioactivity was detected in the corresponding ipsilateral spinal cord segments 48 h following the toxin injection. However, these studies could not demonstrate that the enzymatically active BoNT/A reached the CNS. Other studies also reported that the peripherally injected BoNT/A is transported retrogradely within the axonal compartment (Black and Dolly, 1986). Nevertheless, due to the slow axonal traffic it was argued that the toxin is likely to be inactivated before it reaches the CNS (Black and Dolly, 1986). More recently, Tang-Liu et al. (2003) injected radioiodinated neurotoxin complex into the gastrocnemius muscle of rats (70 U) and eyelids of rabbits (24 U). The diffusion of radioactivity from the site of injection was measured at different time-points (0.5, 2, 6, 24 and 48 h post-injection). The authors reported that the majority of neurotoxin remained localized at the site of injection. Following the intramuscular injection, radioactivity was detected in sites distal to the injection site. In particular, significant amounts were recovered from thyroid gland and contralateral muscles. However, the authors suggested that this radioactivity did not appear to represent an intact neurotoxin and it was assumed that the signal may be associated with non-toxic proteins of the complex, or ^{125}I which dissociated from the complex (Tang-Liu et al., 2003).

6.2. Behavioral and pharmacological evidence for the necessity of BoNT/A axonal transport for its antinociceptive activity

6.2.1. Axonal transport necessary for BoNT/A antinociceptive activity occurs in sensory neurons

The importance of axonal transport of BoNT/A for its antinociceptive activity was demonstrated behaviorally for the first time in a model of acidic saline-induced bilateral mechanical hyperalgesia (Bach-Rojecky and Lacković, 2009). In line with the proposed central site of action, injection of small dose BoNT/A (0.5 U/kg) into the stump of a distally transected sciatic nerve reduced the acidic saline-induced hyperalgesia on the contralateral side. This experiment excluded the involvement of peripheral nerve endings as the indirect site of BoNT/A action on the contralateral side. In addition, it suggested BoNT/A retrograde axonal movement within peripheral nerve. Indeed, the axonal transport blocker colchicine injected into the sciatic nerve ipsilaterally to BoNT/A peripheral treatment eliminated the antinociceptive effect on both sides. This observation suggested that BoNT/A requires axonal transport along the sciatic nerve in order to exhibit its antinociceptive effect. Colchicine injected into the contralateral sciatic nerve did not affect BoNT/A action on either side, ruling out the possibility of toxin spread to nerve endings of contralateral hind-limb. This set of observations indicated that BoNT/A axonal transport occurs through peripheral nerves and is directed to CNS, but it has remained unknown whether the transport occurs in motor neurons or sensory neurons (Bach-Rojecky and Lacković, 2009).

To investigate whether the BoNT/A traffic occurs through sensory neurons, its axonal transport was further studied in trigeminal system. Injections of low doses of BoNT/A into the whisker pad (3.5 U/kg) reduced formalin-induced orofacial pain and allodynia induced by experimental trigeminal neuropathy (Filipović et al., 2012; Matak et al., 2011). Effect on pain was prevented by axonal transport blocker colchicine injected into the trigeminal ganglion (Filipović et al., 2012; Matak et al., 2011) Sensory character of trigeminal ganglia suggests that axonal transport occurs indeed in sensory axons. In line with that suggestion, antinociceptive effect in orofacial formalin test occurred also after direct BoNT/A injection (1 U/kg) into the trigeminal sensory ganglion (Matak et al., 2011). The onset of antinociceptive action of intraganglionic BoNT/A was, however, delayed. It occurred 2 days after toxin delivery into the ganglion. Intraganglionic colchicine again prevented the BoNT/A antinociceptive effect when toxin was delivered directly into the ganglion. Delayed action upon ganglionic delivery and necessity of axonal transport suggested that BoNT/A, in order to reduce pain, must be anterogradely transported further from ganglion into the CNS.

Interestingly, peripherally induced pain in trigeminal area was accompanied by neurogenic extravasation of plasma proteins (measured spectrophotometrically with Evans blue dye) in the cranial dura mater. Peripherally delivered BoNT/A prevented this phenomenon and the action was, again, colchicine sensitive *i.e.* axonal transport dependent (Filipović et al., 2012).

6.2.2. Effects of BoNT/A in sensory ganglia

Kitamura et al. (2009) showed that BoNT/A inhibits the vesicular release from trigeminal ganglion neurons acutely isolated from animals with experimental trigeminal neuropathy and pretreated with BoNT/A peripherally. As the explanation for the effect on vesicular release (measured by FM4-64 dye), the authors proposed BoNT/A axonal transport from periphery and transcytosis within the trigeminal ganglion. Novel study reported reduction of TRPV1 expression within the trigeminal ganglion neurons projecting to cerebral dura mater after BoNT/A facial injection (Shimizu et al., 2012). Since neurons innervating the dura and periphery are different, the authors suggested axonal transport of BoNT/A to trigeminal ganglion, but, also, a possible transcytosis of BoNT/A between different sensory neurons in the ganglion. It was proposed that BoNT/A may reduce the TRPV1-expression in sensory neurons which innervate dura by modulating the SNARE-mediated TRPV1 translocation to plasma membrane (Shimizu et al., 2012).

BoNT/A effects at the level of sensory ganglion were also reported in a model of ventral root transection-induced neuropathy. It was found that BoNT/A prevented the up-regulation of pain receptors purinergic receptor P2X3 and transient receptor TRPV1. The authors proposed that BoNT/A-mediated pain reduction may be linked to reduced expression of receptors and ion channels involved in pain pathophysiology (Xiao et al., 2011, 2013).

6.3. Neurophysiological evidence for axonal transport of BoNT/A

6.3.1. Studies in humans

Studies performed in animals suggest that retrograde axonal transport of BoNT/A occurs regularly after low-dose peripheral injections and *via* different routes of administration (Bach-Rojecky et al., 2008; Filipović et al., 2012; Matak et al., 2011). Although the BoNT/A axonal transport in humans has never been assessed directly, several neurophysiological studies reported central effects after peripheral BoNT/A application. Already in 1963 Tyler reported alterations of the H reflex in a man with botulism, indicative of alterations at the spinal level (Tyler, 1963). Similar observations suggested that the axonal transport of BoNT/A to CNS might also exist in humans treated clinically for neuromuscular disorders or intoxicated with BoNT/A (Garner et al., 1993; Marchand-Pauvert et al., 2013; Santini et al., 1999; Wohlfarth et al., 2001; reviewed recently by Caleo and Schiavo, 2009; Palomar and Mir, 2012). By employing single fiber electromyography, Garner et al. (1993) reported reduced activity of the distant, non-injected muscle (extensor digitorum brevis) in patients treated with BoNT/A for focal dystonia in the head and neck region. Authors discussed the possibility of a very efficient local uptake of BoNT/A and retrograde axonal transport to the CNS (Garner et al., 1993). In another study performed in patients treated with BoNT/A for spasmodic torticollis and writer's cramps, the remote changes in motoneuronal excitability (F-wave changes) were observed (Wohlfarth et al., 2001). Authors ruled out possible BoNTA diffusion to remote muscles or distant muscle spindle afferents. As a possible explanation, BoNT/A action on the level of motoneuronal bodies in CNS was proposed (Wohlfarth et al., 2001).

Recently, possible existence of BoNT/A axonal transport *via* motoneuronal axons to their recurrent axonal collaterals in the CNS was reported by Marchand-Pauvert et al. (2013). They measured recurrent inhibition in the injected and non-injected leg muscles in patients treated for spasticity. The authors observed depressed recurrent inhibition of distant, non-injected muscles. Although it cannot be completely ruled out, they argued against indirect peripheral or systemic BoNT/A effect. Distant changes of recurrent inhibition suggest a direct BoNT/A effect at the cholinergic synapse between recurrent collaterals of primary motoneurons and Renshaw interneurons in the ventral horn (Marchand-Pauvert et al., 2013). Possibility that peripherally delivered BoNT/A may target the SNAP-25 in ventral horn cholinergic synapses was experimentally confirmed by immunohistochemistry in rats (Matak et al., 2012).

6.3.2. Neurophysiological evidence for BoNT/A axonal transport in animals

Few neurophysiological studies in experimental animals employing high doses of BoNT/A reported indicated a possibility of BoNT/A retrograde spread into the CNS. BoNT/A 3 ng injection into the cat abducens muscle produced alterations in the discharge pattern of abducens motoneurons. This was accompanied by ultrastructural synaptic alterations at the level of motoneuronal cell bodies (Moreno-López et al., 1997; Pastor et al., 1997). The authors suggested retrograde and possible transsynaptic spread of high-dose BoNT/A.

Recently, possible retrograde and transsynaptic traffic of BoNT/A was reported (Torii et al., 2011; Akaike et al., 2013). Injection of BoNT/A subtypes A1 and A2 at high doses into rat forelimb or hindlimb exhibited a dose-dependent bilateral muscle relaxation. Interestingly, contralateral effects of BoNT/A1 (commercially available onabotulinumtoxin A) were partially mediated by axonal transport. Contralateral effects of BoNT/A2 serotype, occurring only at very high doses, were mediated by systemic diffusion. The authors suggested that BoNT/A1 is retrogradely transported to the CNS, and then anterogradely into the contralateral muscle (Akaike et al., 2013; Torii et al., 2011). The authors also reported BoNT/A distant effect on glycinergic transmission in the dorsal horn, and suggested toxin's transcytosis to glycinergic synapses (Akaike et al., 2013).

6.4. Axonal transport of enzymatically active BoNT/A in the CNS and motoneurons

By employing BoNT/A enzymatic activity detection, axonal transport of active BoNT/A molecules was found in the rodent visual system and facial nerve using an antibody specific to the product of BoNT/A proteolytic activity (Antonucci et al., 2008). Unlike radioactively labeled toxin, cleaved SNAP-25 detection suggests presence of enzymatically active BoNT/A protease. Single BoNT/A molecule may enzymatically cleave many SNAP-25 molecules, leading to higher sensitivity of detection. Cleaved SNAP-25 can then be detected by immunohistochemistry and Western blot.

Antonucci et al. (2008) demonstrated that the unilateral hippocampal injection of BoNT/A resulted in the toxin traffic to contralateral hippocampus and ipsilateral entorhinal cortex *via* direct axonal projections. Moreover, BoNT/A applied into the unilateral hippocampus reduced the neuronal hippocampal activity in contralateral hemisphere. Additionally, BoNT/A injected into the superior colliculus was axonally transported to the contralateral retina and ipsilateral visual cortex (Antonucci et al., 2008). BoNT/A injected into the facial whisker muscles resulted in occurrence of cleaved SNAP-25 in the facial motor nucleus 3 days after the peripheral injection. This observation suggested that peripherally administered BoNT/A may be retrogradely transported to the brainstem *via* facial motor neurons (Antonucci et al., 2008).

Study of Antonucci et al. (2008) was, however, criticized due to the use of high doses of non-commercial preparation of BoNT/A (Alexiades-Armenakas, 2008). Additional questions were raised regarding the ability of antibody-based detection method to discriminate between cleaved and non-cleaved SNAP-25 protein (Aoki and Francis, 2011). In later studies which employed the same antibody to cleaved SNAP-25 as in study from Antonucci et al.

(2008), these questions were answered (Matak et al., 2011, 2012). Central SNAP-25 cleavage was detected in CNS even at low doses of peripherally injected commercially available onabotulinumtoxinA (Matak et al., 2011, 2012). When BoNT/A was injected into the gastrocnemius muscle (5 U/kg), cleaved SNAP-25 was detected in corresponding ipsilateral segments of lumbar spinal cord. These studies indicated that BoNT/A axonal transport to CNS occurs regularly at low peripheral doses of commercially available BoNT/A, comparable to doses used clinically (Matak et al., 2011, 2012). Antibody specificity for BoNT/A-cleaved form of SNAP-25 was verified by comparing Western blot signals of control and BoNT/A-injected rat hippocampus (Matak et al., 2011). 24 kDa signal belonging to cleaved SNAP-25 appeared only in toxin-injected brain tissue. Position of the 24 kDa signal, detected first by the antibody to cleaved SNAP-25, was subsequently confirmed by a well-characterized antibody which binds both intact and cleaved SNAP-25. This experiment demonstrated that the antibody specifically targets the BoNT/A-cleaved sequence of C-terminal SNAP-25 and not the whole SNAP-25 protein (Matak et al., 2011).

Based on an *in vitro* study of BoNT/A movement in sympathetic neurons, Lawrence et al. (2012) suggested that BoNT/A and BoNT/E spread within cell bodies and distal neuronal processes may occur due to passive diffusion. However, it was demonstrated *in vivo* that BoNT/A enzymatic activity in ipsilateral lumbar spinal cord, occurring after its injection into the sciatic nerve, can be prevented by intrasciatic colchicine. Prevention of occurrence of central cleaved SNAP-25 by colchicine demonstrated that the traffic of BoNT/A within peripheral nerves to CNS involves a colchicine-sensitive, microtubule-dependent axonal transport (Matak et al., 2012). Time required for occurrence of truncated SNAP-25 product in the CNS after peripheral injections in rats (3–5 days), and the long distance from the injection site to central regions where the toxin's proteolytic activity was observed, rule out the possibility of passive intraneuronal diffusion (Antonucci et al., 2008; Matak et al., 2012).

Studies involving cleaved SNAP-25 detection lacked the direct evidence for toxin traffic *via* axonal transport. Immunohistochemistry of BoNT/A-truncated SNAP-25 in regions distant from the injection site provided only the indirect evidence of toxin's axonal traffic. Theoretically, instead of BoNT/A, truncated SNAP-25 could have been transported along nerves. Strong *in vivo* evidence in favor of BoNT/A traffic was provided by experiments demonstrating the long-term activity of BoNT/A protease in different parts of optic system distant from injection site (Antonucci et al., 2008; Restani et al., 2011). After tectal injection, toxin's proteolytic activity was detected in retina (Antonucci et al., 2008). The authors then cut the optic nerve to prevent the additional axonal transport from toxin-injected tectum. Subsequently, they employed eye intravitreal injection of transiently active BoNT/E, which cleaves SNAP-25 at a cleavage site further from C-terminal compared to BoNT/A. BoNT/E is therefore able to cleave both intact and BoNT/A-cleaved SNAP-25 and convert them to a single population of BoNT/E-cleaved SNAP-25 (Keller et al., 1999). Intravitreal BoNT/E transiently reduced the immunoreactivity of BoNT/A-truncated SNAP-25 in retina. Re-appearance of BoNT/A-truncated SNAP-25 upon completion of BoNT/E effects demonstrated that the BoNT/A protease itself had been axonally transported to the retina. BoNT/A-truncated SNAP-25 re-occurred due to longer intracellular duration of proteolytic action of BoNT/A LC in comparison to BoNT/E LC (Antonucci et al., 2008; Keller et al., 1999). In a similar experiment it was demonstrated that BoNT/A protease was anterogradely transported within the optic nerve and transcytosed to second-order synapses in the superior colliculus (Restani et al., 2011). BoNT/A proteolytic activity was demonstrated in optic tectum 3 days after BoNT/A application in the eye. The optic nerve was then transected to prevent the additional axonal transport from retina. Subsequent BoNT/E application into the superior colliculus transiently depleted the immunoreactivity of BoNT/A-truncated SNAP-25. Re-occurrence of BoNT/A-truncated SNAP-25 after completion of BoNT/E effects demonstrated the long-term presence of anterogradely transported and transcytosed BoNT/A.

Further studies on the BoNT/A activity in the optic system provided the evidence for axonal transport and transcytosis to higher order synapses in CNS, with resulting blockage of neurotransmitter release in distant synapses (Restani et al., 2012a). When BoNT/A was injected into the optic tectum, ultrastructural analysis demonstrated swelling and accumulation of synaptic vesicles inside retinal terminals, indicative of impaired neuroexocytosis (Restani et al., 2012a). Additionally, BoNT/A injected into the tectum of rat pups induced an inhibition of cholinergic-driven wave activity in retina. This study demonstrated BoNT/A transcytosis from retinal ganglion neurons to cholinergic amacrine cells, and subsequent inhibition of acetylcholine release (Restani et al., 2012a).

In addition to the indirect *in vivo* evidence involving immunodetection of BoNT/A-cleaved SNAP-25, axonal transport of BoNTs was directly visualized *in vitro* by examining the traffic of Alexa Fluor-fluorescently labeled BoNT/A and BoNT/E within the compartmentalized culture of primary motor neurons (Restani et al., 2012b). It was observed that both full-length toxins, as well as their heavy chains, were internalized into the neuronal non-acidic vesicles. Vesicles containing toxins were then redirected to the fast retrograde axonal transport machinery in motoneuronal axons (Restani et al., 2012b). The authors suggested that BoNT/A and BoNT/E share similar intra-vesicular axonal trafficking pathways with different neurotrophic factors, viral pathogens and TeNT.

6.5. Immunohistochemical evidence for axonal transport of enzymatically active BoNT/A to central nociceptive regions

Behavioral data involving colchicine and intraneural or intraganglionic BoNT/A injections suggested that BoNT/A-mediated antinociceptive activity involves axonal transport of active toxin molecule within peripheral sensory neurons, possibly to the CNS (Section 6.2.1). Evidence that the axonally transported BoNT/A molecules are directed to central sensory regions was provided by immunohistochemistry of cleaved SNAP-25. By employing the same antibody as Antonucci et al. (2008), central cleaved SNAP-25 was detected in spinal trigeminal nucleus caudalis after toxin injection into the trigeminal area (Fig. 3; Matak et al., 2011). Cleaved SNAP-25 in CNS was observed starting from 3 days after toxin peripheral injection. BoNT/A-truncated SNAP-25 occurred in trigeminal nucleus caudalis even at 3.5 U/kg, the lowest peripheral dose able to induce the antinociceptive effect in rats (Bach-Rojecky and Lacković, 2005; Matak et al., 2011). Similarly, hind-limb injection of BoNT/A resulted in cleavage of SNAP-25 in lumbar dorsal horn at low peripheral dose (5 U/kg) (Matak et al., 2012), suggesting a long-distance axonal traffic in spinal sensory neurons. BoNT/A enzymatic activity in lumbar dorsal horn and trigeminal sensory nuclei indicated that BoNT/A may interfere with nociceptive neurotransmission between peripheral and central sensory neurons, most likely by preventing the SNARE-mediated vesicular neurotransmitter release (Matak et al., 2011).

In mice, it was reported that BoNT/A-mediated SNAP-25 cleavage is present along the nociceptive pathway: in the injected hind-paw skin, along the sciatic nerve, in peripheral ganglia and within the spinal cord (Marinelli et al., 2012). Most recent study suggested that BoNT/B, applied at high peripheral dose, may also be axonally transported in mouse sensory neurons. Marino et al. (2014) reported reduction of VAMP/synaptobrevin immunoreactivity in dorsal root ganglia after BoNT/B intraplantar injection in

mice, suggesting BoNT/B enzymatic activity in the ganglia. In addition, the authors showed reduced spinal neurokinin 1 receptor internalization evoked by intraplantar formalin or intrathecal capsaicin, and suggested that BoNT/B may reduce the spinal substance P presynaptic release (Marino et al., 2014).

7. What is the mechanism of BoNT/a antinociceptive action in CNS? Possible role of opioidergic and GABA-ergic neurotransmission

In this review we have discussed the evidence that BoNT/A antinociceptive effect is centrally mediated. However, up to now, the mechanism of central antinociceptive action has remained unknown. Recently, it was found that it might be connected with opioid and GABA-ergic system in the CNS.

Few experimental studies demonstrated the synergistic activity of ineffective doses of morphine and BoNT/A on inflammatory pain induced by carrageenan and formalin, and neuropathic pain evoked by chronic constriction sciatic injury (Auguet et al., 2008; Vacca et al., 2012, 2013). In addition, peripherally applied BoNT/A prevented the development of morphine-induced tolerance and associated glial activation in lumbar spinal cord (Vacca et al., 2012, 2013). These studies indirectly suggested that BoNT/A's antinociceptive action might be connected with the endogenous opioid system. Study from our laboratory (Drinovac et al., 2013) showed that opioid antagonist naltrexone, injected both systemically or intrathecally, dose-dependently prevented the BoNT/A-mediated reduction of second phase nocifensive behavior (paw licking and flinching) in a model of formalin-induced pain. Demonstrated efficacy of low intrathecal dose of naltrexone suggested that the effect is mediated at the spinal level. BoNT/A activity was also prevented by more selective μ-antagonist naloxonazine. Reduction of dorsal horn c-Fos expression by BoNT/A in a model of formalin-induced pain was also prevented by naltrexone. Additionally, systemically injected naltrexone prevented the BoNT/A's antinociceptive effects on mechanical hypersensitivity and cold allodynia in a model of partial nerve transection-induced neuropathic pain. These observations suggested that BoNT/A's antinociceptive activity in acute inflammatory and neuropathic pain is associated with the enhanced function of endogenous opioid system involving central μ-opioid receptors (Drinovac et al., 2013).

In addition, we examined the possible role of GABA-ergic inhibitory neurotransmission in BoNT/A action because of its wide involvement in chronic pain patophysiology (Drinovac et al., 2014). Similarly to opioid antagonists, GABA-A receptor antagonist bicuculline prevented the BoNT/A-mediated antinociceptive effect in formalin test and sciatic nerve transection-induced neuropathy. Intrathecal bicuculline prevented the BoNT/A action similar to systemic bicuculline, while intracisternal application had no effect, suggesting that BoNT/A interacts with GABA-ergic transmission at the spinal cord level. Enhancement of inhibitory neurotransmission might be associated with BoNT/A efficacy in relieving chronic pain and hypersensitivity (Drinovac et al., 2014).

BoNT/A effect in CNS, along with proposed inhibition of SNARE-mediated central neurotransmitter release (Matak et al., 2011, 2012; Fig. 4), seems to involve enhanced endogenous opioidergic and GABA-ergic transmission (Drinovac et al., 2013, 2014). These data at first might seem counter-intuitive, since BoNT/A is a selective blocker of neurotransmitter release, with preference for excitatory synapses. We speculate that BoNT/A-mediated blockage of excitatory synapses might lead indirectly to enhanced inhibitory neurotransmission *via* yet unknown mechanism.

8. Concluding overview

8.1. *Central* vs. *peripheral action of BoNT/A*

Main arguments for the peripheral site of BoNT/A action on pain are based on its inhibitory effects on peripheral glutamate and neuropeptide release, and its presumed analogy to widely known BoNT/A anticholinergic effects on peripheral motor nerve endings and autonomic synapses (Table 1, left column). This hypothesis was modified with the assumption that repeated stimulation, inflammation or nerve injury may sensitize peripheral nerve endings resulting in excess stimulation of CNS leading to central sensitization (Aoki and Francis, 2011). Accordingly, BoNT/A "may directly inhibit primary sensory fibers, leading to a reduction of peripheral sensitization, and an indirect reduction in central

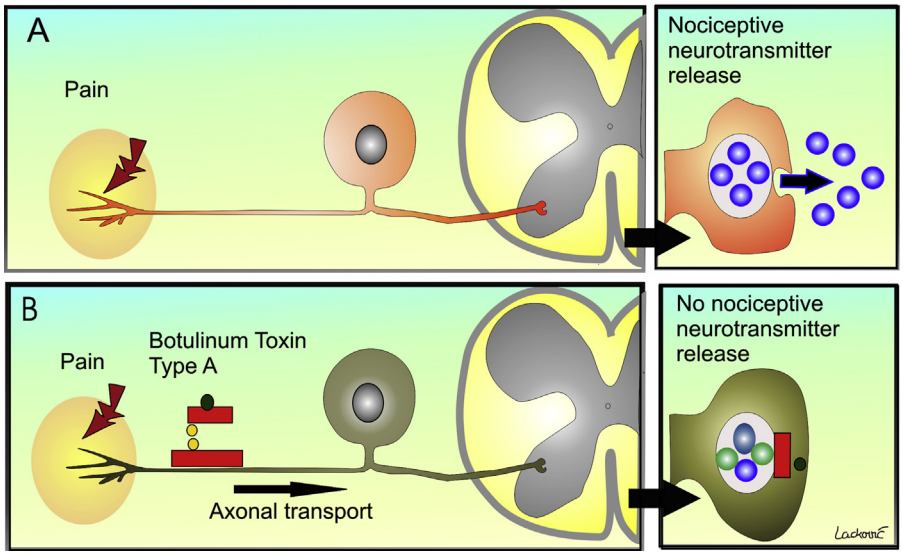

Fig. 4. Suggested mechanism of BoNT/A analgesic activity on the level of central sensory afferent terminals. (A) Neurotransmission of lasting inflammatory and chronic pain. Painful signal from the stimulated peripheral nerve endings (red) is transmitted along pseudounipolar sensory neuron to the spinal cord dorsal horn central afferent terminals (intensive red), where it induces central sensitization, leading to hyperalgesic responses. (B) By entering sensory neurons and axonal transport to the dorsal horn, followed by subsequent central SNAP-25 cleavage, BoNT/A prevents nociceptive neurotransmitter release from central afferent terminals. Different color (gray *vs.* red) indicates possible change in neuronal activity.

Table 1
Systemic reviews and meta-analyses of BoNT/A efficacy in treatment of pain disorders.

Clinical condition	*N* (trials included)	*N* (patients included)	Outcome	Reference
Myofascial pain syndromes	4	233	Inconclusive evidence for effectiveness.	Soares et al. (2012)
Subacute/chronic neck pain	9	503	Lack of benefit.	Langevin et al. (2011)
Low-back pain and sciatica	3	123	Low-quality evidence that BoNT/A is beneficial.	Waseem et al. (2010)
Shoulder pain due to spastic hemiplegia or arthritis	6	164	BoNT/A reduces pain and improves shoulder function.	Singh and Fitzgerald (2010)
Postoperative pain after subpectoral breast implants	7	427	Low-quality evidence that BoNT/A is beneficial.	Winocour et al. (2014)
Trigeminal neuralgia	6	101	BoNT/A may be beneficial in treatment of TN.	Hu et al. (2013)
Tension-type headache	7	675	No reduction in the number of headaches in comparison to placebo.	Jackson et al. (2012)
Episodic migraine	9	1838	No reduction in the number of headaches in comparison to placebo.	Jackson et al. (2012)
Chronic migraine	5	1508	Significant reduction in the number of headaches in comparison to placebo.	Jackson et al. (2012)
Chronic daily headache	3	1115	Significant reduction in the number of headaches in comparison to placebo.	Jackson et al. (2012)

sensitization, receptor field expansion, and allodynia" (Aoki and Francis, 2011).

Evidence for central site of BoNT/A action are based on behavioral studies which reported distant BoNT/A effect on bilateral pain of different origins, necessity of axonal transport, increased potency after intraneuronal or central application, and immunohistochemical evidence of toxin's enzymatic activity in central sensory nociceptive nuclei after peripheral application (Table 2, right column).

The assumption that BoNT/A has only a primary peripheral site of action fails to explain some clinical data, too. For example, migraine is by definition a CNS disease and it remains unclear why peripherally acting BoNT/A can have a long lasting beneficial effect on it. Few reports on the beneficial effect of BoNT/A on phantom pain (Jin et al., 2009; Wu et al., 2012) additionally indicate the limitations of the peripheral hypothesis.

Some contradictory experimental data regarding the time-course of onset of BoNT/A action and the anti-inflammatory activity in different inflammatory pain models is summarized in Table 3.

8.2. Is there any predictive value of preclinical discoveries about the central mechanism of BoNT/A action?

Targeting nerves and ganglia for pain treatment. BoNT/A injected directly into peripheral nerves or sensory ganglia has an increased potency and similar efficacy on pain compared to peripheral injections in rats (Sections 5.3 and 6.2). In humans, by employing a procedure similar to classical nerve blocks, Kapural et al. (2007) found that the BoNT/A injection into the perineural space of greater occipital nerve reduced the pain associated with occipital neuralgia. Thus, BoNT/A perineural use may be an effective

Table 2
Summary of experimental data supporting peripheral hypothesis of BoNT/A action on pain (left) and experimental data supporting central antinociceptive activity of BoNT/A.

Evidence supporting peripheral hypothesis	References	Evidence supporting central hypothesis	References
Analogy with the effect on neuromuscular junction and autonomous synapses.	Aoki (2005), Aoki and Francis (2011)	Bilateral effect of unilateral injection in polyneuropathic and mirror pain models.	Favre-Guilmard et al. (2009), Bach-Rojecky and Lacković (2009), Bach-Rojecky et al. (2010), Xiao et al. (2011), Filipović et al. (2012)
Reduction of formalin-induced increase in peripheral glutamate concentration.	Cui et al. (2004).	Prevention of antinociceptive effect of peripheral BoNT/A by intraneural or intraganglionic colchicine.	Bach-Rojecky and Lacković (2009), Matak et al. (2011), Filipović et al. (2012)
Decreased TRPV1 and P2X3 sensory receptor expression in neurogenic bladder.	Apostolidis et al. (2005)	Contralateral effect after BTX-A injection into the distally transected sciatic nerve in a model of bilateral pain.	Bach-Rojecky and Lacković (2009)
Reduction of peripheral neuropeptide release in iris muscle and urinary bladder.	Ishikawa et al. (2000), Rapp et al. (2006), Lucioni et al. (2008)	Evidence of SNAP-25 cleavage in caudal medulla and spinal cord sensory regions after low dose peripheral BTX-A injection.	Matak et al. (2011,2012), Marinelli et al. (2012)
Decreased glutamate-evoked mechanical sensitivity of craniofacial muscle nociceptors.	Gazerani et al. (2010)	Abolishment of trigeminal pain-evoked dural neurogenic inflammation, dependently on axonal transport in trigeminal nerve.	Filipović et al. (2012)
		Effectiveness of central intracerebroventricular and intrathecal BoNT/A injections. Efficacy of low dose injection into the sensory ganglion. Higher potency of intraneuronal and centrally delivered BoNT/A in comparison to peripheral delivery.	Luvisetto et al. (2006), Bach-Rojecky et al. (2010), Lee et al. (2011), Matak et al. (2011)
		Blockage of neurotransmitter release from distant synapses after retrograde axonal transport.	Restani et al. (2012a)
		Inhibition of antinociceptive activity of peripherally administered BTX-A by intrathecally applied opioid or GABA-A antagonists, prevention of morphine-induced tolerance.	Drinovac et al. (2013, 2014), Vacca et al. (2012, 2013)

Table 3
Contradictory experimental data favoring either peripheral or central site of BoNT/A action (left *vs.* right).

Supporting peripheral hypothesis	References		Supporting central hypothesis	References
Reduction of formalin-induced pain and edema, reduction of capsaicin-induced pain and vasomotor reactions	Cui et al. (2004), Tugnoli et al. (2007), Gazerani et al. (2006, 2009)	*vs.*	No significant antiinflammatory action of effective antinociceptive doses in carrageenan and capsaicin-evoked pain	Bach-Rojecky and Lacković (2005), Bach-Rojecky et al. (2008), Favre-Guilmard et al. (2009), Shin et al. (2013)
Fast onset of antinociceptive action following peripheral application (within 24 h)	Cui et al. (2004), Marinelli et al. (2010), Mika et al. (2011)	*vs.*	Delayed onset of antinociceptive action after peripheral application (several days), in comparison to intrathecal injection (within 24 h).	Chuang et al. (2004), Bach-Rojecky et al. (2005a,b, 2010), Bach-Rojecky and Lacković (2005), Filipović et al. (2012)

alternative for treatment of focal neuropathies attributable to a locally damaged nerve branch. Treatment of neuromas and nerve stumps in damaged or transected nerves has also been clinically reported, suggesting a possible use in dentistry, treatment of phantom limb pain, *etc.* (Climent et al., 2013). These preclinical and clinical observations suggest that a more proximal (intraneuronal or intraganglionic), or even central delivery of low dose BoNT/A may be a useful therapeutic strategy for pain treatment. However, these options should be carefully considered before proceeding with further clinical research. There are unresolved issues in human and animal research regarding the BoNT/A traffic and potential consequences of its action in the CNS (Lacković et al., 2009). Experimental data suggests that BoNT/A, following axonal transport, may be transcytosed within the CNS and reach second-order or even third-order synapses (Akaike et al., 2013; Restani et al., 2012a). Since the possible transcytosis and traffic to distant regions in the CNS have not been characterized in sensory and motor system of animals or humans, their potential clinical relevance and safety issues need to be examined.

Synergism with analgesics. We reported a connection between BoNT/A action and endogenous opioid system involving μ-opioid receptors (Drinovac et al., 2013). Additionally, experimental studies suggested that BoNT/A acts synergistically with morphine and may counteract the tolerance associated with use of high doses of opioids (Auguet et al., 2008; Vacca et al., 2012, 2013). These observations suggest that BoNT/A may be combined with lower doses of opioids for increased clinical efficacy. In addition, it prevents the development of tolerance to opioid analgesics. This is in line with clinical observations that BoNT/A is effective in treatment of chronic migraine in patients with reported medication overuse (Silberstein et al., 2013). BoNT/A even reduced the amount of triptans used in migraine patients (Silberstein et al., 2013). Potential beneficial pharmacological interactions of BoNT/A with opioids, but also with other types of drugs in chronic pain patients need to be assessed in the future.

Chronic migraine is at the moment the only approved indication for BoNT/A use in non-muscular pain conditions. However, the mechanism of BoNT/A action on migraine is unknown. It was hypothesized that BoNT/A may reduce the tension of pericranial muscles and reduce the mechanical sensitivity of muscular nociceptors (Gazerani et al., 2010). In contrast to the suggested peripheral site of BoNT/A action, it is widely believed that the patophysiologically most important cause of migraine pain is the neurogenic vasodilation of dural blood vessels (Geppetti et al., 2012). This is supported by clinical effectiveness of antimigraine drugs like triptans and CGRP antagonists, which target dural neurogenic vasodilatation (Geppetti et al., 2012).

Recently, we found that neuropathic and inflammatory pain in trigeminal area, evoked by infraorbital nerve constriction and formalin, was shown to be accompanied by dural neurogenic inflammation (Filipović et al., 2012, 2014). Single peripheral BoNT/A injection completely resolved the pain-evoked dural plasma protein extravasation. Similarly to the reduction of allodynia, BoNT/A's suppressive action on dural neurogenic inflammation was found to be mediated by toxin's axonal transport in trigeminal nerve (Filipović et al., 2012). In addition, few studies reported the action of peripherally applied BoNT/A at the level of trigeminal ganglion (Section 4.3).

During migraine attack, peripheral sensitization occurs due to activation of trigeminal nerve fibers innervating dura mater and blood vessels, leading to throbbing pain (Mathew, 2011). Prolonged peripheral sensitization leads to central sensitization of second order trigeminal nucleus caudalis neurons, where the extracranial and intracranial inputs converge. This in turn induces cutaneous allodynia and scalp hypersensitivity in the pericranial area (Mathew, 2011). BoNT/A-mediated reduction of neurogenic inflammation suggests that it might be active on the level of peripheral dural afferent terminals, thus, preventing intracranial peripheral sensitization. In addition, after peripheral delivery BoNT/A may be axonally transported centrally to spinal trigeminal nucleus caudalis (Matak et al., 2011). Cleaved SNAP-25 occurrence in TNC suggest that BoNT/A might be active also at the level of second order neurons in the TNC, which receive the convergent nociceptive input from trigeminal nerve and mediate central sensitization (Filipović et al., 2012; Matak et al., 2011).

Hyperalgesia and allodynia. BoNT/A does not alter normal nociceptive thresholds or acute nociceptive pain in humans or animals, as well as immediate painful response to formalin injection (phase I). In contrast, there is a long-lasting BoNT/A induced reduction of thermal and mechanical hyperalgesia and allodynia, associated with central sensitization (Section 4.3). It can be hypothesized that, in patients, pain (including migraine) associated with hyperalgesia and allodynia might be more sensitive to BoNT/A action.

8.3. What we do not know about BoNT/A and CNS

Discovery of axonal transport of BoNT/A to the CNS after peripheral application of very small amount raises many new questions about the significance of BoNT/A action in the brain:

- Clinical significance of axonal transport of BoNT/A in motoneurons is not known. At present there is not even a hypothesis about that.
- Possible transsynaptic transport of BoNT/A inside the brain after peripheral injection. Up to now, such transport is clearly shown only after application of BoNT/A directly to some brain areas (including retina).
- The mechanism of BoNT/A bilateral effect in mirror and polyneuropathic pain models remains unknown.
- After BoNT/A application in the craniocervical region, BoNT/A might be axonally transported to brainstem/cervical sensory and motor regions, depending on the innervation of injected sites. After it reaches sensory or motor regions, the metabolism of BoNT/A in the CNS remains unclear.

- In neuromuscular junction, BoNT/A induces a denervation accompanied by sprouting of new synapses (Duchen et al., 1975; reviewed by Meunier et al., 2002; Wright et al., 2007). In contrary to sprouting, in neuronal cultures BoNT/A prevents SNAP-25-mediated axonal and dendritic outgrowth (Grosse et al., 1999; Morihara et al., 1999). It is not known whether similar BoNT/A-induced morphological changes occur in the brain *in vivo*. Beyond BoNT/A pharmacology, those issues might be important since synaptic plasticity is suggested to be the underlying mechanism of chronic pain, phantom pain, dystonia, *etc.*
- The role of SNAP-25 away from classical synapses along axons or dendrites is not clear. Accordingly, potential significance of BoNT/A effects outside of synaptic zones *in vivo* needs to be further characterized.
- Are there any additional targets/mechanisms of BoNT/A action in the CNS, not necessarily connected with SNAP-25?

9. Conclusion

The dominant opinion suggests that the inhibition of peripheral neurotransmitter/inflammatory mediator release is the underlying primary mechanism of BoNT/A's antinociceptive action, with the secondary effects on central sensitization. However, present literature overview suggests that the existing experimental and clinical data on BoNT/A antinociceptive action cannot be adequately explained by this hypothesis. Several reports demonstrated that BoNT/A induces bilateral effects after unilateral injection in mirror or polyneuropathic pain of different origins. In addition, the antinociceptive effect of peripherally applied BoNT/A is shown to be dependent on axonal transport in sensory nerves. Enzymatic activity of BoNT/A has been immunohistochemically visualized in the spinal cord or brainstem areas receiving sensory input from the toxin's peripheral injection site. Additionally, BoNT/A's antinociceptive activity is shown to be associated with central μ-opioid and GABA-A receptors. The discovery that the BoNT/A action on pain is dominantly a central effect raises many new questions requiring additional research concerning the mechanisms of toxin action in CNS. Elucidation of antinociceptive mechanisms would be invaluable for further development of BoNT/A use in pain, and possibly some other clinical indications. In addition, it might contribute to better understanding of chronic pain pathophysiology.

Conflict of interest

The authors declare that they have no conflict of interest.

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