BOTULINUM TOXIN A, BRAIN AND PAIN

[1]Ivica Matak, [1]Zdravko Lacković

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

Corresponding author:

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

Address: Šalata 11, 10000 Zagreb, Croatia;

tel. no. : 00385 1 4566843;

e-mail: lac@mef.hr

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 for treatment of certain types of pain is increasing, and its long-term effectiveness 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 versus 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.

Keywords: botulinum toxin type A; pain; axonal transport; sensory nervous system, synaptosomal-associated protein of 25 kDa

Highlights:

- BoNT/A is therapeutically useful in certain chronic pain conditions and migraine

- Dominant hypotheses assume its exclusive activity on peripheral nerve endings
- However, BoNT/A axonal transport from periphery to CNS has been discovered
- Recent research revealed that BoNT/A action on pain involves central action

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 (y-aminobutyric acid; CGRP, calcitonin gene-related peptide; TRPV1, transient receptor potential vanilloid 1; PC-12, pheochromocytoma-12

Table of contents:

1. INTRODUCTION

In the last 15-20 years, 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 effectiveness has been clinically investigated in chronic migraine treatment (Dodick et al., 2010) and approved in USA in 2010. Additionally, BoNT/A beneficial effect has been reported in off-label studies 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., 2009; 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 Gram-positive rod-shaped anaerobic bacterium *Clostridium botulinum* and few similar *Clostridia* (*C. butyricum*, *C. baratii* and *C. argentinense)* (Popoff and Bouvet, 2005). 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. 2013; Barash and Arnon, 2013). 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 median lethal dose in humans is only 1 ng/kg or 70 ng/70 kg person (Gill, 1982). One gram of inhaled toxin could kill more than 1 million people, 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 long-term flaccid paralysis of skeletal muscles, and impairment of gastrointestinal and autonomic nervous system. 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 to 36 hours following the exposure to toxin, but sometimes 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, 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 et al., 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 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 recently 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 J. 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; Thengannat et al., 2012). Apart from movement disorders, BoNT/A has been used for treatment of autonomous 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). 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; FDA 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. The larger, heavy chain (100kDa) contains a carboxy 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 haemaglutinins and non-haemaglutinins 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).

Figure 1

3.1.2 Absorption and distribution

In the gut, BoNT/A 150 kDa neurotoxin is transcytosed from lumen of small intestine across the epithelial lining. Similarly, inhaled BoNT/A may penetrate the bloodstream across lung alveolar epithelium. BoNT/A is transported from apical to basolateral surface of epithelial cells, from where it enters the bloodstream or lymph vessels. It then reaches extracellular fluid in various tissues and targets peripheral nerve endings (Simpson et al., 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) (Sobel et al., 2005) 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) (Sobel et al., 2005) 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 intravenously-administered BoNT/A was performed in rodents (Ravichandran et al., 2006). The elimination half-life of active, non-metabolized toxin was around 4 hours. Majority of the toxin was unbound to plasma proteins (85-95 %).

The time course of BoNT/A poisoning was studied using intravenous (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 et al., 2007). 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 et al., 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 isoforms of SV2 (SV2 A-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 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; Keller et al., 2004; Südhof et al., 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. 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; Pelizzari 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., 1993), 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 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 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 (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 single synaptic vesicle with plasma membrane (Meghighian 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 (LD_{50} is 70 ng/70 kg person). Effective therapeutic doses in different clinical conditions are much lower- in some 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, it was subsequently discovered that 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 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).

3.3.2. Preferential effect on excitatory vs. inhibitory neurons.

BoNT/A is more efficient in impairing the release of excitatory neurotransmitters, such as 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., 2007), 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., 2007; 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. 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 axons and dendrites (Fig 1) (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 (Figure 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; Ray et al., 1999; Zhang et al., 2013).

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; Ray et al., 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

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 as the native toxin consisting of both light and heavy chain. 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 BoNT/A injection into bladder reduces the number of sensory neurons containing substance P, CGRP, calbindin, somatostatin, and neuronal nitric oxide synthase in ganglia innervating the bladder. 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 indirect consenquence of peripheral chemical denervation (Humm et al., 2000; Jung et al., 1997; Palomar 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; Thengannat et al., 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; Jankovic et al., 1990; 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 necessary 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 Schwarz, 2003). Additionally, based on the effectiveness of BoNT/A injections into the myofascial trigger points in treatment of myofascial pain, BoNT/A effect was proposed to be linked to either indirect or direct effects on pain fibres 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, tension type headache and different types of neuropathic pain (Argoff et al., 2002; Relja et al., 1997; Silberstein et al., 2000). Along with experimental knowledge obtained from pre-clinical *in vitro* and *in vivo* studies (Section 5. 2), it is accepted that BoNT/A effects on pain may be mediated by its direct effects on sensory system.

3. 1. 2. Current clinical experience

Migraine. Based on the 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 lasting >15 days per month) in 2010. Therapeutic outcome was significantly reduced mean number of migraine attacks per month, 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, organized by Allergan (onabotulinum toxin A 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., 2005; Silberstein 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) (Silberstein et al., 2009). It was also suggested that pericranial allodynia, unilaterality of migraine pain and pericranial muscle tenderness in chronic migraine may be predictive markers for BoNT/A responsiveness (Matthew 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 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. Although some double-blind, placebo controlled studies have been performed (review by Jabbari and Machado, 2011), clinical reports on BoNT/A effectiveness are dominated by studies based on a small number of patients or individual case reports. In the literature there is an increasing number of rare conditions with reported BoNT/A effectiveness on pain 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.

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 myofacial 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 et al., 2004).

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

- *Benign masseter muscle hypertrophy.* Fedorowicz et al., (2013) assessed 23 full text articles but were unable to identify any randomized clinical trial or controlled clinical trial investigating the efficacy and safety of intra-masseteric injections of BoNT/A for people with bilateral benign masseter hypertrophy.

- Winocour et al., (2013) 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. (2011) 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 meta-analysis.

- Singh and Fitzgerald (2010) analysed BoNT/A efficacy in *shoulder pain*. They analysed 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 a large meta-analysis involving 23 studies and more than 5000 patients, Jackson et al. (2012) analyzed BoNT/A efficacy in episodic and 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 appropriate definition of study primary outcomes (Jabbari and Machado, 2011).

In spite of controversies, clinical reports on the use of BoNT/A suggest unique long-lasting pain reduction after a single local use, lasting 3-6 months. Lasting reduction of pain after single BoNT/A application 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 cases of refractory chronic pain where other treatments have failed.

4. 2 Preclinical studies

Up to now, the clinical use of BoNT/A has occurred largely empirically. In PubMed database hundreds of clinical reports on the action of BoNT/A on pain (including migraine) can be found. 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 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 *ex vivo* rat bladder preparation, incubation with BoNT/A prevented the release of SP and CGRP after acute incubation with HCl or cyclophosphamide-induced chronic cystitis (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). BoNT/A 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 et al., 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 is suggested to 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; Meng et al., 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* direct 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; Luvisetto et al., 2006; Vacca et al., 2012; Drinovac et al., 2013). 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 has also been reported (Lee et al., 2011). Efficacy of BoNT/A in reducing thermal and mechanical hyperalgesia was reported in other 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 partial sciatic nerve transection model (Bach-Rojecky et al., 2005), 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 the injured sciatic nerve in a model of sciatic constriction injury-induced peripheral neuropathy, measured 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 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 (Kitamura et al., 2009; Filipović et al., 2012; 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 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; Xiao et al., 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; Chuang et al., 2008; Chuang et al., 2009). Additionally, BoNT/A efficacy was reported in postsurgical pain (Filipović et al., 2009) 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. BoNT/A 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., 2005).

Together with the observed lack of effect on acute nociceptive pain, another important difference between the classical 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., 2005; Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2010). 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). In formalin test similar

antinociceptive effect of 3.5, 7 and 15 U/kg BoNT/A doses was reported (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 was reported in a human model of capsaicin-induced pain (Gazerani et al., 2006; Gazerani et al., 2009; Tugnoli et al., 2007). Gazerani et al. (2006, 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. (2003) and Voller et al. (2007) 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; Gazerani et al., 2009; Schulte-Mattler et al., 2003; Tugnoli et al., 2007; Voller et al., 2007). 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 is causal – BoNT/A indirectly reduces the pain and central sensitization by reducing peripheral neurotransmitter/inflammatory mediator release. However, the relation between the effects of BoNT/A on peripheral inflammation and pain could not be confirmed in subsequent reports (Bach-Rojecky et al., 2005; Bach-Rojecky et al., 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 by BoNT/A (Bach-Rojecky et al., 2005; Bach-Rojecky et al., 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), 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 similar antinociceptive effect without significant difference (Cui et al., 2004). Although the authors suggested that BoNT/A-mediated pain reduction is associated with decrease of peripheral inflammatory mediator release (Cui et al., 2004), there is a difference between the minimal BoNT/A doses needed to produce the anti-inflammatory and antinociceptive effects.

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 neurogenic flare evoked by cutaneous electrical stimulation, however, with very limited analgesic effect. The authors suggested that the neuropeptide-mediated reduction of peripheral neurogenic inflammation does not contribute significantly to BoNT/A analgesic effects observed in clinical pain syndromes (Krämer et al., 2003).

Independently of 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 3. 4) (Sluka et al., 2001; Bach-Rojecky et al., 2009).

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., 2005; Lacković et al., 2006; Bach-Rojecky and Lacković, 2009; Favre Guilmard et al., 2009; Bach Rojecky et al. 2010; 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. 2005; 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 (Sluka et al., 2001; Da Silva et al., 2010). Hypothetically, if the BoNT/A antinociceptive action is mediated by the prevention of peripheral neurotransmitter release, then it shouldn’t reduce the contralateral acidic saline-induced bilateral hyperalgesia. Toxin’s effect on 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 models of peripheral polyneuropathies induced by chemotherapeutic drug paclitaxel (Favre-Guilmard et al., 2009), and beta-cytotoxic drug streptozotocin which induces type I diabetes (Bach-Rojecky et al., 2010). 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 streptozootocin-induced diabetes accompanied by increased sensitivity to pain (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 (Bach-Rojecky et al., 2010).

Due to systemic polyneuropathic effect of paclitaxel, and widespread neuropathy occurring in experimental diabetes type I, pathological neuronal 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; Xiao et al., 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. 2005, Bach-Rojecky and Lacković, 2009; Bach-Rojecky et al., 2010; 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 toxin movement and direct activity in the CNS. Hematogenous route as the underlying pathway for penetration into the CNS is unlikely since low doses of BoNT/A used did not induce systemic poisoning. In addition, BoNT/A is a large protein (150 kDa) which cannot cross the blood-brain barrier. The 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 limited, and toxin 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 largely been 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 et al., 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-radiolabelled 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's injection. However, these studies could not demonstrate that 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, an injection of small dose BoNT/A (0.5 U/kg) into the stump of a distally transected sciatic nerve was still able to reduce 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 d after toxin delivery into the ganglion. Intraganglionic colchicine also 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 treated 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 dura and periphery are different, the authors suggested axonal transport of BoNT/A in primary sensory neurons but, also, a possible transcytosis of BoNT/A between different sensory neurons in the ganglion. It was proposed that BoNT/A may modulate SNARE-mediated TRPV1 translocation to the plasma membrane of sensory neurons (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; Xiao et al., 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., 2009, 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., 2012; Santini et al., 1999; Wohlfart et al., 2001; reviewed recently by Caleo and Schiavo, 2009; Palomar, 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 (Wohlfart 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 (Wohlfart et al., 2001).

Recently, possible existence of BoNT/A axonal transport via motoneuronal axons and recurrent axonal collaterals to CNS in humans 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 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 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-Lopez 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 toxin traffic from injection site via direct axonal projections: namely, to contralateral hippocampus and ipsilateral entorhinal cortex. 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 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; Matak et al., 2012). Central SNAP-25 cleavage was detected in CNS even at low doses of peripherally injected commercially available onabotulinum toxin A (Matak et al., 2011; Matak et al., 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; Matak et al., 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 binding 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. (2011) argued 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 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 long-term presence of BoNT/A protease in retina after toxin's application into the optic tectum (Antonucci et al., 2008). After tectal injection, toxin's proteolytic activity was detected in retina. 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 and Neale, 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 and Neale, 1999). In a similar experiment it was demonstrated that BoNT/A protease was anterogradely transported and transcytosed to second-order synapses in superior colliculus (Restani et al., 2011). BoNT/A was injected into the retina and its proteolytic activity was demonstrated in optic tectum after 3 days. The optic nerve was transected to prevent 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 BoNT/A.

Novel studies on the BoNT/A activity in the optic system provided 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 in optic tectum, ultrastructural analysis revealed 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 recently 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 and 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 the motoneuronal axons (Restani et al., 2012b). The authors suggested that BoNT/A and BoNT/E share similar axonal intra-vesicular 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 (Figure 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 neurokinin 1 receptor internalization evoked by intraplantar formalin or intrathecal capsaicin, and suggested that BoNT/B may reduce spinal substance P presynaptic release (Marino et al., 2014).

Figure 3

Figure 4

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, we have 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; Vacca et al., 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; Vacca et al., 2013). These studies indirectly suggested that BoNT/A's antinociceptive action might be connected with the endogenous opioid system. In our experiments (Drinovac et al., 2013) 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. Effectiveness of low intrathecal dose of naltrexone suggested that the effect is mediated at the spinal level. BoNT/A activity was demonstrated to be mediated by the μ-opioid receptor by 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 effects on thermal and mechanical thresholds, as well as 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 via 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). The effect of GABA antagonism was similar to opioid antagonists: GABA-A antagonist bicuculline prevented the BoNT/A-mediated antinociceptive effect in formalin-induced inflammatory pain, as well as in 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 may explain 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, Matak et al., 2012) (Figure 4), seems to involve increased endogenous opioidergic and GABA-ergic transmission (Drinovac et al., 2013; Drinovac et al., 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). Later, 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 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 central application and immunohistochemical studies involving selective toxin's enzymatic activity in central sensory nociceptive nuclei after peripheral application (Table 1, right column).

The assumption that BoNT/A has 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 anti-inflammatory activity in different inflammatory pain models is summarized in Table 2.

Table 1

Table 2

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 compared to peripheral injections in rats (Sections 5. 3 and 6. 2). 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 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 (Restani et al., 2012; Akaike et al., 2012). 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 of BoNT/A action with the endogenous opioid system and 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; Vacca et al., 2013). These observations suggest that BoNT/A may be combined with low dose morphine and prevent 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 need to be assessed in the future.

Chronic migraine is at the moment the only approved indication for BoNT/A use in 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., 2011). This is supported by clinical effectiveness of antimigraine drugs like triptans and CGRP antagonists, which target dural neurogenic vasodilatation (Geppetti et al., 2011).

In trigeminal pain models we found that BoNT/A may reduce the neurogenic inflammation in dural meninges (Filipović et al., 2012; Matak et al., 2011). 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; Filipović et al., 2014). Single peripheral BoNT/A injection completely resolved the pain-evoked dural plasma protein extravasation. Similarly to 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). Few studies also reported the action of peripherally applied BoNT/A at the level of trigeminal ganglion (Section 4. 5).

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 suggest 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 to central 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 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 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.
- Why does BoNT/A act only on some types of pain primarily accompanied with hyperalgesia and allodynia? Does BoNT/A act only on the subset of sensory neurons or only on some aspects of central neurotransmission?
- 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 denervation accompanied by sprouting of new synapses (Duchen et al., 1975; reviewed by Meunier et al., 2002; Wright et al., 2007). On the contrary, 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, etc.
- The role of SNAP-25 away from classical synapses along the 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-mediated release of neurotransmitters?

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. A modification of this opinion includes the secondary effects on central sensitization. However, a literature overview presented here 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 can be 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.

Acknowledgements

We would like to thank Ingeborg Remich for text revision. Our work was funded by Croatian Ministry of Science, Education and Sports (Project no. 101-1010003-0001), and Deutscher Academischer Austauch Dienst (DAAD). The authors declare no conflict of interest.

REFERENCES:

Akaike, N., Shin, M. C., Wakita, M., Torii, Y., Harakawa, T., Ginnaga, A., Kato, K., Kaji, R., Kozaki, S. 2013 Transsynaptic inhibition of spinal transmission by A2 botulinum toxin. J. Physiol. 591, 1031-1043.

Alexiades-Armenakas, M. 2008 Retrograde transport and transcytosis of botulinum toxin serotypes to the brain: analysis of potential neurotoxicity. J. Drugs Dermatol. 7, 1006-1007

Al-Saleem, F.H., Ancharski, D.M., Joshi, S.G., Elias, M., Singh, A., Nasser, Z., Simpson, L.L. 2012 Analysis of the mechanisms that underlie absorption of botulinum toxin by the inhalation route. Infect. Immun. 80, 4133-4142

Al-Saleem, F.H., Nasser, Z., Olson, R.M., Cao, L., Simpson, L.L. 2011 Identification of the factors that govern the ability of therapeutic antibodies to provide postchallenge protection against botulinum toxin: a model for assessing postchallenge efficacy of medical countermeasures against agents of bioterrorism and biological warfare. J. Pharmacol. Exp. Ther. 338, 503-517.

Anderson, S., Krug, H., Dorman, C., McGarraugh, P., Frizelle, S., Mahowald, M. 2010 Analgesic effects of intra-articular botulinum toxin Type B in a murine model of chronic degenerative knee arthritis pain. J. Pain Res. 3, 161-168.

Antonucci, F., Rossi, C., Gianfranceschi, L., Rossetto, O., Caleo, M. 2008 Long distance retrograde effects of botulinum neurotoxin A. J. Neurosci. 28, 3689-3696.

Aoki, K. R. 2005 Review of a proposed mechanism for the antinociceptive action of botulinum toxin type A. Neurotoxicology 26, 785–793.

Aoki, K. R., Francis, J. 2011 Update on antinociceptive mechanism hypothesis of botulinum toxin A. J. Parkinsonism Relat. Disord. 117, S27-S33.

Apostolidis, A., Popat, R., Yiangou, Y., Cockayne, D., Ford, A.P., Davis, J.B., Dasgupta, P., Fowler, C.J., Anand, P. 2005. Decreased sensory receptors P2X3 and TRPV1 in

suburothelial nerve fibers following intradetrusor injections of botulinum toxin for human detrusor overactivity. J. Urol. 174, 977-982.

Arnon, S. M., Schechter, R., Inglesby, T. V., Henderson, D. A., Bartlet, D. J., Michael, S., Ascher, M. S., Eitzen, E., Fine, A. D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M. T., O'Toole, T., Parker, G., Perl, T. M., Philip, K., Russell, F. K., Swerdlow, D. L., Tonat, K., Working Group on Civilian Biodefense. 2001 Botulinum toxin as a biological weapon: medical and public health management. JAMA. 285, 1059-1070.

Arezzo, J. C. 2002 Possible mechanisms for the effects of botulinum toxin on pain. Clin J Pain. 18, S125-S132.

Argoff, C. E. 2002 A focused review on the use of botulinum toxins for neuropathic pain. Clin J Pain. 18, S177-S181.

Auguet, M., Favre-Guilmard, C., Chabrier, P. E. 2008 Analgesic effects of botulinum toxin A in an inflammatory pain model in rats: comparison of Dysport and Botox; synergistic interaction with morphine. Abstract Toxins. Toxicon 51, Suppl 9.

Bach-Rojecky, L., Lacković, Z. 2005 Antinociceptive effect of botulinum toxin type A in rat model of carrageenan and capsaicin induced pain. Croat. Med. J. 46, 201–208

Bach-Rojecky, L., Relja, M., Lacković, Z. 2005 Botulinum toxin type A in experimental neuropathic pain. J. Neural Transm. 112, 215–219.

Bach-Rojecky L, Relja M, Lacković Z. Botulinum toxin type A in rat model of muscle hyperalgesia. 16th International Congress on Parkinson's disease and Related Disorders, Berlin 2005. J. Parkinsonism Relat. Disord. 11, 106.

Bach-Rojecky, L., Dominis, M., Lacković, Z., 2008 Lack of anti-inflammatory effects of botulinum toxin A in experimental models of inflammation. Fundam. Clin. Pharmacol. 22, 503–509.

Bach-Rojecky, L., Lacković, Z. 2009 Central origin of the antinociceptive action of botulinum toxin type A. Pharmacol. Biochem. Behav. 94, 234–238.

Bach-Rojecky L, Šalković-Petrišić M, Lacković Z. 2010 Botulinum toxin type A reduces pain supersensitivity in experimental diabetic neuropathy: bilateral effects after unilateral injection. Eur. J. Pharmacol 633, 10–14.

Bandala, C., Perez-Santos, J. L., Lara-Padilla, E., Delgado Lopez, G., Anaya-Ruiz, M. 2013 Effect of botulinum toxin a on proliferation and apoptosis in the T47D breast cancer cell line. Asian Pac. J. Cancer Prev. 14, 891-894.

Barash, J. R., Arnon, S. S. 2014 A novel strain of Clostridium botulinum that produces type B and type H botulinum toxins. J. Infect. Dis. 209, 183-91

Barnes, M., 2003. Botulinum toxin-mechanisms of action and clinical use in spasticity. J. Rehabil. Med. Suppl 41, 56-59.

Bartels, F., Bergel, H., Bigalke, H., Frevert J., Halpern J., Middlebrook J. 1994 Specific antibodies against the Zn2+-binding domain of clostridial neurotoxins restore exocytosis in chromaffin cells treated with tetanus or botulinum A neurotoxin. J. Biol. Chem. 269:8122–8127.

Bigalke, H., Rummel, A., 2005 Medical aspects of toxin weapons. Toxicology 214, 210-220

Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Südhof, T. C., Jahn, R., Niemann, H. 1994 Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J. Biol. Chem. 269, 1617-1620.

Black, J., Dolly, O. 1986 Interaction of 125I-labeled botulinum neurotoxins with nerve terminals. I. Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. J. Cell. Biol. 103, 521–534.

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Südhof, T. C., Niemann, H., Jahn, R. 1993 Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature 365, 160-163.

Borodic, G. E., Caruso, P., Acquadro, M., Chick, S. 2013 Parry-Romberg syndrome vasculopathy and its treatment with botulinum toxin. Ophthal. Plast. Reconstr. Surg. Doi: 10. 1097/IOP. 0b013e31828de9c0

Bossowska, A., Majewski, M. 2012 Botulinum toxin type A-induced changes in the chemical coding of dorsal root ganglion neurons supplying the porcine urinary bladder. Pol. J. Vet. Sci. 15, 345-353.

Brin, M.F., Fahn, S., Moskowitz, C., Friedman, A., Shale, H.M., Greene, P.E., Blitzer, A., List, T., Lange, D., Lovelace, R.E., McMahon, D. 1987 Localized injections of botulinum toxin for the treatment of focal dystonia and hemifacial spasm. Mov Disord. 2, 237-254.

Brodsky, M. A., Swope, D. M., Grimes, D. 2012 Diffusion of botulinum toxins. Tremor Other Hyperkinet. Mov. (N Y). 2, tre-02-85-417-1.

Brook, I. 2007 Infant botulism. J. Perinatol. 27, 175-180.

Burstein, R., Dodick, D., Silberstein, S. 2009 Migraine prophylaxis with botulinum toxin A is associated with perception of headache. Toxicon 54, 624-627.

Caleo, M., Schiavo, G. 2009 Central effects of tetanus and botulinum neurotoxins. Toxicon 54, 593-599.

Carli, L., Montecucco, C., Rossetto, O. 2009 Assay of diffusion of different botulinum neurotoxin type a formulations injected in the mouse leg. Muscle Nerve 40, 374-380.

Chapman, M. 2012 The global botox market forecast to reach $2. 9 billion by 2018. Companies and Markets (accessed Sept 2013) http://www. companiesandmarkets. com/News/Healthcare-and-Medical/The-global-botox-market-forecast-to-reach-2-9-billion-by-2018/NI2991

Chen, F., Kuziemko, G.M., Stevens, R.C. 1998 Biophysical characterization of the stability of the 150-kilodalton botulinum toxin, the nontoxic component, and the 900-kilodalton botulinum toxin complex species. Infect Immun. 66, 2420-2425.

Chen, W. T., Yuan, R. Y., Chiang, S. C., Sheu, J. J., Yu, J. M., Tseng, I. J., Yang, S. K., Chang, H. H., Hu, C. J. 2013 OnabotulinumtoxinA improves tactile and mechanical pain perception in painful diabetic polyneuropathy. Clin. J. Pain 29, 305-310.

Cheng, J., Liu, W., Duffney, L.J., Yan, Z. SNARE proteins are essential in the potentiation of NMDA receptors by group II metabotropic glutamate receptors. J Physiol. 591, 3935-3947.

Chertow, D. S., Tan, E. T., Maslanka, S. E., Schulte, J., Bresnitz, E. A., Weisman, R. S., Bernstein, J., Marcus, S. M., Kumar, S., Malecki, J., Sobel, J., Braden, C. R. 2006 Botulism in 4 adults following cosmetic injections with an unlicensed, highly concentrated botulinum preparation. JAMA. 296, 2476-2479.

Cheshire, W. P., Abashian, S. W., Mann, J. D. 1994 Botulinum toxin in the treatment of myofascial pain syndrome. Pain 59, 65–69

Chou, C. L., Lee, S. H., Lu, S. Y., Tsai, K. L., Ho, C. Y., Lai, H. C. 2010 Therapeutic effects of intra-articular botulinum neurotoxin in advanced knee osteoarthritis. J. Chin. Med. Assoc. 73, 573-580.

Chuang, Y., Yoshimura, N., Huang, C., Po, H., Chancellor, M. B. 2004 Intravesical botulinum toxin A administration produces analgesia against acetic acid induced bladder pain responses in rats. J. Urol. 172, 1529-1532.

Chuang, Y. C., Yoshimura, N., Huang, C. C., Wu, M., Chiang, P. H., Chancellor, M. B. 2008 Intraprostatic botulinum toxin a injection inhibits cyclooxygenase-2 expression and suppresses prostatic pain on capsaicin induced prostatitis model in rat. J. Urol. 180, 742-748.

Chuang, Y. C., Yoshimura, N., Huang, C. C., Wu, M., Chiang, P. H., Chancellor, M. B. 2009 Intravesical botulinum toxin A administration inhibits COX-2 and EP4 expression and suppresses bladder hyperactivity in cyclophosphamide-induced cystitis in rats. Eur. Urol. 56, 159-166.

Climent, J. M., Mondéjar-Gómez, F., Rodríguez-Ruiz, C., Díaz-Llopis, I., Gómez-Gallego, D., Martín-Medina, P. 2013 Treatment of Morton neuroma with botulinum toxin A: a pilot study. Clin. Drug Investig. 33, 497-503.

Coffield, J. A., Yan, X. 2009 Neuritogenic actions of botulinum neurotoxin A on cultured motor neurons. J. Pharm. Exp. Ther. 330, 352–358

Cohen, L. G., Hallett, M., Geller, B. D., Hochberg, F. 1989 Treatment of focal dystonias of the hand with botulinum toxin injections. J. Neurol. Neurosurg. Psychiatry 52, 355-363.

Colasante, C., Rossetto, O., Morbiato, L., Pirazzini, M., Molgó, J., Montecucco, C. 2013 Botulinum neurotoxin type A is internalized and translocated from small synaptic vesicles at the neuromuscular junction. Mol. Neurobiol. 48, 120-127.

Crowner, B. E., Brunstrom, J. E., Racette, B. A. 2007 Iatrogenic botulism due to therapeutic botulinum toxin A injection in a pediatric patient. Clin. Neuropharmacol. 30, 310-313.

Cui, M., Khanijou, S., Rubino, J., Aoki, K. R. 2004 Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. Pain 107, 125–133.

Da Silva, L.F., Desantana, J.M., Sluka, K.A. 2010 Activation of NMDA receptors in the brainstem, rostral ventromedial medulla, and nucleus reticularis gigantocellularis mediates mechanical hyperalgesia produced by repeated intramuscular injections of acidic saline in rats. J. Pain. 11, 378-387.

Dodick, D.W., Turkel, C.C., DeGryse, R.E., Aurora, S.K., Silberstein, S.D., Lipton, R.B., Diener, H.C., Brin, M.F.; PREEMPT Chronic Migraine Study Group. 2010 OnabotulinumtoxinA for treatment of chronic migraine: pooled results from the double-blind, randomized, placebo-controlled phases of the PREEMPT clinical program. Headache 50, 921-936.

Dolly, J. O., Wang, J., Zurawski, T. H., Meng, J. 2011 Novel therapeutics based on recombinant botulinum neurotoxins to normalize the release of transmitters and pain mediators. FEBS J. 278, 4454-4466.

Dong, M., Yeh, F., Tepp, W. H., Dean, C., Johnson, E. A., Janz, R., Chapman, E. R. 2006 SV2 is the protein receptor for botulinum neurotoxin A. Science. 312, 592-596.

Dover, N., Barash, J. R., Arnon, S. S. 2009 Novel Clostridium botulinum toxin gene arrangement with subtype A5 and partial subtype B3 botulinum neurotoxin genes. J. Clin. Microbiol. 47, 2349-2350.

Dover, N., Barash, J.R., Hill, K.K., Xie, G., Arnon, S.S. 2014 Molecular characterization of a novel botulinum neurotoxin type H gene. J. Infect. Dis. 209, 192-202

Dressler, D. 2013 Botulinum toxin therapy: its use for neurological disorders of the autonomic nervous system. J. Neurol. 260, 701-713.

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.

Drinovac, V., Bach-Rojecky, L., Lacković, Z. 2014 Association of antinociceptive action of botulinum toxin type A with GABA-A receptor. J. Neural Transm. doi: 10.1007/s00702-013-1150-6

Duc, C., Catsicas, S., 1995. Ultrastructural localization of SNAP-25 within the rat spinal cord and peripheral nervous system. J. Comp. Neurol. 356, 152-163.

Duchen, L. W., Rogers, M., Stolkin, C., Tonge, D. A. 1975 Proceedings, Suppression of botulinum toxin-induced axonal sprouting in skeletal muscle by implantation of an extra nerve. J. Physiol. 248, 1P-2P.

Durham, P. L., Cady, R. 2004 Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by botulinum toxin type A: Implications for migraine therapy. Headache 44, 35–42.

Erbguth, F. J. 2008 From poison to remedy: the chequered history of botulinum toxin. J. Neural Transm. 115, 559–565

Fabregat, G., Asensio-Samper, J. M., Palmisani, S., Villanueva-Pérez, V. L., De Andrés, J. 2013 Subcutaneous botulinum toxin for chronic post-thoracotomy pain. Pain Pract. 13, 231-234.

Favre-Guilmard, C., Auguet, M., Chabrier, P.E. 2009 Different antinociceptive effects of botulinum toxin type A in inflammatory and peripheral polyneuropathic rat models. Eur J Pharmacol 617, 48–53.

FDA; US FOOD AND Drug Information: FDA Gives Update on Botulinum Toxin Safety Warnings; Established Names of Drugs Changed August 3, 2009, http://www. fda. gov/NewsEvents/Newsroom/PressAnnouncements/2009/ucm175013. htm (accessed Sept 2013)

FDA; US FOOD AND Drug Information: Information for Healthcare Professionals: 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 2013)

Fedorowicz, Z., van Zuuren, E.J., Schoones, J. 2013 Botulinum toxin for masseter hypertrophy. Cochrane Database Syst Rev. 9;9:CD007510. doi: 10. 1002/14651858. CD007510.

Ferrari, E., Gu, C., Niranjan, D., Restani, L., Rasetti-Escargueil, C., Obara, I., Geranton, S.M., Arsenault, J., Goetze, T.A., Harper, C.B., Nguyen, T.H., Maywood, E., O'Brien, J., Schiavo, G., Wheeler, D.W., Meunier, F.A., Hastings, M., Edwardson, J.M., Sesardic, D.,

Caleo, M., Hunt, S.P., Davletov, B. 2013 Synthetic self-assembling clostridial chimera for modulation of sensory functions. Bioconjug. Chem. 24,1750-1759

Filipović, B., Bach-Rojecky, L., Lacković, Z. 2010 Lasting reduction of postsurgical hyperalgesia after single injection of botulinum toxin type A in rat. Fundam Clin Pharmacol. 24, 43-45

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

Filipović, B., Matak, I., Lacković, Z. 2014 Dural neurogenic inflammation induced by neuropathic pain is specific to cranial region. J. Neural Transm. 121, 555-563

Fischer, A., Montal, M. 2007 Single molecule detection of intermediates during botulinum neurotoxin translocation across membranes. Proc. Natl. Acad. Sci. U S A. 104,10447-10452

Foran, P., Lawrence, G.W., Shone, C.C., Foster, K.A., Dolly, J.O. 1996 Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. Biochemistry. 35, 2630-2636.

Francisco, G.E., Tan, H., Green, M. 2012 Do botulinum toxins have a role in the management of neuropathic pain?: a focused review. Am. J. Phys. Med. Rehabil. 91, 899-909.

Frampton, J.E. 2012 OnabotulinumtoxinA (BOTOX®): a review of its use in the prophylaxis of headaches in adults with chronic migraine. Drugs 72, 825-845.

Franz, D.R., Peter, B., Jahrling, P.B., Friedlander, A.M., McClain, D.J., Hoover, D.L., Bryne, R., Julie, A., Pavlin, J.A., Christopher, G.W., Eitzen, E.M. 1997 Clinical recognition and management of patients exposed to biological warfare agents. JAMA. 278, 399-411.

Freund, B., Schwartz, M. 2003 Temporal relationship of muscle weakness and pain reduction in subjects treated with botulinum toxin A. J. Pain 4, 159-165.

Gady, J., Ferneini, E. M. 2013 Botulinum toxin A and headache treatment. Conn. Med. 77, 165-166.

Galli, T., Garcia, E. P., Mundigl, O., Chilcote, T. J., De Camilli, P. 1995 v- And t-SNAREs in neuronal exocytosis: a need for additional components to define sites of release. Neuropharmacology 34, 1351–1360.

Garner, C. G., Straube, A., Witt, T. N., Gasser, T., Oertel, W. H. 1993 Time course of distant effects of local injections of botulinum toxin. Mov. Disord. 8:33-37.

Gassner, H. G., Brissett, A. E., Otley, C. C., Boahene, D. K., Boggust, A. J., Weaver, A. L., Sherris, D. A. 2006 Botulinum toxin to improve facial wound healing: a prospective, blinded, placebo-controlled study. Mayo Clin. Proc. 81, 1023–1028.

Gazerani, P., Staahl, C., Drewes, AM., Arendt-Nielsen, L. 2006 The effects of Botulinum Toxin type A on capsaicin-evoked pain, flare, and secondary hyperalgesia in an experimental human model of trigeminal sensitization. Pain 122, 315-325.

Gazerani, P., Pedersen, N. S., Staahl, C., Drewes, A. M., Arendt-Nielsen, L., 2009. Subcutaneous botulinum toxin type A reduces capsaicin-induced trigeminal pain and vasomotor reactions in human skin. Pain 141, 60-69

Gazerani, P., Au, S., Dong, X., Kumar, U., Arendt-Nielsen, L., Cairns, B. E. 2010 Botulinum neurotoxin type A (BoNTA) decreases the mechanical sensitivity of nociceptors and inhibits neurogenic vasodilation in a craniofacial muscle targeted for migraine prophylaxis. Pain 151, 606–616.

Geppetti, P., Rossi, E., Chiarugi, A., Benemei, S. 2012 Antidromic vasodilatation and the migraine mechanism. J. Headache Pain 13, 103-111.

Gerachshenko, T., Blackmer, T., Yoon, E. J., Bartleson, C., Hamm, H. E., Alford, S. 2005 Gbetagamma acts at the C terminus of SNAP-25 to mediate presynaptic inhibition. Nat. Neurosci. 8, 597-605.

Gerona, R. R., Larsen, E. C., Kowalchyk, J. A., Martin, T. F. 2000 The C terminus of SNAP25 is essential for Ca(2+)-dependent binding of synaptotagmin to SNARE complexes. J. Biol. Chem. 275, 6328-6336.

Gerwin, R. 2012 Botulinum toxin treatment of myofascial pain: a critical review of the literature. Curr. Pain Headache Rep. 16, 413-422

Giladi, N. 1997 The mechanism of action of botulinum toxin type A in focal dystonia is most probably through its dual effect on efferent (motor) and afferent pathways at the injected site. J Neurol Sci. 152, 132-135.

Gill, D.M. 1982 Bacterial toxins: a table of lethal amounts. Microbiol Rev. 46, 86-94.

Göbel, H., Heinze, A., Reichel, G., Hefter, H., Benecke, R. 2006 Dysport Myofascial Pain Study Group. Efficacy and safety of a single botulinum type A toxin complex treatment (Dysport) for the relief of upper back myofascial pain syndrome: Results from a randomized double-blind placebo-controlled multicentre study. Pain 125, 82–8.

Goode, G.B., Shearn, D.L. 1982 Botulism. A case with associated sensory abnormalities. Arch. Neurol. 39, 55.

Grosse, G., Grosse, J., Tapp, R., Kuchinke, J., Gorsleben, M, Fetter, I., Höhne-Zell, B., Gratzl, M., Bergmann, M. 1999 SNAP-25 requirement for dendritic growth of hippocampal neurons. J Neurosci Res. 56, 539-546.

Grumelli, C., Corradini, I., Matteoli, M., Verderio, C. 2010 Intrinsic calcium dynamics control botulinum toxin A susceptibility in distinct neuronal populations. Cell Calcium. 47, 419-424.

Gu, S., Jin, R. 2013 Assembly and function of the botulinum neurotoxin progenitor complex. Curr Top Microbiol Immunol. 364, 21-44.

Habermann, E., 1974 125I-labeled neurotoxin from Clostridium botulinum A: preparation, binding to synaptosomes and ascent to the spinal cord. Naunyn. Schmiedebergs. Arch. Pharmacol. 281, 47–56.

Harper, C.B., Martin, S., Nguyen, T.H., Daniels, S.J., Lavidis, N.A., Popoff, M.R., Hadzic, G., Mariana, A., Chau, N., McCluskey, A., Robinson, P.J., Meunier, F.A. 2011 Dynamin inhibition blocks botulinum neurotoxin type A endocytosis in neurons and delays botulism. J. Biol. Chem. 286, 35966-35976.

He, Y., Elias, C.L., Huang, Y.C., Gao, X., Leung, Y.M., Kang, Y., Xie, H., Chaddock, J.A., Tsushima, R.G., Gaisano, H.Y. 2008 Botulinum neurotoxin A and neurotoxin E cleavage products of synaptosome-associated protein of 25 kd exhibit distinct actions on pancreatic islet beta-cell Kv2. 1 channel gating. Pancreas 36, 10-17.

Hu, Y., Guan, X., Fan, L., Li, M., Liao, Y., Nie, Z., Jin, L. 2013 Therapeutic efficacy and safety of botulinum toxin type A in trigeminal neuralgia: a systematic review. J. Headache Pain. 14, 72.

Huang, P.P., Khan, I., Suhail, M.S., Malkmus, S., Yaksh, T.L. 2011 Spinal botulinum neurotoxin B: effects on afferent transmitter release and nociceptive processing. PLoS One 6, e19126.

Huang, X., Wheeler, M.B., Kang, Y.H., Sheu, L., Lukacs, G.L., Trimble, W.S., Gaisano, H.Y. 1998 Truncated SNAP-25 (1-197), like botulinum neurotoxin A, can inhibit insulin secretion from HIT-T15 insulinoma cells. Mol. Endocrinol. 12, 1060-1070.

Humm, A.M., Pabst, C., Lauterburg, T., Burgunder, J.M. 2000 Enkephalin and aFGF are differentially regulated in rat spinal motoneurons after chemodenervation with botulinum toxin. Exp. Neurol. 161,361-372.

Ishida, H., Zhang, X., Erickson, K., Ray, P. 2004 Botulinum toxin type A targets Rho B to inhibit lysophosphatidic acid-stimulated actin reorganization and acetylcholine release in nerve growth factor treated PC12 cells. J. Pharm. Exp. Ther. 310, 881–889.

Ishikawa, H., Mitsui, Y., Yoshitomi, T., Mashimo, K., Aoki, S., Mukuno, K., Shimizu, K. 2000 Presynaptic effects of botulinum toxin type A on the neuronally evoked responses of albino and pigmented iris sphincter and dilator muscles. Jpn. J. Ophthalmol. 44, 106-109

Jabbari, B., Machado, D., 2011 Treatment of refractory pain with botulinum toxins-an evidence-based review. Pain Med. 12, 1594-1606.

Jackson, J.L., Kuriyama, A., Hayashino, Y. 2012 Botulinum toxin A for prophylactic treatment of migraine and tension headaches in adults: a meta-analysis. JAMA. 307, 1736-1745.

Jacky, B.P., Garay, P.E., Dupuy, J., Nelson, J.B., Cai, B., Molina, Y., Wang, J., Steward, L.E., Broide, R.S., Francis, J., Aoki, K.R., Stevens, R.C., Fernández-Salas, E. 2013 Identification of fibroblast growth factor receptor 3 (FGFR3) as a protein receptor for botulinum neurotoxin serotype A (BoNT/A). PLoS Pathog. 9, e1003369.

Jakubowski, M., McAllister, P.J., Bajwa, Z.H., Ward, T.N., Smith, P., Burstein, R. 2006 Exploding vs. imploding headache in migraine prophylaxis with Botulinum Toxin A. Pain 125, 286-295.

Ji, J., Salapatek, A.M., Lau, H., Wang, G., Gaisano, H.Y., Diamant, N.E. 2002 SNAP-25, a SNARE protein, inhibits two types of K channels in esophageal smooth muscle. Gastroenterology 122, 994-1006.

Jin, L., Kollewe, K., Krampfl, K., Dengler, R., Mohammadi, B. 2009 Treatment of phantom limb pain with botulinum toxin type A. Pain Med. 10, 300-303

Jung, H.H., Lauterburg, T., Burgunder, J.M.. 1997 Expression of neurotransmitter genes in rat spinal motoneurons after chemodenervation with botulinum toxin. Neuroscience 78,469-479.

Kalandakanond, S., Coffield, J.A. 2001 Cleavage of SNAP-25 by botulinum toxin type A requires receptor-mediated endocytosis, pH-dependent translocation, and zinc. J. Pharmacol. Exp. Ther. 296, 980-986.

Kalb, S.R., Santana, W.I., Geren, I.N., Garcia-Rodriguez, C., Lou, J., Smith, T.J., Marks, J.D., Smith, L.A., Pirkle, J.L., Barr, J.R. 2011Extraction and inhibition of enzymatic activity of botulinum neurotoxins /B1, /B2, /B3, /B4, and /B5 by a panel of monoclonal anti-BoNT/B antibodies. BMC Biochem. 15; 12, 58.

Kanno, T., Nishizaki, T. 2012 A(2a) adenosine receptor mediates PKA-dependent glutamate release from synaptic-like vesicles and Ca efflux from an IP(3)- and ryanodine-insensitive intracellular calcium store in astrocytes. Cell. Physiol. Biochem. 30, 1398-1412.

Kapural, L., Stillman, M., Kapural, M., McIntyre, P., Guirgius, M., Mekhail, N. 2007Botulinum toxin occipital nerve block for the treatment of severe occipital neuralgia: a case series. Pain Pract. 7, 337-340.

Karsenty, G., Rocha, J., Chevalier, S., Scarlata, E., Andrieu, C., Zouanat, F.Z., Rocchi, P., Giusiano, S., Elzayat, E.A., Corcos, J. 2009 Botulinum toxin type A inhibits the growth of LNCaP human prostate cancer cells in vitro and in vivo. Prostate 69, 1143-1150.

Keller, J.E., Neale, E.A., Oyler, G., Adler, M. 1999 Persistence of botulinum neurotoxin action in cultured spinal cord cells. FEBS Lett. 456, 137–142

Keller, J.E., Neale, E.A. 2001 The role of the synaptic protein SNAP-25 in the potency of botulinum neurotoxin type A. J Biol Chem 276,13476-13482

Kerner, J. 1822 Das Fettgift oder die Fettsaure und ihre Wirkungen auf den thierischen Organismus, ein Beytrag zur Untersuchung des in verdorbenen Wursten giftig wirkenden Stoffes. Cotta, Stuttgart; Tübingen

Kitamura, Y., Matsuka, Y., Spigelman, I,. Ishihara, Y., Yamamoto, Y., Sonoyama, W., Kamioka, H., Yamashiro, T., Kuboki, T., Oguma, K. 2009 Botulinum toxin type A (150 kDa) decreases exaggerated neurotransmitter release from trigeminal ganglion neurons and relieves neuropathy behaviors induced by infraorbital nerve constriction. Neuroscience 59, 1422–1429.

Koltzenburg, M., Wall, P.D., McMahon, S.B. 1999 Does the right side know what the left is doing? Trends Neurosci. 22, 122-127.

Krämer, H.H., Angerer, C., Erbguth, F., Schmelz, M., Birklein, F. 2003 Botulinum Toxin A reduces neurogenic flare but has almost no effect on pain and hyperalgesia in human skin. J. Neurol. 250,188-193.

Krug, H.E., Frizelle, S., McGarraugh, P., Mahowald, M.L. 2009 Pain behavior measures to quantitate joint pain and response to neurotoxin treatment in murine models of arthritis. Pain Med. 10, 1218-1228.

Kumada, A., Matsuka, Y., Spigelman, I., Maruhama, K., Yamamoto, Y., Neubert, J.K., Nolan, T.A., Watanabe, K., Maekawa, K., Kamioka, H., Yamashiro, T., Kuboki, T., Oguma, K. 2012 Intradermal injection of Botulinum toxin type A alleviates infraorbital nerve constriction-induced thermal hyperalgesia in an operant assay. J Oral Rehabil. 39, 63-72.

Kuo, H.C. 2013 Repeated onabotulinumtoxin-A injections provide better results than single injection in treatment of painful bladder syndrome. Pain Physician. 16, E15-23.

Kuruoğlu, R., Cengiz, B., Tokçaer, A. 1996 Botulism with sensory symptoms diagnosed by neuromuscular transmission studies associated with edrophonium responsiveness. Electromyogr. Clin. Neurophysiol. 36, 477-480.

Lacković, Z., Bach-Rojecky, L., Šalković-Petrišić, M. 2006 Antinociceptive effect of botulinum toxin type A in experiemtal diabetic neuropathy // 58th Annual American Academy of Neurology, San Diego, USA. Neurology. 66, A376

Lacković, Z., Rebić, V., Riederer,P.F. 2009 Single intracerebroventricular injection of botulinum toxin type A produces slow onset and long-term memory impairment in rats. J Neural Transm. 116, 1273-1280.

Láinez-Andrés, J.M. 2012 Onabotulinumtoxin A in the treatment of chronic migraine. Rev. Neurol. 54 Suppl 2:S39-50.

Langevin, P., Peloso, P.M., Lowcock, J., Nolan, M., Weber, J., Gross, A., Roberts, J., Goldsmith, C.H., Graham, N., Burnie, S.J., Haines, T. 2011 Botulinum toxin for subacute/chronic neck pain. Cochrane Database Syst. Rev. 7, CD008626. doi: 10. 1002/14651858. CD008626. pub2.

Lawrence, G.W., Foran, P., Oliver Dolly, J., 2002. Insights into a basis for incomplete inhibition by botulinum toxin A of Ca2+-evoked exocytosis from permeabilised chromaffin cells. Toxicology 181-182, 249-253.

Lawrence, G.W., Ovsepian, S.V., Wang, J., Aoki, K.R., Dolly, J.O. 2012 Extravesicular intraneuronal migration of internalized botulinum neurotoxins without detectable inhibition of distal neurotransmission. Biochem. J. 441, 443-452.

Lawrence, G.W., Ovsepian, S.V., Wang, J., Aoki, K.R., Dolly, J.O. 2013 Therapeutic effectiveness of botulinum neurotoxin A: potent blockade of autonomic transmission by targeted cleavage of only the pertinent SNAP-25. Neuropharmacology 70, 287-295.

Lee, W.H., Shin, T.J., Kim, H.J., Lee, J.K., Suh, H.W., Lee, S.C., Seo, K. 2011 Intrathecal administration of botulinum neurotoxin type A attenuates formalin-induced antinociceptive responses in mice. Anesth. Analg. 112, 228–233.

Lee, K., Gu, S., Jin, L., Le, T.T., Cheng, L.W., Strotmeier, J., Kruel, A.M., Yao, G., Perry, K., Rummel, A., Jin, R. 2013 Structure of a bimodular botulinum neurotoxin complex provides insights into its oral toxicity. PLoS Pathog. 9, e1003690.

Lucioni, A., Bales, G.T., Lotan,T.L., McGehee, D.S., Cook, S.P., Rapp, D.E. 2008 Botulinum toxin type A inhibits sensory neuropeptide release in rat bladder models of acute injury and chronic inflammation. BJU Int. 101(3), 366-70.

Luvisetto, S., Marinelli, S., Lucchetti, F., Marchi, F., Cobianchi, S., Rossetto, O., Montecucco, C., Pavone, F. 2006 Botulinum neurotoxins and formalin-induced pain: central vs. peripheral effects in mice. Brain Res. 1082, 124-31.

Luvisetto, S., Marinelli, S., Cobianchi, S., Pavone, F. 2007 Anti-allodynic efficacy of botulinum neurotoxin A in a model of neuropathic pain. Neuroscience. 145, 1-4.

Mahrhold, S., Rummel, A., Bigalke, H., Davletov, B., Binz, T., 2006. The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. FEBS. Lett. 580, 2011-2014.

Maksymowych, A.B., Reinhard, M., Malizio, C.J., Goodnough, M.C., Johnson, E.A., Simpson, LL. 1999 Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade. Infect. Immun. 67, 4708-4712.

Majlesi, G. 2008 GaAs laser treatment of bilateral eyelid ptosis due to complication of botulinum toxin type A injection. Photomed. Laser Surg. 26, 507-509.

Marchand-Pauvert, V., Aymard, C., Giboin, L.S., Dominici, F., Rossi, A., Mazzocchio, R. 2013 Beyond muscular effects: depression of spinal recurrent inhibition after botulinum neurotoxin A. J. Physiol. 591, 1017-1029

Marinelli, S., Luvisetto, S., Cobianchi, S., Makuch, W., Obara, I., Mezzaroma, E., Caruso, M., Straface, E., Przewlocka, B., Pavone, F. 2010 Botulinum neurotoxin type A counteracts

neuropathic pain and facilitates functional recovery after peripheral nerve injury in animal models. Neuroscience 171, 316–328.

Marinelli, S., Vacca, V., Ricordy, R., Uggenti, C., Tata, A.M., Luvisetto, S., Pavone, F., 2012. The analgesic effect on neuropathic pain of retrogradely transported botulinum neurotoxin A involves Schwann cells and astrocytes. PLoS ONE 7, e47977.

Marino, M.J., Terashima, T., Steinauer, J.J., Eddinger, K.A., Yaksh, T.L., Xu, Q. 2014 Botulinum toxin B in the sensory afferent: transmitter release, spinal activation and pain behavior. Pain 155, 674-684.

Martínez-Castrillo, J.C., Del Real, M.A., Hernandez Gonzalez, A., De Blas, G., Alvarez-Cermeño J.C. 1991 Botulism with sensory symptoms: a second case. J Neurol. Neurosurg. Psychiatry. 54, 844-845.

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.

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

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.

Mathew, N.T., Kailasam, J., Meadors, L. 2008 Predictors of response to botulinum toxin type A (BoNTA) in chronic daily headache. Headache. 48, 194-200.

Mathew, N.T. 2011 Pathophysiology of chronic migraine and mode of action of preventive medications. Headache. 51 Suppl 2, 84-92.

McMahon, H. T Foran, P. Dolly, J. O. Verhage, M. Wiegant, V. M. Nicholls, D. G. 1992 Tetanus toxin and botulinum toxins type A and B inhibit glutamate, gamma-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. Clues to the locus of action. *J.* Biol. Chem. *267*, 21338–21343.

Megighian, A., Scorzeto, M., Zanini, D., Pantano, S., Rigoni, M., Benna, C., Rossetto, O., Montecucco, C., Zordan, M., 2010. Arg206 of SNAP-25 is essential for neuroexocytosis at the Drosophila melanogaster neuromuscular junction. J. Cell. Sci. 123, 3276-3283.

Meng, J., Wang, J., Lawrence, G., Dolly, J.O., 2007. Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential. J. Cell. Sci. 120, 2864-2874.

Meng. J., Ovsepian, S.V., Wang, J., Pickering, M., Saase, A., Aoki, K.R., Lawrence, G.W. 2009 Activation of TRPV 1 mediates calcitonin gene-related peptide release, which excites trigeminal sensory neurons and is attenuated by a retargeted botulinum toxin with anti-nociceptive potential. J. Neurosci. 29, 4981–4992.

Mense, S. 2004. Neurobiological basis for the use of botulinum toxin in pain therapy. J. Neurol. 251 Suppl 1:I1-7.

Meunier, F.A., Schiavo, G., Molgó, J. 2002 Botulinum neurotoxins: from paralysis to recovery of functional neuromuscular transmission. J Physiol. Paris. 96, 105-113.

Meunier, F.A., Lisk, G., Sesardic, D., Dolly, J.O., 2003. Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. Mol. Cell. Neurosci. 22, 454-466.

Mika, J., Rojewska, E., Makuch, W., Korostynski, M., Luvisetto, S., Marinelli, S., Pavone, F., Przewlocka, B. 2011 The effect of botulinum neurotoxin A on sciatic nerve injury-induced neuroimmunological changes in rat dorsal root ganglia and spinal cord. Neuroscience 175, 358–366

Moreno-López, B, de la Cruz, R.R., Pastor, A.M., Delgado-García, J.M. 1997 Effects of botulinum neurotoxin type A on abducens motoneurons in the cat: alterations of the discharge pattern. Neuroscience. 81, 437-455.

Morenilla-Palao, C., Planells-Cases, R., García-Sanz, N., Ferrer-Montiel, A., 2004. Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. J. Biol. Chem. 279, 25665-25672.

Morihara, T., Mizoguchi, A., Takahashi, M., Kozaki, S., Tsujihara, T., Kawano, S., Shirasu, M., Ohmukai, T., Kitada, M., Kimura, K., Okajima, S., Tamai, K., Hirasawa, Y., Ide, C. 1999 Distribution of synaptosomal-associated protein 25 in nerve growth cones and reduction of neurite outgrowth by botulinum neurotoxin A without altering growth cone morphology in dorsal root ganglion neurons and PC-12 cells. Neuroscience 91, 695-706.

Morris, J.L., Jobling, P., Gibbins, I.L. 2002 Botulinum neurotoxin A attenuates release of norepinephrine but not NPY from vasoconstrictor neurons. Am. J. Physiol. Heart Circ. Physiol. 283, H2627-2635.

Nakano, H., Okabe, T., Hashimoto, H., Sakaguchi, G. 1990 Incidence of Clostridium botulinum in honey of various origins. Jpn. J. Med. Sci. Biol. 43, 183-195.

Nakov, R., Habermann, E., Hertting, G., Wurster, S., Allgaier, C. 1989 Effects of botulinum A toxin on presynaptic modulation of evoked transmitter release. Eur. J. Pharmacol. 164, 45-53.

Naumann, M., Dressler, D., Hallett, M., Jankovic, J., Schiavo, G., Segal, K.R., Truong, D. 2013 Evidence-based review and assessment of botulinum neurotoxin for the treatment of secretory disorders. Toxicon 67, 141-152

Palomar, F. J., Mir, P. 2012 Neurophysiological changes after intramuscular injection of botulinum toxin. Clin. Neurophysiol. 123, 54-60.

Park, H.J., Lee, Y., Lee, J., Park, C., Moon, D.E. 2006 The effects of botulinum toxin A on mechanical and cold allodynia in a rat model of neuropathic pain. Can. J. Anaesth. 53, 470-477.

Pastor, A.M., Moreno-López, B., De La Cruz, R.R., Delgado-García, J.M. 1997 Effects of botulinum neurotoxin type A on abducens motoneurons in the cat: ultrastructural and synaptic alterations. Neuroscience 81, 457-478.

Pavone, F., Luvisetto, S. 2010 Botulinum neurotoxin for pain management: insights from animal models. Toxins (Basel) 2, 2890-2913.

Pellizzari, R., Rossetto, O., Schiavo, G., Montecucco, C., 1999. Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 354, 259-268.

Popoff, M.R., Bouvet, P. 2013 Genetic characteristics of toxigenic Clostridia and toxin gene evolution. Toxicon 75, 63-89

Pozzi, D., Condliffe, S., Bozzi, Y., Chikhladze, M., Grumelli, C., Proux-Gillardeaux, V., Takahashi, M., Franceschetti, S., Verderio, C., Matteoli, M. 2008 Activity-dependent phosphorylation of Ser187 is required for SNAP-25-negative modulation of neuronal voltage-gated calcium channels. Proc. Natl. Acad. Sci. USA. 105, 323-328.

Proietti, S., Nardicchi, V., Porena, M., Giannantoni A. 2012 Attivita della tossina botulinica A in linee cellulari di cancro prostatico. Urologia. 79, 135-141.

Purkiss, J., Welch, M., Doward, S., Foster, K., 2000. Capsaicin-stimulated release of substance P from cultured dorsal root ganglion neurons: involvement of two distinct mechanisms. Biochem. Pharmacol. 59, 1403-1406.

Ranoux, D., Attal, N., Morain, F., Bouhassira, D. 2008 Botulinum toxin type a induces direct analgesic effects in chronic neuropathic pain. Ann. Neurol. 64, 274–283.

Rapp, D.E., Turk, K.W., Bales, G.T., Cook, S.P. 2006 Botulinum toxin type A inhibits calcitonin gene-related peptide release from isolated rat bladder. J. Urol. 175, 1138-1142.

Ravichandran, E., Gong, Y., Al Saleem, F.H., Ancharski, D.M., Joshi, S.G., Simpson, L.L. 2006 An initial assessment of the systemic pharmacokinetics of botulinum toxin. J. Pharmacol. Exp. Ther. 318, 1343-1351

Ray, P., Berman, J.D., Middleton, W., Brendle, J. 1993 Botulinum toxin inhibits arachidonic acid release associated with acetylcholine release from PC12 cells. J. Biol. Chem. 268, 11057-11064

Ray, P., Ishida, H., Millard, C. B., Petrali, J. P. & Ray, R. 1999 Phospholipaise A2 and arachidonic acid-mediated mechanism of neuroexocytosis: a possible target of botidinum neurotoxin A other then SNAP-25. J. Appl. Toxicol. Suppl 1, S27-8.

Relja, M. 1997 Treatment of tension-type headache by local injection of botulinum toxin. Eur J Neurol 4, S71–S73.

Relja, M., Klepac, N. 2002 Different doses of botulinum toxin A and pain responsiveness in cervical dystonia. Neurology 58 , A474

Relja, M., Telarović, S. 2004 Botulinum toxin in tension-type headache. J. Neurol. 251, Suppl 1, I12-14.

Relja, M., Miletić, V. 2006 Botulinum toxin type-A in the treatment of painful diabetic neuropathy, A prospective study. Neurology 66, A70

Relja, M., Poole, A.C., Schoenen, J., Pascual, J., Lei, X., Thompson, C.; European BoNTA Headache Study Group. 2007 A multicentre, double-blind, randomized, placebo-controlled, parallel group study of multiple treatments of botulinum toxin type A (BoNTA) for the prophylaxis of episodic migraine headaches. Cephalalgia. 27, 492-503.

Restani, L., Antonucci, F., Gianfranceschi, L., Rossi, C., Rossetto, O., Caleo, M., 2011 Evidence for anterograde transport and transcytosis of botulinum neurotoxin A (BoNT/A). J. Neurosci. 31, 15650-15659.

Restani, L., Novelli, E., Bottari, D., Leone, P., Barone, I., Galli-Resta, L., Strettoi, E., Caleo, M. 2012 Botulinum neurotoxin A impairs neurotransmission following retrograde transynaptic transport. Traffic 13, 1083-1089.

Restani, L., Giribaldi, F., Manich, M., Bercsenyi, K., Menendez, G., Rossetto, O., Caleo, M., Schiavo, G. 2012 Botulinum neurotoxins A and E undergo retrograde axonal transport in primary motor neurons. PLoS Pathog. 8(12):e1003087.

Rodriguez, R.L., Fernandez, H.H. 2013 Sustained benefit of painful legs moving toes syndrome with botulinum toxin type A. J. Clin. Neurosci. 20, 1031-1032.

Russell, A., Kavia, R., Dasgupta, P., Sahai, A. 2013. The use of botulinum toxin for the treatment of urologic pain. Curr Opin Urol. 23, 570-578

Santini, M., Fabri, S., Sagnelli, P., Manfredi, M., Francia, A. 1999 Botulism: a case associated with pyramidal signs. Eur. J. Neurol., 6, 91–93.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., Montecucco, C. 1992 Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature 359, 832-835.

Schulte-Mattler, W.J., Opatz, O., Blersch, W., May, A., Bigalke, H., Wohlfahrt ,K. 2007 Botulinum toxin A does not alter capsaicin-induced pain perception in human skin. J. Neurol. Sci. 260, 38-42.

Schulte-Mattler, W.J., Krack, P. 2004 BoNTTH Study Group. Treatment of chronic tension-type headache with botulinum toxin A: a randomized, double-blind, placebo-controlled multicenter study. Pain 109, 110-114.

Scott, A.B., Rosenbaum, A., Collins, C.C. 1973 Pharmacologic weakening of extraocular muscles. Invest. Ophthalmol. 12, 924-927.

Scott, A.B. 1980 Botulinum toxin injection into extraocular muscles as an alternative to strabismus surgery. J. Pediatr. Ophthalmol. Strabismus. 17, 21-25.

Seth, J., Khan, M.S., Dasgupta, P., Sahai, A. 2013 Botulinum toxin-what urologic uses does the data support? Curr Urol Rep. 14, 227-234

Shimizu, T., Shibata, M., Toriumi, H., Iwashita, T., Funakubo, M., Sato, H., Kuroi, T., Ebine, T., Koizumi, K., Suzuki, N., 2012. Reduction of TRPV1 expression in the trigeminal system by botulinum neurotoxin type-A. Neurobiol. Dis. 48, 367-378.

Shin, M.C., Yukihira, T., Ito, Y., Akaike, N. 2013 Antinociceptive effects of A1 and A2 type botulinum toxins on carrageenan-induced hyperalgesia in rat. Toxicon 64, 12-19.

Silberstein, S., Mathew, N., Saper, J., Jenkins, S., for the BOTOX® Migraine Clinical Research Group. 2000 Botulinum toxin type A as a migraine preventive treatment. Headache 40, 445-450.

Silberstein, S.D., Blumenfeld, A.M., Cady, R.K., Turner, I.M., Lipton, R.B., Diener, H.C., Aurora, S.K., Sirimanne, M., DeGryse, R.E., Turkel, C.C., Dodick, D.W. 2013

OnabotulinumtoxinA for treatment of chronic migraine: PREEMPT 24-week pooled subgroup analysis of patients who had acute headache medication overuse at baseline. J. Neurol. Sci. 331, 48-56.

Silva, J., Pinto, R., Carvallho, T., Coelho, A., Avelino, A., Dinis, P., Cruz, F. 2009 Mechanisms of prostate atrophy after glandular botulinum neurotoxin type a injection: an experimental study in the rat. Eur Urol. 56,134-140.

Simpson L. 2013 The life history of a botulinum toxin molecule. Toxicon 68, 40-59.

Singh, J.A., Fitzgerald, P.M. 2010 Botulinum toxin for shoulder pain. Cochrane Database Syst. Rev. 9, CD008271. doi: 10. 1002/14651858. CD008271. pub2.

Sluka, K.A., Kalra, A., Moore, S.A., 2001. Unilateral intramuscular injections of acidic saline produce a bilateral, long-lasting hyperalgesia. Muscle Nerve 24, 37-46.

Soares, A., Andriolo, R.B., Atallah, A.N., da Silva, E.M. 2012 Botulinum toxin for myofascial pain syndromes in adults. Cochrane Database Syst. Rev. 4, CD007533. doi: 10. 1002/14651858. CD007533. pub2.

Sobel, J. 2005 Botulism. Clin. Infect. Dis. 41, 1167-1173.

Stell, R., Thompson, P. D., Marsden, C.D. 1988 Botulinum toxin in spasmodic torticollis. J. Neurol. Neurosurg. Psychiatry 51, 920–923.

Sudhof, T.C. 2013 A molecular machine for neurotransmitter release; synaptotagmin and beyond. Nat. Med. 19, 8-13

Sugii, S., Ohishi, I., Sakaguchi, G., 1977. Correlation between oral toxicity and in vitro stability of Clostridium botulinum type A and B toxins of different molecular sizes. Infect. Immun. 16, 910-914.

Sycha, T., Samal, D., Chizh, B., Lehr, S., Gustorff, B., Schnider, P., Auff, E., 2006. A lack of antinociceptive or antiinflammatory effect of botulinum toxin A in an inflammatory human pain model. Anesth. Analg. 102, 509-516.

Tang-Liu, D.D., Aoki, K.R., Dolly, J.O., de Paiva, A., Houchen, T.L., Chasseaud, L.F., Webber, C. 2003 Intramuscular injection of 125I-botulinum neurotoxin-complex versus 125I-botulinum-free neurotoxin: time course of tissue distribution. Toxicon 42,461-469.

Tarsy, D., First, E.R. 1999 Painful cervical dystonia: clinical features and response to treatment with botulinum toxin. Mov Disord. 14,1043-1045.

Thenganatt, M.A., Fahn, S., 2012 Botulinum toxin for the treatment of movement disorders. Curr. Neurol. Neurosci. Rep. 12, 399-409.

Thyssen, A., Hirnet, D., Wolburg, H., Schmalzing, G., Deitmer, J.W., Lohr, C. 2010 Ectopic vesicular neurotransmitter release along sensory axons mediates neurovascular coupling via glial calcium signaling. Proc. Natl. Acad. Sci. U S A. 107, 15258-15263.

Torii, Y., Akaike, N., Harakawa, T., Kato, K., Sugimoto, N., Goto, Y., Nakahira, S., Kohda, T., Kozaki, S., Kaji, R., Ginnaga, A. 2011 Type A1 but not type A2 botulinum toxin decreases the grip strength of the contralateral foreleg through axonal transport from the toxin-treated foreleg of rats. J. Pharmacol. Sci. 117, 275-285.

Truong, D.D., Stenner. A., Reichel, G. 2009 Current clinical applications of botulinum toxin. Curr Pharm Des.15, 3671-3680.

Tsai, Y.C., Maditz, R., Kuo, C.L., Fishman, P.S., Shoemaker, C.B., Oyler, G.A., Weissman, A.M. 2010 Targeting botulinum neurotoxin persistence by the ubiquitin-proteasome system. Proc Natl Acad Sci U S A. 107, 16554-16559.

Tsui, J.K., Eisen, A., Stoessl, A.J., Calne, S., Calne, D.B. 1986 Double-blind study of botulinum toxin in spasmodic torticollis. Lancet. 2, 245-247.

Tugnoli, V., Capone, J.G., Eleopra, R., Quatrale, R., Sensi, M., Gastaldo, E., Tola, M.R., Geppetti, P. 2007 Botulinum toxin type A reduces capsaicin-evoked pain and neurogenic vasodilatation in human skin. Pain 130, 76-83.

Tyler, H.R. 1963 Botulinus toxin: effect on the central nervous system of man. Science 139, 847–848

Upile T, Elmiyeh B, Jerjes W, Prasad V, Kafas P, Abiola J, Youl B, Epstein R, Hopper C, Sudhoff H, Rubin J. 2009 Unilateral versus bilateral thyroarytenoid Botulinum toxin injections in adductor spasmodic dysphonia: a prospective study. Head. Face. Med. 5,20.

Vacca, V., Marinelli, S., Eleuteri, C., Luvisetto, S., Pavone, F. 2012 Botulinum neurotoxin A enhances the analgesic effects on inflammatory pain and antagonizes tolerance induced by morphine in mice.Brain. Behav. Immun. 26, 489-499

Vacca, V., Marinelli, S., Luvisetto, S., Pavone, F. 2013 Botulinum toxin A increases analgesic effects of morphine, counters development of morphine tolerance and modulates glia activation and μ opioid receptor expression in neuropathic mice.
Brain Behav. Immun. 32, 40-50.

Verderio, C., Grumelli, C., Raiteri, L., Coco, S., Paluzzi, S., Caccin, P., Rossetto, O., Bonanno, G., Montecucco, C., Matteoli, M. 2007 Traffic of botulinum toxins A and E in excitatory and inhibitory neurons. Traffic 8, 142–153.

Verderio, C., Pozzi, D., Pravettoni, E., Inverardi, F., Schenk, U., Coco, S., Proux-Gillardeaux, V., Galli, T., Rossetto, O., Frassoni, C., Matteoli, M. 2004 SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. Neuron 41, 599-610.

Voller, B., Sycha, T., Gustorff, B., Schmetterer, L., Lehr, S., Eichler, H.G., Auff, E., Schnider, P. 2003 A randomized, double-blind, placebo controlled study on analgesic effects of botulinum toxin A. Neurology 61, 940-944.

Waseem, Z., Boulias, C., Gordon, A., Ismail, F., Sheean, G., Furlan, A.D. 2010 Botulinum toxin injections for low-back pain and sciatica. Cochrane Database Syst. Rev. 1, CD008257. doi: 10. 1002/14651858. CD008257. pub2.

Welch, M.J., Purkiss, J.R., Foster, K.A., 2000. Sensitivity of embryonic rat dorsal root ganglia neurons to Clostridium botulinum neurotoxins. Toxicon 38, 245-258.

Wenham, T.N. 2008 Botulism: a rare complication of injecting drug use. Emerg. Med. J. 25, 55-56

Wheeler, A.H., Goolkasian, P., Gretz, S.S. 1998 A randomized, double-blind, prospective pilot study of botulinum toxin injection for refractory, unilateral, cervicothoracic, paraspinal, myofascial pain syndrome. Spine 23, 1662–1666.

Wheeler, A., Smith, H.S. 2013 Botulinum toxins: mechanisms of action, antinociception and clinical applications. Toxicology 306, 124-146. .

WHO: World Health Organisation: Botulism**.** Fact sheet N°270 Updated August 2013 http://www. who. int/mediacentre/factsheets/fs270/en/

Wiegand, H., Erdmann, G., Wellhoner, H. H. 1976 125I-labelled botulinum A neurotoxin: pharmacokinetics in cats after intramuscular injection. Naunyn. Schmiedebergs. Arch. Pharmacol. 292, 161–165.

Wiegand, H., Wellhöner, H.H.. 1977 The action of botulinum A neurotoxin on the inhibition by antidromic stimulation of the lumbar monosynaptic reflex. Naunyn. Schmiedebergs. Arch. Pharmacol. 298, 235-238.

Winocour, S., Murad, M.H., Bidgoli-Moghaddam, M., Jacobson, S.R., Bite, U., Saint-Cyr, M., Tran, N.V., Lemaine, V. 2014 A systematic review of the use of Botulinum toxin type A with subpectoral breast implants. J. Plast. Reconstr. Aesthet. Surg. 67-34-41.

Wohlfarth, K., Schubert, M., Rothe, B., Elek, J., Dengler, R., 2001. Remote F-wave changes after local botulinum toxin application. Clin. Neurophysiol. 112, 636-640.

Wright, M.C., Cho, W.J., Son, Y.J. 2007 Distinct patterns of motor nerve terminal sprouting induced by ciliary neurotrophic factor vs. botulinum toxin. J. Comp. Neurol. 504, 1-16.

Wu, H., Sultana, R., Taylor, K.B., Szabo, A. 2012 A prospective randomized double-blinded pilot study to examine the effect of botulinum toxin type A injection versus Lidocaine/Depomedrol injection on residual and phantom limb pain: initial report. Clin J Pain. 28, 108-112.

Xiao, L., Cheng, J., Dai, J., Zhang, D. 2011 Botulinum toxin decreases hyperalgesia and inhibits P2X3 receptor over-expression in sensory neurons induced by ventral root transection in rats. Pain Med. 12, 1385-94.

Xiao, L., Cheng, J., Zhuang,Y., Qu, W., Muir, J., Liang, H., Zhang, D. 2013 Botulinum toxin type A reduces hyperalgesia and TRPV1 expression in rats with neuropathic pain. Pain Med. 14, 276-86.

Yiangou, Y., Anand, U., Otto, W.R., Sinisi, M., Fox, M., Birch, R., Foster, K.A., Mukerji, G., Akbar, A., Agarwal, S.K., Anand, P. 2011 Increased levels of SV2A botulinum neurotoxin receptor in clinical sensory disorders and functional effects of botulinum toxins A and E in cultured human sensory neurons. J Pain Res. 4, 347-55.

Yuan, R.Y., Sheu, J.J., Yu, J.M., Chen, W.T., Tseng, I.J., Chang, H.H., Hu, C.J. 2009 Botulinum toxin for diabetic neuropathic pain: a randomized double-blind crossover trial. Neurology 72, 1473-1478.

Zamponi, G.W. 2003 Regulation of presynaptic calcium channels by synaptic proteins. J Pharmacol Sci 92, 79–83.

Zhang, P., Ray, R., Singh, B.R., Ray, P., 2013. Mastoparan-7 rescues botulinum toxin-A poisoned neurons in a mouse spinal cord cell culture model. Toxicon 76, 37-43.

Zhang, X., Verge, V.M., Wiesenfeld-Hallin, Z., Piehl, F., Hökfelt, T.1993 Expression of neuropeptides and neuropeptide mRNAs in spinal cord after axotomy in the rat, with special reference to motoneurons and galanin. Exp. Brain. Res. 93,450-461.

Zúñiga, C., Díaz, S., Piedimonte, F., Micheli, F., 2008. Beneficial effects of botulinum toxin type A in trigeminal neuralgia. 2008. Arq. Neuropsiquiatr. 66, 500-503.

Figure 1 Schematic representation of 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.

Figure 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 (grey 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. Size of of schematic representations of proteins, atoms and neurotransmitter is not shown in scale compared to synaptic structures and vesicles.

Figure 3. Fluorescent microphotograph of cleaved SNAP-25 in the ipsilateral trigeminal nucleus caudalis 5 d 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

Figure 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 (grey *vs* red) indicates possible change in neuronal activity.

Figure 1

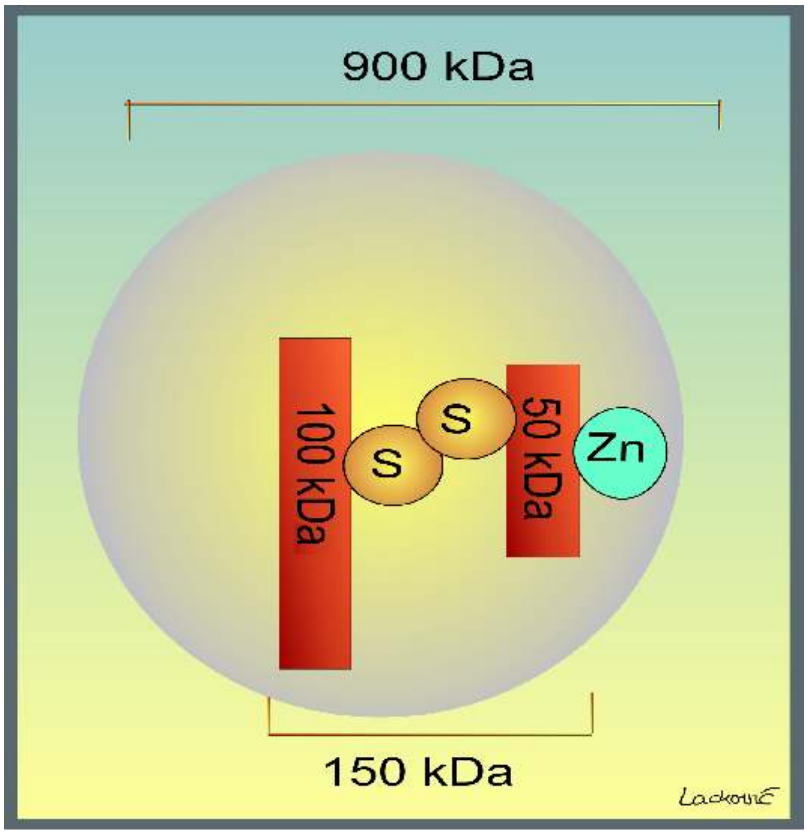

Figure 2

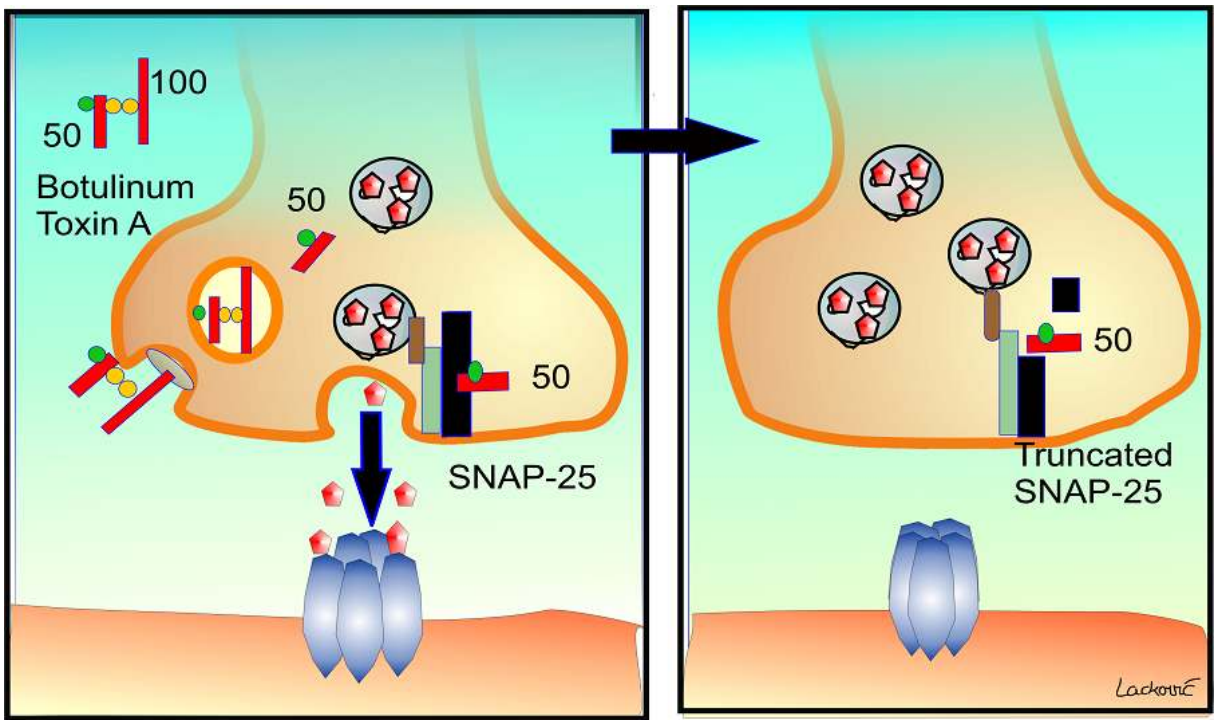
100
50
Botulinum
Toxin A
50
50
SNAP-25
50
Truncated
SNAP-25
Lackovic

Figure 3

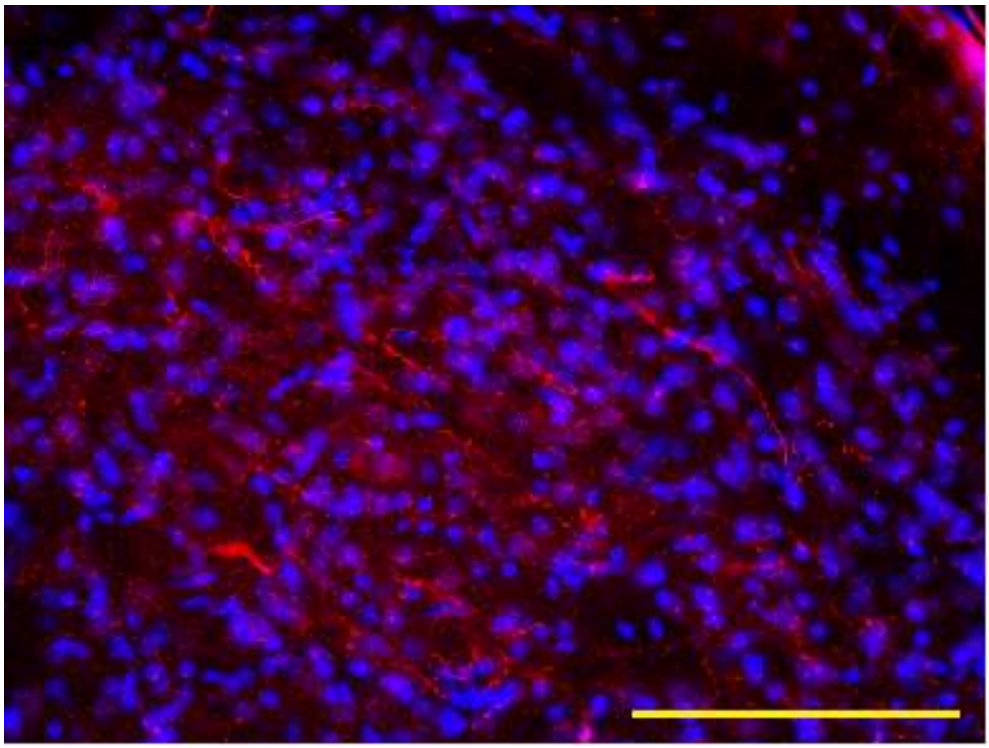

Figure 4

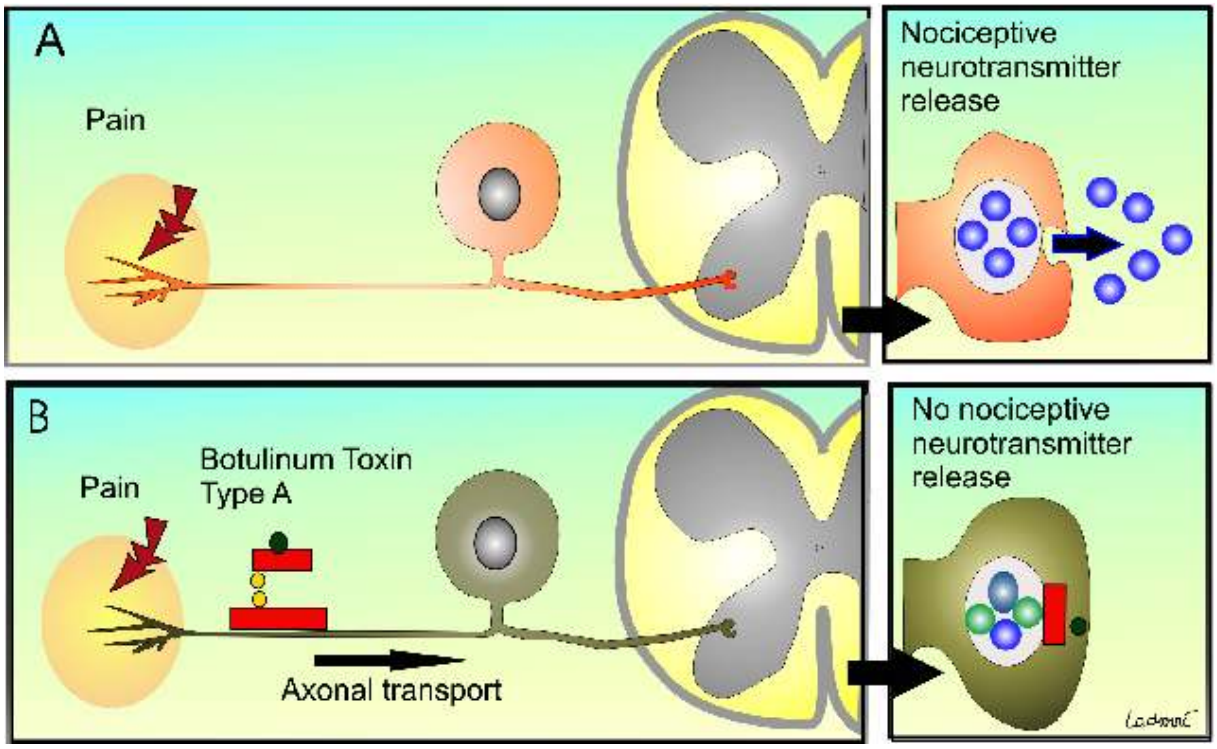
A
Pain
Nociceptive
neurotransmitter
release
B
Pain
Botulinum Toxin
Type A
Axonal transport
No nociceptive
neurotransmitter
release

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., 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)

Table 2 Summary of experimental data supporting peripheral hypothesis of BoNT/A action on pain (left) and experimental data supporting central antionociceptive 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 neuropathic and mirror pain models	*(Favre Guilmard., 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	*(Qui 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 sensoryreceptor expression in neurogenic bladder	*(Apostolidis et al., 2005)*	Contralateral effect after BTX-A injection into the distallytransected sciatic nerve in a model of bilateral pain	*(Bach-Rojecky and Lacković, 2009)*
Reduction of peripheral neuropeptide release in iris muscle and urinarybladder	*(Ishikawa et al., 2000; Rapp et al., 2006; Lucioni et al., 2008)*	Evidence of SNAP-25 cleavage in caudal medulla and spinal cord sensoryregions after low dose peripheral BTX-A injection	*(Matak et al., 2011; Matak et al., 2012; Marinelli et al., 2012)*
Decreased glutamate-evoked mechanical sensitivityof craniofacial muscle nociceptors	*(Gazerani et al., 2010)*	Abolishment of trigeminal pain-evoked dural neurogenic inflammation, dependentlyon axonal transport in trigeminal nerve	*(Filipovic et al., 2012)*

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; Gazerani et al., 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., 2012)*
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., 2005; Bach-Rojecky and Lacković, 2005; Bach-Rojecky et al., 2010; Filipović et al., 2012)*