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The effect of Botulinum neurotoxin type B on neuropathic pain

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“Rare sono le persone che usano la mente,
poche coloro che usano il cuore
e uniche coloro che usano entrambi.”

Rita Levi-Montalcini

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Introduction

Botulinum neurotoxins (BoNTs) are the most potent toxins known and are the causative agent of botulism, a serious and potentially fatal paralytic disease. The main effect of BoNTs is predominantly exerted as inhibitor of acetylcholine (ACh) release from cholinergic nerve endings at the skeletal neuromuscular junction (NMJ). The action of BoNTs at the NMJ has been extensively investigated and knowledge gained in this field laid the basis for the use of BoNTs in human pathologies characterized by excessive muscle contractions. Over the past few years, therapeutic applications of BoNTs were extended to a wide variety of neurological disorders ranging from treatment of overactive skeletal and smooth muscles to management of hypersecretory disorders. Pain conditions, and particularly neuropathic and intractable pain, are some of the pathological states that have been recently treated with BoNTs with beneficial effects. Despite both BoNT/A and BoNT/B are commercially available, in clinical practice the therapeutic use of BoNT/A is prevalent. Development of immunogenicity is an important problem concerning the long-term use of BoNT/A. Studies indicate that neutralizing antibodies develop in more than 2% of BoNT/A treated patients. The high number of patients not responding to this treatment makes it necessary to test other BoNT serotypes in clinical practice and BoNT/B was proposed as a possible alternative treatment to BoNT/A. However, only a smaller number of studies have focused on clinical use of BoNT/B. Therefore, to understand the molecular mechanisms whereby BoNT/B alter pain processing, and the possible differences between the two serotypes in these mechanisms, has become urgent and indispensable.

Chapter 1

Botulinum neurotoxins

Historical overview and classification

Botulinum neurotoxins (BoNTs) are produced by different species of anaerobic Gram-positive bacteria belonging to the genus *Clostridium*. These bacteria, and in particular their spores, are spread in soil, water, vegetation and in the intestinal tract of many animals (Rossetto et al., 2014). In 1895 van Ermengem identified and isolated for the first time the bacterium responsible for botulism, *Clostridium botulinum*. Five years later two different bacterial neurotoxins, subsequently nominated as type A and B, have been discovered and in the following years other serotypes of BoNT produced by different species of clostridia have been identified (Bentivoglio et al., 2015). BoNTs have been classified on the basis of specific antibody recognition and each serotype is identified by a letter (from A to G). Recently, an additional BoNT serotype (H) has been discovered, however this result must be confirmed (Montecucco & Rasotto, 2015). BoNT/G, isolated by Gimenez and Ciccarelli in 1969 from a cornfield in Argentina (Ciccarelli et al., 1977), is the only one BoNT produced by *Clostridium argentinense*; the other BoNT serotypes are produced by *Clostridium botulinum*, *Clostridium baratii* and *Clostridium butyricum*. These bacterial species were divided into 6 groups according to the fermentation process used and to toxin produced. Although most strains of *Clostridium botulinum* express a single toxin serotype, some strains can produce a mixture of BoNT subtypes or mosaic toxins (see table 1).

Clostridium species						
	Group I **Proteolytic C.botulinum**	**Group II** **Non-proteolytic C.botulinum**	**Group III** **C.botulinum**	**Group IV** **C.argentinense**	**Group V** **C.butyricum**	**Group VI** **C.baratii**
Type	**A-B-(H)*** ***serotype H remains to be experimentally verified**	**B-E-F**	**C/D**	**G**	**E**	**F**
Subtype	**A_1→A_{10}** **B_1→B_3** **B_5(B)/B_6** **B_7/A(B)** **Ab/Af/Af_8** **Bf/F_1→F_5**	**B_4;E_1→E_3** **E_6→E_1** **F_6**	**C/D** **CD/DC**		**E_4/E_5**	**F_7**

Table 1. Serotypes and subtypes of BoNT produced by different classes of neurotoxigenic *Clostridium* species. (Adapted from Rossetto et al., 2014).

BoNTs specifically affect vertebrates but different animal species show a great range of sensitivity to the different BoNTs. Serotypes /A, /B, and /E are those more frequently associated with human botulism. The most common forms of human botulism are *food-borne botulism*, which occurs following the ingestion of food containing the pre-formed toxin, and *infant botulism*, caused by the ingestion of food contaminated with clostridia spores that germinate into the gastrointestinal tract where the bacterium can proliferate due to the reduced resident microbiota in infants (Aureli et al., 1986; Johnson & Montecucco, 2008; Koepke et al., 2008). Other forms of human botulism are much rarer and include *inhalational botulism*, owing to inhalation of BoNT contained in

aerosols, *iatrogenic botulism*, caused by injections of excessive clinical doses of BoNT, and *wound botulism*, which is almost exclusively associated with drug injections (Rossetto et al., 2014). After breaching the intestinal epithelial barrier, BoNTs reach the lymphatic and blood circulation. Through the circulation the toxins can reach the peripheral nervous system (PNS) but cannot enter the central nervous system (CNS) because BoNTs are unable to cross the blood-brain barrier (Rossetto et al., 2014). The cause of the high toxicity of BoNTs is their absolute neurospecificity. In PNS the toxins contact the peripheral cholinergic nerve endings and acting inside nerve block acetylcholine release from the presynaptic nerve terminal at the neuromuscular junction (Simpson, 2013). The block of neurotransmitter release, *via* their activity directed specifically on SNARE proteins, induce flaccid paralysis and autonomic dysfunctions and, in severe form of botulism the death occurs due to respiratory failure (Montecucco & Rasotto, 2015). During the time that the toxin is present in blood it is highly stable. Clinical trials have provided impressive evidence for stability of the toxin in the human body. Fagan et al. (2009) have carried out toxicity assays on serum samples from patients diagnosed with type A oral botulism. They were able to detect active toxin in the circulation of patients as much as 11 days after ingestion of tainted food. Sheth et al. (2008) reported the presence of active toxin in the serum of a patient even 25 days after onset of illness. These clinical trials showed that the toxin enters the circulation and remains there intact and biologically active until it is delivered to the target cells, or until you delete it from the body. The very long systemic half-life of botulinum neurotoxin in the human body can probably be attributed to the fact that, even at multi-lethal doses (the 50% lethal dose, $LD_{50,}$ in mice and human varies between 0,1 ng and 1 ng of toxin per kg

of body weight), the circulating titer of toxin is too low to be easily or efficiently revealed by any metabolic system (Simpson, 2013).

Structure

Different species of clostridia *(botulinum, argentinense, butyricum and baratii)* produce eight different BoNT serotypes (from A to H). The neurotoxins, released *via* bacterial lysis, are synthesized as a single inactive polypeptide chain of 150 kDa. Inactive polypeptide, subjected to the action of trypsin or a trypsin-like protease, is cleaved (nicked) and transformed into an activated di-chain molecule (DasGupta & Sugiyama 1972a). Clostridia possess the proteases necessary to activate only some toxin serotypes (for example, BoNT/A is released from bacteria as the fully active toxin). For some BoNT serotypes, clostridia do not have this ability, so, through the bacterial lysis is exclusively released the inactive protoxins (BoNT/E is the most well studied of the unnicked toxins, it is between one and two orders of magnitude less potent than the nicked variant) (Simpson, 2013) or, as type B, only a portion of active neurotoxin are produced (Das Gupta & Sugiyama 1972b). Active di-chain neurotoxin is formed by a 50 kDa light chain (LC) linked by a disulphide bond with a 100 kDa heavy chain (HC) (Montecucco et al., 1994). Structural and biochemical data (Robinson et al., 1988; Schiavo et al., 1993) showed that the active neurotoxin di-chain possesses different domains that play different roles during cell intoxication. LC domain is the catalytic and toxic domain; this is a metalloprotease domain that specifically cleaves the necessary proteins to the neurotransmitter exocytosis, the SNARE proteins. The HC domain is functionally divided into two distinct domains: the translocation HC_N domain (the 50 kDa

amino-teminal half of the HC chain), required to transport LC across the membrane of endocytic vesicles into the neuronal cytosol, and the receptor binding HC_c domain (the 50 kDa carboxyl-terminal half of the HC chain), divided in turn in two different sub-domains, necessary for the binding of the toxin to the nerve cell surface (Pellett et al., 2015; Rossetto et al., 2014) (see figure 1).

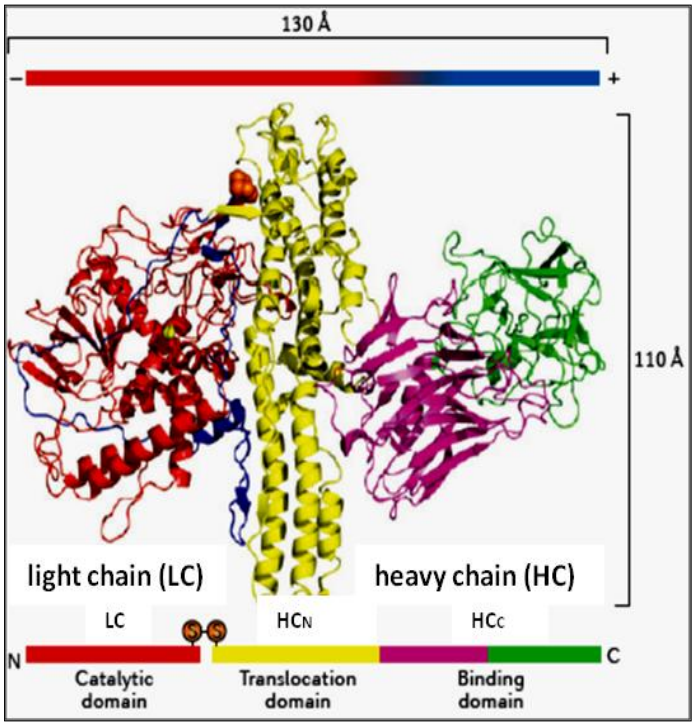


Figure 1. Botulinum Neurotoxin serotype A. The 50 kDa light chain (LC, red) is linked to the 100 kDa heavy chain (HC, yellow, violet and green) by a disulphide bond. The LC chain is the catalytic and toxic domain; The HC chain is functionally divided into the translocation domain (HC_N, yellow), required for transport of the LC from the endosome into the cell cytosol, and the receptor binding domain (HCc), divided in turn in two different sub-domains (violet and green), necessary for the binding of the toxin to the cell surface (modified from Rossetto et al., 2014).

The absolute neurospecificity of BoNTs is the cause of their high toxicity. BoNTs only bind to peripheral nerve terminals, particularly those of skeletal and autonomic cholinergic nerves (Dolly et al., 1984). To bind the presynaptic membrane of peripheral nerves, BoNTs use two independent receptors: a polysialoganglioside (PSG) and a protein receptor included in the lumen of synaptic vesicles membrane (see figure 2). PSG molecules are present at a high density on the presynaptic membrane and are organized in microdomains that include glycoproteins and glycolipids. PSGs act as 'magnets' that attract toxins that pass close and concentrate them on the nerve terminal surface. The binding of BoNTs to the PSG molecule is rapid: BoNTs bind to the most distal part of the PSG sugar head *via* a PSG-binding site that is located in the HC domain of the neurotoxin. Studies of the rat NMJ have shown that hundreds of BoNT/A or BoNT/B molecules can bind per square micrometer of the presynaptic membrane (Black & Dolly 1986). Following attachment to PSG, BoNT/B, /DC and /G bind the secretory vesicle protein synaptotagmin (Syt, isoforms I, II,) *via* a binding site in the HC_C domain, close to the PSG-binding. The two binding sites of PSG and Syt are structurally separated and independent of each other (Dong et al., 2003; Rummel et al., 2007). It has been noted that, unlike the mouse and rat, human Syt-II is not a high affinity receptor for BoNT/B and /G due to a mutation present only in humans and chimpanzees. This mutation eliminates one of three main points of interaction between Syt-II and BoNT/B and it would explain the disparity in potency of BoNT/B in humans compared to mice (Strotmeier et al., 2012). BoNT/A and /E bind the synaptic vesicle protein SV2 (isoforms A, B, and C) (Dong et al., 2006). Although isoform SV2C seems to be the main receptor that is involved in BoNT/A binding in vitro *via* an interaction with the HC

domain, both SV2A and SV2B can also mediate BoNT/A entry, and all three isoforms are expressed on motor nerve terminals (Dong et al., 2006, 2008). Glycosylated residues are present in the toxin-binding site of SV2 and this could be potentially clinically relevant because different pattern of glycosylation among individuals would provide a simple explanation for the variable sensitivity of different patients to BoNT/A1 injection, which is often observed in clinical settings (Rossetto et al., 2014). Syt and SV2 are integral proteins of the synaptic vesicle membrane and expose their BoNT-binding sites inside the lumen of the vesicle. Unlike PSG, these protein receptors are not directly accessible to BoNTs. Syt and SV2 become available only after the fusion of the synaptic vesicle with the presynaptic membrane, when the vesicular lumen is exposed to the extracellular environment. The binding of the toxin to its two receptors is needed to the subsequent step of intoxication: the endocytosis of BoNTs (Rossetto et al., 2014).

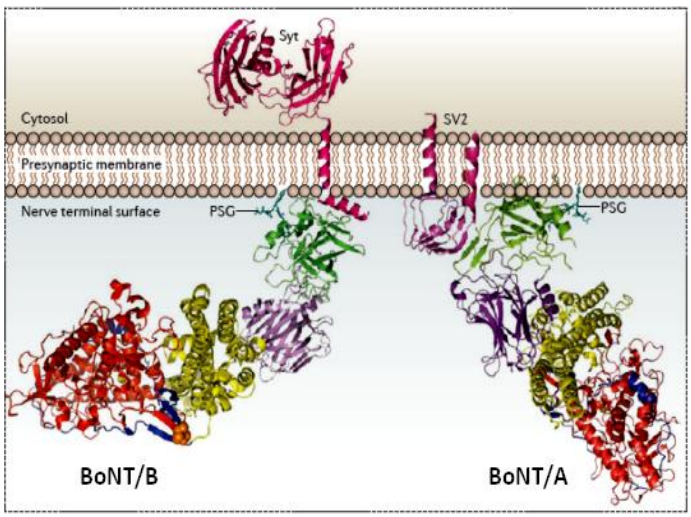


Figure 2. Binding of botulinum neurotoxins on the surface of peripheral terminals nerve (modified from Rossetto et al., 2014).

Following binding to the membrane receptors, the neurotoxin is internalized in vesicles. To reach its target the toxin has to move his catalytic LC domain from the vesicular lumen into the cytosol of nerve cells. Acidification of the vesicles, generated by the vesicular ATPase proton pump that drives the reentry of neurotransmitter into the synaptic vesicle after exocytosis, triggers a conformational change of the HC_N domain which inserted into the vesicle membrane. The HC_N domain forms in this way a transmembrane protein channel that allows translocation of the LC metalloprotease domain from the vesicular lumen inside the cytosol (Montal, 2010). The release of the LC domain into the cytosolic side, requires the reduction, and so the cleavage, of the inter-chain disulphide bond (Fischer & Montal, 2007). The reduction of the disulphide bond at any stage before its exposure to the cytosol prevents LC translocation, so this domain must emerge on the cytosolic side before reduction takes place (Fischer & Montal, 2007). The cleavage of inter-chain disulphide bonds is catalyzed in the cytosol cell by different enzymatic systems and when this occurs the LC domain translocation is irreversible and the toxin is so free to interact with its target, the SNARE proteins.

SNARE proteins

SNARE proteins, (**S**oluble **N**-ethylmaleimide-sensitive factor **A**ttachment protein **RE**ceptor), are membrane-associated proteins that participate in the fusion of synaptic vesicles with the plasmatic membrane. These proteins, on the basis of their locations, were originally classified into two categories: vesicle-associated or v-SNARE proteins, incorporated into the membranes of transport vesicles, and target-membrane or t-SNARE proteins, that are proteins associated with nerve terminal membranes. A more recent classification, based on the presence of arginine (R) or glutamine (Q) residue in a conserved 60-70-amino-acid motif, has divided these proteins into: R-SNAREs (generally, but not always, on vesicles) and Q-SNAREs (usually at the target membrane) (Stow et al., 2006). The synaptic vesicles membrane contains a single R-SNARE (the synaptobrevin, SYB, also called vesicle-associated membrane protein or VAMP) while the plasmatic membrane of the neurons contains two different Q-SNAREs (syntaxin and SNAP-25). When the vesicles are approaching the plasmatic membrane, SNARE proteins interact with each other to form a four-helix parallel bundle known as the core complex of SNARE (Sutton et al., 1998). The SNARE core complex is formed by ionic interactions between amino acids of SNARE motifs of one syntaxin, one VAMP, and two SNAP-25 (Meng & Wang, 2015). The core complex is remarkably stable and can be separated only by boiling in the presence of sodium dedocyl sulfonate (SDS) (Fasshauer et al., 2002). The assembly of synaptobrevin, syntaxin, and SNAP-25 into the SNARE complex creates a bridge between the two membranes destined to fuse. (see figure 3).

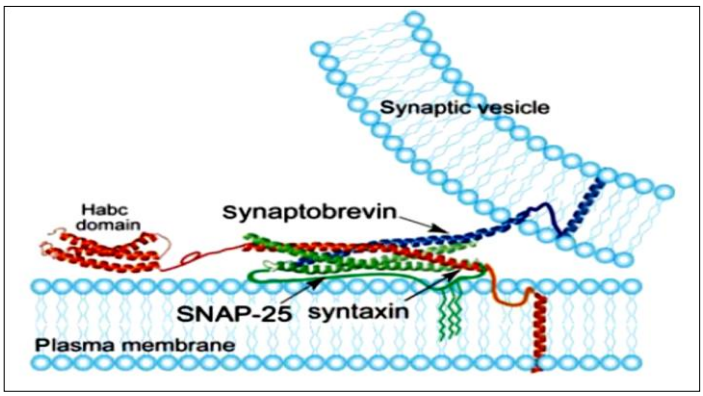


Figure 3. Description of molecular assembly of synaptobrevin, syntaxin and SNAP-25 into the SNARE core complex (modified from the book: 'Molecular Mechanisms of Neurotransmitter Release', edited by Zhao Wen Wang).

The cleavage of these proteins by BoNTs causes a failure of core complex assembly and a synaptic vesicle exocytosis block (Hayashi et al., 1994). Each SNARE protein possesses particular characteristics that make it the target of a specific BoNT serotype.

Synaptobrevin (SYB, also known as VAMP) is a protein of synaptic vesicles of 13 kDa. VAMP was originally identified and cloned from Torpedo nervous system (Trimble et al., 1988). Later it was found in a variety of species including mammals and non-vertebrates species, as squid, Aplysia and Drosophila showing a high degree of conservation in evolution (Südhof et al., 1989; Elferink et al., 1989; Yamasaki et al., 1994). The VAMP family consists of many isoforms but only three of these have been extensively characterized: VAMP1, VAMP2, and VAMP3 (Schiavo et al., 2000). Each of them has distinct functions and their expression varies between different species and tissues. For example, VAMP1 is expressed in sensory neurons to regulate pain-

peptide exocytosis and in motor neurons to mediate acetylcholine release (Meng et al., 2007; Peng et al., 2014). VAMP2 is mainly expressed in the CNS and regulates exocytosis of neurotransmitters from small vesicles (Schoch et al., 2001). VAMP3 is widespread outside the nervous system and is involved in integrin-mediated cell adhesion (Luftman et al., 2009). VAMP proteins contain a short carboxyl-terminal segment located inside the vesicle lumen, while most of the molecule is exposed to the cytosol. BoNT serotypes /B, /D, /F and /G cleave the cytosolic segment of VAMP1, -2 and -3 and release its amino-terminal part into the cytosol (see figure 4).

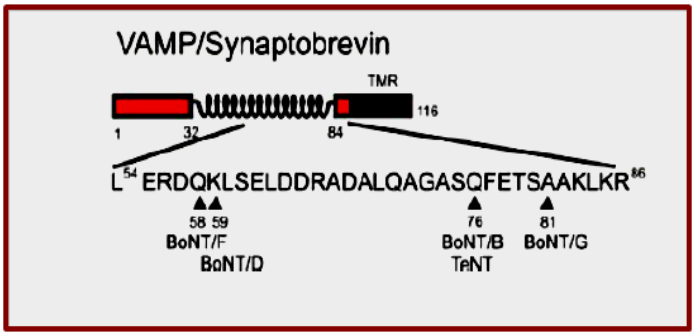


Figure 4. BoNT/B, /D, /F and /G cleave VAMP1-2 and -3 on the conserved central portion of the protein and release its amino-terminal part into the cytosol (modified from Binz et al.,2010).

It has been observed that VAMP1 of chicken and rat is insensitive to BoNT/B due to a single amino acid replacement at the site of BoNT/B cleavage. This amino acid substitution has been proposed to be associated with the resistance of rats and chickens to type B botulism, which contrasts with the sensitivity of humans and mice (Montecucco et al., 1994). Further experiments indicate that a polymorphism at position

48 of VAMP1 in rats, renders VAMP1 resistant (I48) or sensitive (M48) to BoNT/D. Also the human VAMP1 contains the same I48 polymorphism, and this may explain why humans are insensitive to BoNT/D (Peng et al., 2014). To confirm this, it has been reported that synaptic transmission in isolated human nerve-muscle biopsy tissues was not blocked by BoNT/D (Coffield et al., 1997) moreover, low doses of BoNT/D into the extensor digitorum brevis muscle of human volunteers showed no significant paralysis, indicating that humans are indeed insensitive to BoNT/D (Sobel, 2005; Eleopra et al., 2013).

Syntaxin was originally identified as the HPC-1 antigen, in the mouse hippocampus and amacrine cells of the retina (Barnstable et al., 1983). Afterwards Bennett, in 1992, called this protein syntaxin (STX) (Tagaya et al., 1995; Yoshida et al., 1992). Syntaxin is an integral membrane protein of 35 kDa, located mainly on the neuronal plasmatic membrane. The NH2-terminal portion is exposed to the cytosol and is followed by a transmembrane domain and few extracellular residues. Two isoforms of different length have been identified in neurons (STX1A and STX1B) and a vast polymorphism is present in several other tissues as in chromaffin granules (Tagaya et al., 1995), in tissues of the immune system, in thymus, in adipose tissue (Rezaei Farimani et al., 2015), in spleen and lymph nodes (Bin et al., 2013). It was observed that STX1A and STX1B, having a differential distribution in some parts of both peripheral and central nervous system (Ruiz-Montasell et al., 1996), play distinct roles in supporting neuronal survival by glial cells (Kofuji et al., 2014).

Several syntaxin isoforms, including STX1, undergo a complex pattern of alternative splicing and this differential expression of the protein could

be important for the formation of distinct SNARE complexes with different SNAP-25 and VAMP isoforms (Schiavo et al., 2000). The syntaxin protein is the target of BoNT/C but various syntaxin isoforms show different sensitivity to the neurotoxin: isoforms 1A, 1B, 2, 3 are sensitive, while isoforms 4 and 5 are resistant to the toxin cleavage (Blasi et al., 1993a; Schiavo et al., 1995).

BoNT/C cleaves STX1, 2 and 3 at a single site near the cytosolic membrane surface causing the release of a large portion of the cytosolic domain of the protein (Blasi et al., 1993b) (see figure 5).

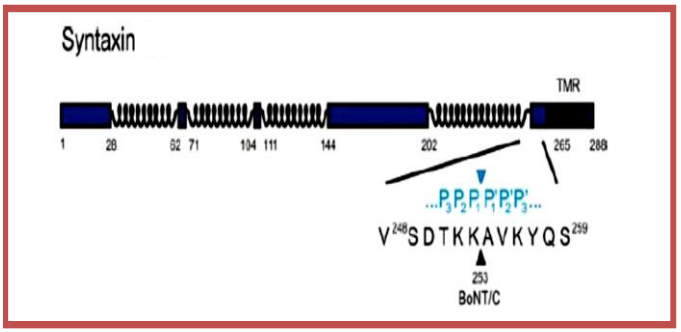


Figure 5. BoNT/C cleaves syntaxin at a single site near the cytosolic membrane surface located 12 aminoacids upstream of the C-terminal transmembrane domain between Lys 253 and Ala 254,. The action of BoNT/ C causes the release of a large portion of the cytosolic domain of the protein (modified from Binz et al., 2010).

SNAP-25 is a membrane associated protein highly conserved in the evolution (Risinger et al., 1993). It was originally characterized and located in the presynaptic terminals of mouse hippocampal mossy fibers and inner molecular layer of the dentate gyrus (Oyler et al., 1989). Subsequently it has been also identified in Drosophila, goldfish, Torpedo (Risinger et al., 1993), chicken (Bark, 1993), mouse (Oyler et al., 1989)

and in different human tissues (Bark & Wilson 1994; Jacobsson et al., 1994). SNAP-25 is a 206 residue protein of 25 kDa and, because of the absence of a transmembrane segment, its membrane localization is mediated by the palmitoylation of cysteine residues located in the middle of the polypeptide chain (Patarnello et al., 1993; Lane & Liu, 1997;Oyler et al., 1989). There are two isoforms of SNAP-25, SNAP-25a and SNAP-25b. They are generated by alternative splicing and differ by only nine amino acids (Bark, 1993). SNAP-25a appears early in the development while the expression of SNAP-25b begins on the onset of synaptogenesis (Oyler et al., 1991; Bark et al., 1995). Low levels of SNAP-25a expression are also found in the adult brain however, the two isoforms localization seems to be different, since SNAP-25a would be localized mainly in the cell-bodies of neurons and SNAP-25b would concentrate in nerve terminals (Oyler et al., 1989; Bark et al., 1995). Three homologous proteins to SNAP-25 have been identified: SNAP-23 (Ravichandran et al., 1996; Wang et al., 1997), SNAP-29 (Steegmaier et al., 1998) and SNAP-47 (Holt et al., 2006). SNAP-29 and SNAP-47 are involved in intracellular vesicle trafficking (Yamamori et al., 2011). In contrast, SNAP-23 is ubiquitously expressed in and out of the nervous system and participates in exocytosis of various cell types (Suh et al., 2010). In some regulated secretory pathways SNAP-23 can replace SNAP-25, thus suggesting a partial overlapping in their functions (Sadoul et al., 1997). SNAP-25 is substrate of the proteolytic activity of Botulinum neurotoxins type /A, /E and /C. Only a small segment of SNAP-25 is released by the selective proteolysis of BoNTs (see figure 6). BoNT/A, /E and /C cleave SNAP-25 at the carboxyl terminus, with the release of nine, twenty-six and eight residues peptides respectively (Blasi et al., 1993a,b; Binz et al., 1994; Williamson & Neale, 1998). Since the

BoNT/A and /C remove only a few residues from the C-terminus of SNAP-25 and this truncated form of SNAP-25 can form a stable SNARE complex, the molecular mechanism of BoNT/A- and /C-induced neuroparalysis remains to be elucidated (Rossetto et al., 2014).

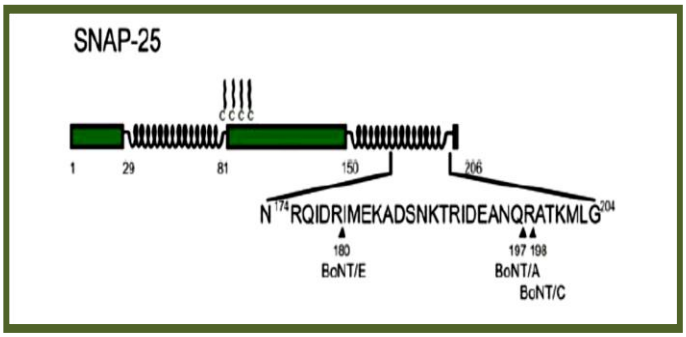


Figure 6. BoNT/A, /C and /E cleave SNAP-25 at the carboxyl terminus with the release of nine, eight and twenty-six residues peptides respectively (modifed from Binz et al.,2010).

The cleavage of SNARE proteins prevents the fusion of the synaptic vesicle membrane with the plasmatic membrane and blocks the transmitter release. The effect of this block causes a local muscle chemodenervation without degeneration of nerve endings (Jankovic, 2004). Muscle denervation promotes a compensatory sprouting of nerve terminals leading to the formation of new synaptic contacts. In approximately 3-4 months, when BoNTs are proteolysed and SNARE proteins are re-synthetized, there is a full recovery of the original neuromuscular junction with regression of the sprouts (Schiavo et al., 2000). One special feature of BoNTs is the reversibility of their action. BoNTs cause flaccid paralysis, which can result in respiratory failure and death, however, if respiration is mechanically assisted, botulism patients

recover completely from the neuroparalysis, though with different time courses depending on the BoNT serotype involved (Johnson & Montecucco, 2008). There is a remarkable diversity in the duration of BoNT-induced neuroparalysis. The lifetime of the metalloprotease within the nerve terminal cytosol is the predominant, but not the only factor that contributes to the time of paralysis (Pantano & Montecucco, 2014; Shoemaker & Oyler, 2013). The time of paralysis varies with the vertebrate species, the activity of the affected muscle, the toxin dose and the BoNT serotype involved. In mice the duration of paralysis is in the following order (with the longest duration of action first): BoNT/A, /C, /B, /D, /F, /G, /E (Naumann et al., 2013).

The pharmacologic potency, high specificity, and long duration of effect of BoNTs make these toxins remarkably effective therapeutic agents for the management of several disorders characterized by muscle hyperactivity.

In the next chapter will be discussed the clinical and experimental use of botulinum neurotoxin.

Chapter 2

BoNTs and neuropathic pain

The medical use of BoNTs as helpful drugs starts in the early 70s during the search for an alternative care to surgery for the treatment of strabismus. In those years several experiments on animals have demonstrated that low doses of BoNT/A produced the desired long-lasting dose-dependent muscle weakening without any systemic toxicity (Scott et al., 1973). Based on these results, in 1979 FDA (Food and Drug Administration) permitted to test BoNT/A in humans for the treatment of strabismus. These tests successfully demonstrated the effectiveness of neurotoxin and the results were published in 1980 (Scott, 1980). With this publication BoNT/A was decreed as a ‘‘novel therapeutic’’ and in 1989 FDA approved the ‘‘Alan Scott's type A neurotoxin preparation’’ as Oculinum, later renamed Botox®. In the 80s other research groups tested the neurotoxin for treatment of blepharospasm and dystonia and, once again, the BoNT/A successes for these medical indications were reported (Brin et al., 1987; Tsui et al., 1985). Afterwards BoNT/A has been used for different human diseases characterized by hyperactivity of the colinergic neuromuscolar fibres such as multiple dystonias (e.g. cranial, cervical, laryngeal, limb, hand) (Brin et al., 1987; Tsui et al., 1986), facial emispasm (Yoshimura et al., 1992), spasticity post stroke (Brashear et al., 2002), clubfoot (Ramachandran & Eastwood, 2006), infantile cerebral paralysis (Wasiak et al., 2004), and various other neurogenic conditions. During the use of BoNT/A for treatment of spasticity disorders it has been accidentally observed that pain associated with muscle spasms was significantly reduced to a level not ascribable to the simple reduction of muscle contracture (Brin et al., 1987). This observation, followed by intensive research, allowed in 2000 the FDA to approve the use of BoNT/A (Botox®) and BoNT/B (Myobloc®) for the treatment of abnormal head position and neck pain associated with

cervical dystonia (Abrams & Hallett, 2013). Subsequent studies, intent to understand the mechanism of action of BoNTs, have helped to determine that the neurotoxin, despite acting preferentially on nerve terminals between motor neurons and muscle fibers, can block the release of different neurotransmitters, including those involved in pain transmission (Arezzo, 2002; Cui et al., 2004; Park et al., 2015; Bittencourt da Silva et al., 2014; da Silva et al., 2014, 2015). These results have stimulated the study of the effects of BoNTs on the mechanism of pain and how and why BoNTs have relevant effects upon a variety of pain states, including neuropathic pain.

Neuropathic Pain

Generally, pain is divided into nociceptive and pathological pain. Nociceptive pain is caused by the sustained activation of peripheral nociceptors in response to peripheral tissue injury. The extent of nociceptive pain normally reflects the extent of tissue damage and when the damage heals it disappears. As opposed to nociceptive pain, pathological pain often depends on a chronic condition in which pain exceeds the extent of tissue damage. One type of chronic pain is neuropathic pain (NP), which is defined as ‘‘pain arising as a direct consequence of a lesion or disease affecting the somatosensory system either at peripheral or central level’’ (Haanpää et al., 2011). Diabetes, infection, nerve trauma and autoimmune conditions are examples of illnesses that may cause neuropathic pain. NP can occur spontaneously or can be evoked by a stimulus. It is described as burning, itching, lancing, and numbness but the symptoms that most characterize NP are two: hyperalgesia and allodynia (Intiso et al., 2015). Allodynia is related to a

condition in which a stimulus that normally does not cause pain becomes painful while hyperalgesia describes a state in which a stimulus that normally causes pain produces increased pain. The pathophysiological mechanisms underlying NP are not fully understood. Various hypotheses have been proposed including sensitization of nociceptors, abnormal ectopic excitability of neurons affected, loss of nociceptive control at the spinal level and CNS reorganization. All these conditions characterize the peripheral and central sensitization (Nickel et al., 2012).

Harmful stimuli (mechanical, thermal or chemical), applied to the skin or underlying tissues, activate nociceptors that are constituted by the free endings of afferent nociceptive fibers (the unmyelinated C and myelinated Aδ fibers) (Costigan et al., 2009). Nociceptor activation causes, among others, the release of calcitonin gene-related peptide (CGRP) and substance P (SP) from nociceptive nerve endings. CGRP modulates the cholinergic system, facilitates glutamatergic transmission and induces vasodilation; SP acts on mast cells inducing the release of histamine and cytokines, which directly excite nociceptors. Inflammatory mediators, produced by inflamed or damaged tissue and by immune system cells, such as bradykinin, prostaglandins and other derivatives of arachidonic acid together with growth factors, neurotransmitters and excitatory substances, can also activate and sensitize nociceptors (Julius & Basbaum, 2001). Nociceptors sensitization, also called peripheral sensitization, is determined by a lowering of activation threshold of free endings of nociceptive fibers (this causes hyperalgesia), until arriving in the most intense cases to the spontaneous activation (ectopic impulse generation) with onset of pain in the absence of stimulus (Costigan et al., 2009). Afferent nociceptive fibers, whose cell bodies are located in the dorsal root ganglia, convey the nerve impulses from periphery to the

dorsal horn of the spinal cord (lamina I-V). In the dorsal horns the majority of primary afferent fibers generates synapses with interneurons, whose axon remains entirely in the spinal cord and ramifies inside it. Here, the central endings of sensory fibers release glutamate and different neuropeptides including substance P, which increases and potentiates the action of glutamate (De Biasi & Rustioni, 1988). It was noted that as a result of persistent tissue damage, C fibers can discharge action potentials in a sustained and repeated manner. This causes a massive glutamate release and a sustained depolarization of dorsal horn neurons. In dorsal horn interneurons the excitatory amino acid glutamate determines the opening of ion channels type N-methyl-D-aspartate (NMDA) that produces an influx of extracellular Ca^{2+}. The intracellular increase of Ca^{2+} induces the expression of *c-fos*, a protein believed to be involved in the transcriptional control of genes that encode a variety of neurotransmitters, their receptors, and neuropeptides, including enkephalin and dynorphin. Enkephalin typically produces antinociceptive effects, while dynorphin has direct excitatory effects on spinal projection neurons but may also produce inhibition *via* a negative feedback mechanism on dynorphin-containing neurons (Hunt et al., 1987; Dubner & Ruda, 1992; Coderre et al., 1993). Therefore, a persistent tissue damage can lead to long-term changes in excitability of the interneurons of the dorsal horn and so generate a central sensitization namely a ‘‘pain memory’’ (Harden, 2005). Additional mechanism that contributes to the development of central sensitization is the loss of inhibitory control projecting to the superficial spinal cord dorsal horn. The spinal pain transmission system is under continuous inhibitory control generated by inhibitory interneurons, which release γ-aminobutyric acid (GABA), glycine, and endogenous opioids (enkephalins), and by descending

inhibitory pathways that release serotonin and norepinephrine (Harden, 2005). Because this inhibition acts as a ‘‘spinal gate’’ for sensory information, reduced inhibition increases the activity of neurons of the dorsal horn and promotes the central sensitization genesis. It was also observed that partial nerve injury may cause GABAergic inhibitory interneuron apoptosis and degeneration of C fibers (Moore et al., 2002). In the spinal cord most of the opioid μ receptors are located on the presynaptic terminal of the fibers C. Therefore, the loss of these afferent fibres results in a failure to analgesic response to endogenous opioids, favoring the maintenance of pain states, and the loss of an important pharmacological approach to neuropathic pain (Dickenson, 1994).

Pre-clinical and clinical use of BoNTs in the treatment of neuropathic pain

Neuropathic pain is a debilitating condition for which there are few effective treatments which can also have significant side effects. BoNTs are used frequently to treat muscle hyperactivity but are emerging more and more evidence in support of its analgesic potential (Walsh, 2010; Luvisetto et al., 2015; Pavone & Luvisetto, 2010; Bittencourt da Silva et al., 2014; da Silva et al., 2014; Cairns & Gazerani, 2014). The majority of the studies have focused on the commercially available BoNT/A (Botox®, Xeomin®, Dysport®) and BoNT/B (Myobloc®) serotypes but only BoNT/A was better evaluated in pre-clinical and clinical studies on neuropathic pain.

Table 2 summarizes the studies that have examined the effectiveness of BoNT/A and BoNT/B in peripheral neuropathic pain conditions.

Neuropathic pain			
	Type of pain	**BoNT serotype**	**References**
Pre-clinical studies (Rat/Mouse)	Mononeuropathy (Nerve ligation and nerve transection)	BoNT/A and BoNT/B	(Park et al., 2006; Luvisetto et al., 2007; Kitamura et al., 2009; Filipović et al., 2012; Huang et al., 2011; Park et al., 2015; Marinelli et al., 2010; Bach-Rojecky et al., 2005)
	Polyneuropathy (Chemotherapeutics, diabetes)	BoNT/A and BoNT/B	(Favre-Guilmard et al., 2009; Bach-Rojecky et at., 2010; Park et al., 2015)
Clinical studies (Human)	Mononeuropathy (Peripheral nerve injury and trigeminal neuralgia)	BoNT/A and BoNT/B	(Ranoux et al, 2008 .; Borodic & Acquadro, 2002; Piovesan et al., 2005; Türk et al, 2005.; Bohluli et al., 2011; H. Wu et al., 2012; Zhang et al., 2014; Breuer et al., 2009)
	Polyneuropathy (Diabetic neuropathy)	BoNT/A	(Ghasemi et al., 2014; Yuan et al., 2009; Jin et al., 2009; H. Wu et al., 2012;)
	Chronic low back pain	BoNT/A	(Jabbari, 2008)
	Myofascial pain	BoNT/A	(De Carli et al., 2016; Cheshire et al., 1994)

Table 2. Pre-clinical and clinical studies that have examined the effectiveness of BoNT/A and BoNT/B in neuropathic pain conditions.(Adapted from Pellet et al., 2015).

The analgesic effect of BoNTs: experimental evidence

Many models of neuropathic pain are currently in use both in rats and mice to mimic human peripheral neuropathic conditions. Mononeuropathy can be generated by Spinal Nerve Ligation (SNL) (Kim & Chung, 1992) and Sciatic Nerve Transection (SNT) (Campbell & Meyer, 2006), which simulate the clinical conditions of the amputation. By Partial Nerve Ligation (PNL) is possible to reproduce a nerve damage trauma-induced (Seltzer et al., 1990) and with Chronic Constriction Injury (CCI) may be recreated the clinical conditions of the chronic nerve compression (Bennett & Xie, 1988). Polyneuropathy can be induced by chemotherapy (vinca alkaloids, platinum drugs) or by diabetes (Siau et al., 2006; Tesch & Allen, 2007). The effect of BoNT/A on neuropathic pain was first analyzed on the peripheral neuropathy induced by partial sciatic nerve transection in rats (Bach-Rojecky et al., 2005). In this study the authors observe that a single peripheral injection of BoNT/A was sufficient to reduce thermal and mechanical hyperalgesia. The antinociceptive effect started 5 days following BoNT/A injection and lasted at least 15 days. Subsequently Park et al. (2006) tested the analgesic efficacy of BoNT/A in rats subjected to spinal nerve ligation. The researchers found that peripherally administered BoNT/A reduces mechanical and cold allodynia in neuropathic animals. By using chronic constriction injury (CCI) of the sciatic nerve in mice and rats other authors found that a single non-toxic dose of BoNT/A into plantar surface of animals injured paws was sufficient to induce antiallodynic effects for at least 3 weeks (Luvisetto et al., 2007; Marinelli et al., 2010). These effects were evident 24 hours after BoNT/A injection and were

dose-dependent. Furthermore, if BoNT/A was injected contralaterally to the lesion, the antiallodynic effects were not observed, demonstrating the absence of a systemic diffusion of the toxin in these experimental conditions. Unilateral infraorbital nerve constriction (IoNC) is an animal model that induces trigeminal neuralgia. By using this pain model, Kitamura et al. (2009) found that intradermal injection of BoNT/A in the area of infraorbital branch of the trigeminal nerve alleviated trigeminal neurophaty in rats. Similar results were obtained by Filipovic and his coworkers (2012). The authors observe that a single unilateral BoNT/A injection reduced allodynia induced by infraorbital nerve constriction and the dural extravasation that accompanies it for more than two weeks. Favre-Guilmard's group investigated the effect of BoNT/A in paclitaxel-induced peripheral neuropathy in rats (Favre-Guilmard et al., 2009). Paclitaxel treatment mimics the peripheral polyneuropathy induced by chemotherapy. The researchers observe that a single subplantar injection of BoNT/A produced a significant antihyperalgesic effect in the ipsi and contralateral injected paw of neuropathic animals 3 days after administration. In another study, animals become diabetic by a single intraperitoneal injection of streptozotocin were injected with BoNT/A either subcutaneously (Bach-Rojecky et al., 2010). As observed with paclitaxel treatment, also with this experimental pain model the authors demonstrate that BoNT/A reduced mechanical and thermal hypersensitivity not only on ipsilateral, but also on contralateral side. The antinociceptive effect started 5 days following BoNT/A injection and lasted at least 15 days. Due to the higher toxicity in mice and the resistance to the toxin in rats (Montecucco et al., 1994; Luvisetto et al., 2003), the effect of BoNT/B on peripheral neuropathic pain only recently was analyzed. In this regard, Huang and colleagues (2011) wanted to

investigate the effects of intrathecal (IT) BoNT/B injection on spinal nerve ligation-induced tactile allodynia in mice. In this study, a single IT BoNT/B injection was administered and pain thresholds were assessed up to 15 days after BoNT/B injection. The analgesic effect of BoNT/B became evident within 6 hours after IT treatment and lasted for the next 15 days. By observation of motor behavior, this experiment also revealed that the animals showed normal motor function and symmetrical ambulation and did not show signs of motor weakness. These results suggested a long-term alleviation of hyperalgesia by IT BoNT/B injection, as well as long-term dose-tolerability of the neurotoxin in mouse. A recent study described for the first time the effects of intraplantar BoNT/B administration on mono and polyneuropathies (Park et al., 2015). In this experiment mononeuropathy (MN) with ipsilateral tactile allodynia in mice is generated by physical injury to a peripheral nerve (L5 nerve ligation) whereas exposure to a chemotherapeutic agent (cisplatin) results in a polyneuropathy (PN) with persistent bilateral allodynia. On day 14, after the left L5 ligation for the MN, or day 15, after the initiation of the intraperitoneal cisplatin injection series for the PN, a single non-toxic dose of intraplantar BoNT/B was administered into the left hind paw. In this study the authors observed that BoNT/B injection prevented the allodynia in the ipsilateral but not in the contralateral paw in MN mice. Similarly, in the bilateral allodynia observed in the PN, the effect of the toxin was most evident in the paw ipsilateral to the intraplantar delivery. The authors emphasize the importance of the homolaterality antiallodynic effect of BoNT/B as it indicates that, as already observed with BoNT/A, the effects of intraplantar injection of the neurotoxin were not the result of a general systemic or contralateral spread of the treatment.

The analgesic effect of BoNTs: clinical evidence

In parallel to pre-clinical studies, the effect of BoNTs on neuropathic pain was also analyzed in clinical trials.

Ranoux' study has been the first double blind trial on the effect of BoNT/A in painful post-traumatic neuropathy (Ranoux et al., 2008). In this study, twenty-nine patients with focal painful neuropathies and mechanical allodynia received intradermal administration of BoNT/A into the painful area. The follow-up was performed at 4, 12 and 24 weeks after injections. BoNT/A treatment was associated with significant pain improvement from 2 weeks after the injection to 14 weeks. In particular, a reduction of the intensity of mechanical allodynia on the painful side, without alteration of the perception thresholds, were observed. These results indicated for the first time that BoNT/A may induce direct analgesic effects in patients with chronic neuropathic pain. Several studies concerned the use of BoNT/A for the treatment of trigeminal neuralgia (TN). In an open-label pilot study, Borodic & Acquadro (2002) evaluated the efficacy of multifocal BoNT/A injections for the treatment of patients with postsurgical pain syndromes, temporomandibular syndrome and idiopathic trigeminal neuralgia. 33 of 44 patients responded positively to treatment, including 8 of 11 patients with TN. The duration of beneficial effect ranged from 2 to 4 months. Thereafter other clinical studies have reported the same results also in patients refractory to classical medical treatments (Piovesan et al., 2005; Türk et al., 2005; Zúñiga et al., 2008; Bohluli et al., 2011). After positive outcomes from these studies, several double-blind and placebo-controlled trials were conducted (Wu et al., 2012; Zhang et al., 2014). In particular,

Zhang and coworkers (2014) treated 56 cases of classical TN with local multi-point injection of BoNT/A while 28 neuralgic patients received placebo. Follow-up visits were conducted every week after the injection, and the overall duration of the study for each patient was 8 weeks. BoNT/A significantly reduced pain intensity from the first week and the duration of beneficial effect was observed for all eight weeks. Another study investigated the effect of intradermal BoNT/A injections on pain symptoms of patients with diabetic neuropathic pain (Ghasemi et al., 2014). In this randomized controlled, double-blind clinical trial study, 40 diabetic patients with neuropathic pain in both feet were enrolled. Intradermal BoNT/A injections across dorsum of feets reduced burning, lancing, and numbness without causing side effects. Few years earlier, similar results had been obtained by Yuan et al. (2009). Another painful condition treated with BoNT/A is the phantom limb pain (PLP). Phantom limb pain and sensations are common in amputees; the pathophysiology is unclear and the treatment is difficult and often unsuccessful. Opioids are frequently used when non-narcotics failed, but are not effective in many cases. Jin et al. (2009) report a study on three patients, resistant to previous therapies, who were successfully treated with BoNT/A. In all three cases, the pain intensity was reduced significantly and no side effects were reported. The duration of response lasted up to 11 weeks.

In 2012, Wu and colleagues (Wu et al., 2012) conducted the first randomized, double-blinded pilot study on PLP. 14 amputees with intractable PLP received BoNT/A injection. BoNT/A injection was compared to lidocaine in patients with PLP and residual limb pain (RLP). RLP was defined as a painful condition that could occur very quickly after amputation due to scar and neuroma formation. Each patient was evaluated at baseline and every month after the injection for 6 months.

Researchers found that both BoNT/A and lidocaine injections resulted in immediate improvements of RLP and pain tolerance. The treatment effect lasted for 6 months in both groups (Wu et al. 2012). The effectiveness of BoNT/A was also evaluated in the treatment of chronic back pain and on myofascial pain syndrome (Jabbari, 2008; De Carli et al., 2016; Cheshire et al., 1994). For both pathologies, BoNT/A has proven effective in reducing pain.

As opposed to BoNT/A, only few studies have focused on clinical use of BoNT/B and of these the majority was concentrated on the therapeutic use the toxin in neurological disorder with muscular hyperactivity. Dry mouth is the most frequently reported side effect of BoNT/B in all the studies, even with very low doses and nausea, constipation, dysphagia and short efficacy have often been noted (Bentivoglio et al., 2015). In respect to the clinical use of BoNT/B on peripheral neuropathic pain, only one study is reported (Breuer et al., 2006). In this randomized, double-blinded pilot study the authors enroll 20 patients with carpal tunnel syndrome with associated hand pain to receive placebo or BoNT/B injections in three muscles anatomically attached to the carpal tunnel. The initial plan was to randomize the patients for each of the following treatments: placebo (normal saline solution); 2.500, 5.000 or 7.500 units of BoNT/B. During the trial, however, the authors were obliged to unblind the treatment of two patients (they had received 7.500 or 5.000 units of BoNT/B) due to adverse events occurred. The results of this experiment showed that the treatment with either BoNT/B or placebo decreases the hand pain. The maximum improvement occurred between weeks 6 and 9 of the trial. Significant decreases in pain were noted in both placebo and BoNT/B groups but there were no significant differences between the two groups. The authors conclude by affirming

that, despite BoNT/B is not dramatically superior to placebo for the relief of carpal tunnel syndrome symptoms, the similarity of improvements for both interventions suggests a strong placebo effect rather than a reduced effectiveness of treatment (Breuer et al., 2006; Singh, 2013).

Mechanism-based evidence for the analgesic action of BoNT/A and BoNT/B

With regard to the underlying pathophysiological mechanisms that operate in reducing pain, although several hypotheses have been suggested, the mechanisms of the analgesic effects of BoNTs remain unclear. A direct involvement of BoNTs in mechanisms involved in pain modulation was described by Cui et al. (2004) and Luvisetto et al. (2006), who analyzed the effects of BoNT/A and BoNT/B in formalin-induced inflammation as an animal model of inflammatory pain. Cui and coworkers noticed that a single peripheral subcutaneous injection of BoNT/A, after formalin solution injection into one hindpaw of rat, was able to reduce licking activity during the second inflammatory phase, which reflects the activation of central sensitization processes. The reduction of licking activity was accompanied by the inhibition of peripheral release of glutamate into the injected paw and by a reduction of paw edema that is usually observed as a consequence of inflammatory processes. This latter effect suggested to the researcher that BoNT/A may reduce not only the release of glutamate, but also of SP and CGRP, two neuropeptides involved in plasma extravasation and vasodilation. The analgesic activity of BoNT/A was considered by Cui and coworkers as a consequence of inhibition of peripheral sensitization, resulting in an indirect reduction of central sensitization processes. Luvisetto et al.

(2006) confirmed these results and extended the analysis on inflammatory pain using two BoNTs serotypes (BoNT/A and BoNT/B) and two different routes of administration (intracerebroventricular and intraplantar). These authors prove that pre-treatment with BoNT/A was able to reduce the second phase of formalin independently from the route of administration and that the neurotoxin could act not only peripherally but also at the central level. On the contrary, BoNT/B did not reduce licking activity and, when centrally injected, had a hyperalgesic effect on the interphase of the formalin test, phase characterized by the lack of licking activity due to inhibitory descending pathways. These results indicated a different mechanism of action of BoNTs and suggested that the two serotypes were not interchangeable, an important point to take into account when evaluating BoNTs as pain pharmaceuticals (Cui et al., 2004; Luvisetto et al., 2006). Previous *in vitro* experiments have already demonstrated that glutamatergic neurons are blocked by BoNT/A and BoNT/B while GABAergic neurons are blocked only by BoNT/B (McMahon et al., 1992; Frassoni et al., 2005; Verderio et al., 2004; Verderio et al., 2007). Considering the different effects of the two neurotoxins of different cell types, the effect of BoNT/B described by Luvisetto and coworkers (2006) on the interphase of the formalin test could be partially attributed to a block of GABA inhibition on the primary afferent fibers in spinal dorsal horn. Subsequent studies have also confirmed the hypothesis about the ability of BoNTs to interfere with the expression of substance P and calcitonin gene-related protein (CGRP), key mediators of neurogenic inflammation (Marino et al., 2014; Ramachandran et al., 2015; Hou et al., 2007; Lucioni et al., 2008; Meng et al., 2009). Different results about the analgesic effect of BoNT/B were published in the following years. In 2011, Yaksh et al (2011) showed that

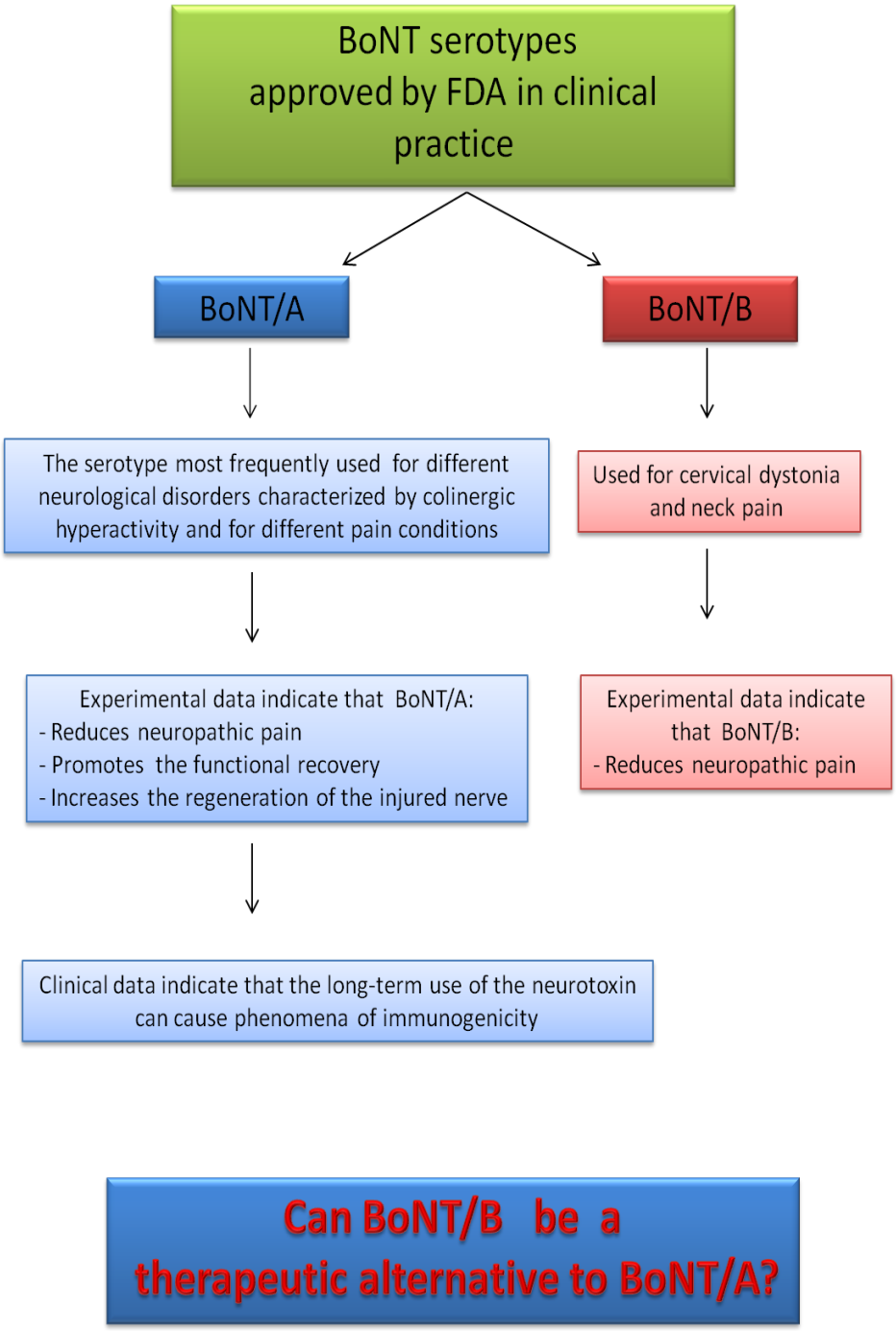
a single IT injection of BoNT/B, 2 or 5 days prior of intraplantar formalin injection, reduces the release of substance P from spinal afferent nociceptors and reduces spinal neuronal activation and nociceptive behavior in mice. In addition, in mice with spinal nerve ligation, IT injection of BoNT/B decreases tactile allodynia within 6 hours after IT treatment and lasted for the next 15 days (Yaksh et al., 2011). This study provided an initial assessment of the ability of IT BoNT/B injections to regulate spinal nociceptive processing. Few years later the results were confirmed using intraplantar injections of BoNT/B in mice (Marino et al., 2014). This experiment also showed that peripherally delivered BoNT/B is taken up at the peripheral afferent terminals and is transported in an active form to central afferent terminals where prevents the transmitter release. In the same years other studies demonstrated that BoNT/A can undergo, as well as an axonal migration, also a neuronal transcytosis (Restani et al., 2011, 2012; Matak et al., 2012; Antonucci et al., 2008; Marinelli et al., 2012). Marinelli and colleagues (2012) demonstrated that a single peripheral administration of BoNT/A in the paw of CCI mice was able not only to induce analgesic action but also to accelerate functional recovery through an acceleration of regenerative processes associated to the Schwann Cells proliferation. With the aim to better define the role of BoNT/A as an analgesic and to investigate the neural mechanisms involved, Mika et al. (2011) wanted to investigate the molecular changes that occur in DRG and spinal cord after CCI to the sciatic nerve in rats. The authors observed that peripheral administration of BoNT/A attenuates neuropathic pain-related behavior by modulating several proteins in DRG and spinal cord and by silencing microglia and macrophages in the same structures. The researchers suggest that the silence of microglia/macrophages after BoNT/A administration could be

secondary to the inhibition of neuronal activity and that this decrease in neuroimmune interactions could be the key to the long-lasting BoNT/A effect on neuropathic pain. Despite Mika et al. (2011) observations, and although the involvement of SNARE proteins in the release of mediators from macrophages has been demonstrated (Stow et al., 2006), the direct effect of BoNT/A and BoNT/B on this release thus far has not been adequately understood, as well as it was not understood the exact mechanism responsible for the analgesic effects of BoNT/A and BoNT/B.

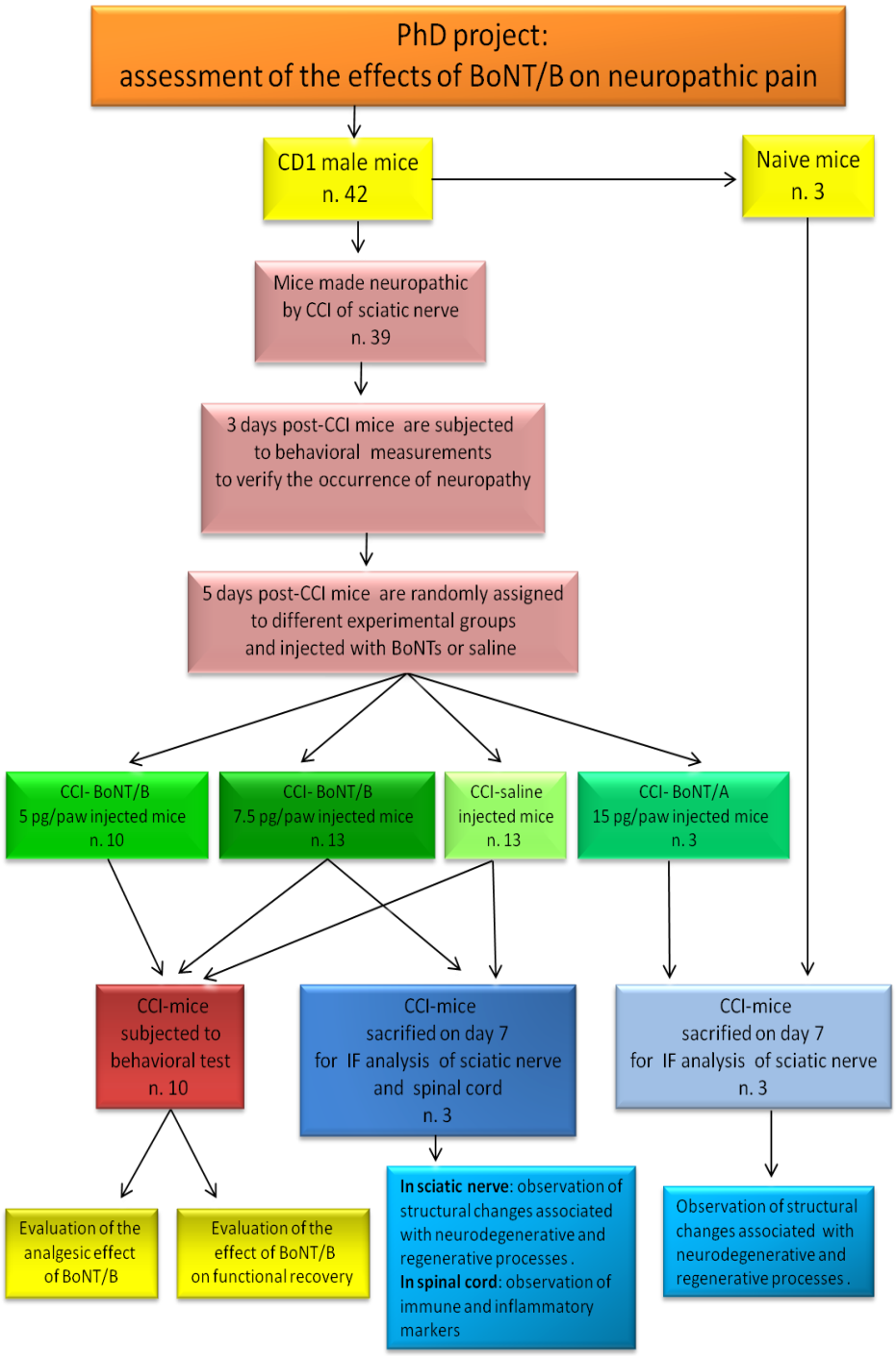
Chapter 3

Experimental section

BoNTs and neuropathic pain flowchart



Study design flowchart



Aim

Therapeutic use of botulinum neurotoxins is well established and is continuously expanding. In clinical practice, BoNT/A has been widely used to treat many pathological conditions characterized by autonomic overactivity. Although much is known about the action of BoNTs on the peripheral system, increasing evidence has demonstrated several effects also at the central level. Current data suggest that BoNTs, given locally into peripheral tissues, alter nociceptive processing initiated by inflammation or nerve injury. This demonstration has recently allowed the use of BoNT/A as a novel treatment option for a variety of pain syndromes including neuropathic pain, pathological pain condition for which no definitive treatments are available. In clinical practice it was observed that an increasing number of patients become non-responding to BoNT/A, in particular when repetitive injections were done. The absence of clinical response could be related to an immune response caused by the production of specific anti-BoNT/A antibodies. It was suggested that subjects who become less and less sensitive to BoNT/A could be treated with alternative BoNT serotypes: BoNT/B was proposed as a potential therapeutic option to BoNT/A. However, nowadays, the cellular and molecular pharmacology and mechanism of action of different BoNT serotypes is still not fully understood. Therefore, to avoid errors and ensure the therapeutic efficacy and safety of treatment, it becomes imperative to compare the mechanism of action adopted by the two neurotoxins, and if necessary to highlight their differences.

In this regard, the general purpose of my PhD project has been to assess the effect of BoNT/B on neuropathic pain. To this aim, mice made neuropathic by Chronic Constriction Injury (CCI) of sciatic nerve, were

injected with a single dose of BoNT/B (5 or 7.5 pg) into the injured hind paw. The time course of the neuropathy was observed for 101 days and the effect of the neurotoxin on both pain and functional recovery was evaluated. For a qualitative analysis of structural alterations and for a comparison with functional data, immunofluorescence (IF) experiments were associated to behavioral tests. By means of the investigative technique of IF, carried out on the sciatic nerve and on the dorsal horn of the spinal cord, we wanted to analyze the expression of several neuronal and glial markers associated with neurodegenerative and regenerative processes. In particular, in order to observe structural changes in damaged nerve, we analyzed the expression of NF200, marker of neurons intermediate filaments, P0 and PMP22, markers of peripheral myelin proteins. Moreover, we analyzed the expression of markers (GFAP, S100β) of Schwann Cells (SCs), and of SCs proliferation, which represent an important event promoting axon regeneration, by studying the co-expression of a SCs marker with the Cdc2 proliferation marker. The study of the expression of CC1 (marker of mast cells) and CD11b (marker of macrophages) gave us the opportunity to investigate the activation of immune cells, after the nerve damage. In spinal cord, to observe microglial cells, members of the monocyte/macrophage family, and their activation, we examined the coexpression of Cd11b and p-p38 markers on ipsilateral-side of dorsal and ventral horn. Microglial recruitment and activation in dorsal and ventral horn is accompanied by proliferation and activation of astrocytes in the ipsilateral-side of spinal cord. Together with microglia astrocytes play a critical role in chronic pain sensitization and their activation is correlated with chronic pain behaviors (Wang et al., 2009; Liu et al., 2012). In order to observe

astrocytes and their activation we used GFAP and p-p38 markers on dorsal and ventral horn of spinal cord.

Materials and methods

Animals

CD1 male mice (Charles River, Como, Italy) were used in the present study. Upon arrival, the animals were housed in groups of four in standard breeding cages (21cm x 21cm x 12 cm) placed in a rearing room at a constant temperature (22±1 °C) under diurnal conditions (light–dark: 08:00–20:00), with food and water ad libitum. At the time of surgery, they were approximately 12 weeks old and their weights ranged from 40 to 45g. The experiments were carried out between 10:00 and 14:00 h. Testing was performed blind as for treatment group to which each subject belonged. All procedures were in strict accordance with the Italian National law (DLGs n.26, 04/03/2014, application of the European Communities Council Directive 2010/63/UE) on care and handling of the animals, with the Institute's Animal Research Committee (Krakow, Poland) and with the guidelines of the Committee for Research and Ethical Issues of IASP (Zimmermann, 1983).

Surgical procedure

Following the procedure originally proposed by Bennett and Xie (Bennett & Xie, 1988) adapted for mice, the Chronic Constriction Injury (CCI) of sciatic nerve was used as model of peripheral nerve injury that can evoke neuropathic pain symptoms.

Surgery was performed under anesthesia induced by intraperitoneal injection (ip) of a mixture of ketamine (100 mg/kg) and xylazine (5

mg/kg) purchased from Sigma-Aldrich (St. Louis, MO). CCI was obtained by three unilateral ligatures of sciatic nerve. The middle third of the right sciatic nerve was exposed through a 1.5 cm longitudinal skin incision. Three ligatures (7-0 prolene, Ethicon) were tied loosely around the nerve. The wound was then closed with silk suture (4-0 vicryl, Ethicon) and the mouse was allowed to recover in a heated cage until all reflexes were normal. The injured and uninjured hindpaws were named as ipsilateral and contralateral hindpaws, respectively. To verify the occurrence of neuropathy, behavioral measurements were made on days 3, 4 and 5 after CCI. Only neuropathic mice were included into the study.

Drugs and peripheral injections

BoNTs, isolated and purified, (Rossetto et al., 1992; Schantz & Johnson, 1992), were a kind gift from Prof. C. Montecucco and Prof. O. Rossetto (University of Padova). The toxins were freezed in liquid nitrogen and stored at −80 °C in 10 mM Na HEPES, 150 mM NaCl, pH 7.2. Stock solutions of purified 150 kDa di-chain molecule of BoNT/A and BoNT/B were tested for activity in the ex vivo mouse hemidiagphram model and in the in vitro cleavage of SNAP-25 and VAMP/synaptobrevin. Injectable solutions of BoNTs were freshly made by dilution in saline (0.9% NaCl). BoNTs or vehicle injections were performed 5 days after CCI. For injections, a volume of 20 μl of either saline (0.9% NaCl) or selected BoNT solutions (BoNT/A: 15 pg/mouse; BoNT/B: 5 and 7.5 pg/mouse) was subcutaneously (sc) injected into plantar surface of the injured hindpaw of mice using a microsyringe equipped with a 26 gauge needle. Dose of BoNT/A (15 pg/paw) was chosen on the basis of neurotoxicity and previous studies (LD_{50}: 0.5–1.0 x 10^{-6} mg/kg) (Luvisetto et al., 2003, 2004, 2006; Marinelli et al., 2010). Dose of

BoNT/B (5 and 7.5 pg/paw) were chosen on the basis of preclinical studies on the safety margins of intramuscular injection of BoNTs in mice (Aoki, 2001). These studies show that BoNT/B subcutaneously administered into paw of mice is more toxic than BoNT/A, therefore, concentrations of BoNT/B higher than 7.5 pg/paw were not tested. The doses of BoNT/A and BoNT /B used in this experiment are the maximal effective doses that can be peripherally injected in mice without causing side effects including neuroparalysis and alteration of spontaneous locomotor activity.

Experimental groups

On day 3 post-CCI, mice were randomly assigned to different experimental groups. Behavioral investigation was conducted on three groups of animals two of which treated with two different doses of BoNT/B (5 and 7.5 pg/paw) and the third was used as control group. Regarding the IF analysis this was carried out on the sciatic nerve sections of four experimental groups: naive, CCI-saline, CC-BoNT/A 15 pg and CCI-BoNT/B 7.5 pg/paw injected animals. For spinal cord IF were considered only naive and CCI-BoNT/B 7.5 pg/paw treated mice because data on CCI-BoNT/A 15 pg injected animals had already been published by our laboratory (table 3 shows the total number of mice assigned to behavioral tests and biochemical assay).

To verify the occurrence of neuropathy, behavioral measurements were made on days 3, 4 and 5 after CCI. BoNTs or saline were injected into the injured hindpaws intraplantar (i.pl.) surface of mice on day 5, 2 hours after behavioral measurement. All groups of animals were examined for mechanical nociceptive thresholds, weight distribution, footprint walking tracks and measurement of sciatic static index (SSI). With the exception

of the animals sacrificed on day 7 post-CCI for IF analysis, all mice were tested until the day 101.

Behavioral tests	**Number of mice**	**Day of test**
CCI-BoNT/B 5 pg/paw (20µl)	N=10	From day 3 until day 101
CCI-BoNT/B 7.5 pg/paw (20µl)	N=10	From day 3 until day 101
CCI-Saline (20µl)	N=10	From day 3 until day 101
Immunofluorescence (IF) analysis		**Day of sacrifice to perform biochemical assay**
CCI-BoNT/B 7.5 pg/paw (20µl)	N=3	Day 7 post-CCI
CCI-BoNT/A 15 pg/paw (20µl)	N=3	Day 7 post-CCI
CCI-Saline (20µl)	N=3	Day 7 post-CCI
Naive (no CCI/injection)	N=3	Day 7 post-CCI

Table 3. Total number of mice assigned to behavioral tests and biochemical assay.

Behavioral tests

Measurement of mechanical nociceptive threshold

The onset of CCI-induced neuropathy was assessed by measuring the threshold of both hindpaws to normally non-noxious punctuate mechanical stimuli. The hindpaw nociceptive threshold, measured by an automatic von Frey apparatus (Dynamic Plantar Aesthesiometer, Ugo Basile, Italy), was expressed as the force (in grams) at which mice withdrew their paws in response to the mechanical stimulus. For habituation, mice were placed in plastic cages with a wire net floor 5 minutes before the experiment. The mechanical stimulus was applied to the mid-plantar surface of the hindpaw to induce a slight pressure to the skin. At each testing day, ipsi- and contra-lateral withdrawal thresholds were taken as mean of three consecutive measurements per paw with 10 seconds interval between each measurement.

Measurement of weight bearing

Weight bearing on the two hindlimbs was determined by incapacitance test (Linton Instrumentation, Norfolk, UK). The apparatus used was adapted for mice with a strain gauge/amplifier resolution of 0.03 grams and a strain gauge/amplifier accuracy of 0.1 grams. Mice were carefully placed in an angled plexiglas chamber positioned with hindpaws on the two separate force plates. Care was taken to ensure that the animal weight was directed onto the force plates and not dissipated through the walls of the chamber. The force exerted by each hindlimb (measured in grams) was automatically averaged over a 5 seconds period and, for each animal, measurement was repeated three times with 5 minutes interval.

According to Helyes et al. (2010), the weight percent distributed onto the ipsilateral hindlimb was calculated by the equation:

[ipsilateral weight/(ipsilateral weight + contralateral weight)] x 100.

Walking track analysis and measurement of sciatic static index

Evaluation of functional recovery was determined using the walking track analysis described by Inserra et al. (1998) and Varejão et al. (2001). The gradual recovery of the paw functionality was monitored through the analysis of individual free-walking patterns and by measuring several footprint parameters to calculate the sciatic static index (SSI). Footprints were recorded by dipping the mouse hindpaws in black ink and having them to freely walk along a Perspex runaway corridor (15 x 5 x 50 cm) lined with white paper (Dijkstra et al., 2000). Mice were tested from day 3 to day 101 post-CCI and, for each mouse, the footprint parameters were calculated from at least five footprints recorded on three different walking track runaways. The SSI was evaluated using the formula proposed by Baptista et al. (2007), calculated considering two parameters: the 1st–5th toe spread (TS) and the distance between the tip of the third toe and the most posterior aspect of the paw (paw length (PL)). These variables were measured and entered into the equation:

$$SSI=+101.3x(ITS-CTS)/CTS-54.03x(IPL-CPL)\ /CPL-9.5$$

where ITS and CTS are the ipsilateral and contralateral toe spreads, while IPL and CPL are the ipsilateral and contralateral paw lengths, respectively. Based on the equation, a value of SSI close to 0 corresponds to normal function while a value of -100 is equivalent to complete functional loss.

Biochemical assay

Sciatic nerve and Spinal cord immunohistochemistry

7 days post-CCI, mice were anaesthetized with chloral hydrate (500 mg/kg i.p.; Sigma-Aldrich, Italy) and the lesioned part of the ipsilateral sciatic nerve including ligatures were removed immediately before the perfusion and kept in immersion for 48 hours in paraformaldehyde (PFA (Sigma-Aldrich, Italy) 4% in 0.1 M phosphate-buffer saline (PBS, Sigma-Aldrich, Italy), pH 7.2, at room temperature. Trans-cardiac perfusion was performed with 100 ml saline, followed by 100 ml of PFA. After the perfusion, the entire spinal cord of each animal was removed and kept in PFA for 24 hours. Both nerves and spinal cord, after cryoprotection with solution of 30% (w/v) sucrose in PBS, were then stored at -80 °C until the sectioning.

Longitudinal sections (25 μm thick) of D7/CCI-sciatic nerves segment including the portion with ligature were cut on cryostat microtome and mounted directly on slides.

For IF staining, sciatic nerves sections were washed three times in PBS and then incubated overnight in Triton (Sigma-Aldrich, Italy) 0.3% in PBS, with different primary antibodies (see table 4):
- rabbit anti-VAMP2 (polyclonal, 1:100, Abcam)
- mouse anti-GFAP (monoclonal, 1:100, Sigma-Aldrich)
- rabbit anti-PMP22 (polyclonal, 1:100, Sigma-Aldrich)
- chicken anti-P0 (polyclonal, 1:100, Millipore)
- rabbit anti-Neurofilament 200 (polyclonal, 1:100, Sigma-Aldrich)
- mouse anti-S100β (polyclonal, 1:100, Sigma-Aldrich)

- rabbit anti-Cdc2 (polyclonal,1:100, Calbiochem)
- rat anti-CD11b (monoclonal, 1:100, Biorad)
- mouse anti-CC1 (monoclonal, 1:100, Santa Cruz)

Afterwards, after three times washing with PBS, sections were incubated, for 2 hours at room temperature, in Triton with secondary antibodies :
- donkey anti-mouse fluorescein-conjugated (FITC, 1:100, Jackson ImmunoResearch);
- goat anti-rabbit fluorescein-conjugated (FITC, 1:100, Santa Cruz);
- goat anti-rabbit rhodamine-conjugated (TRITC, 1:100, Jackson ImmunoResearch);
- donkey anti-chicken rhodamine-conjugated (TRITC, 1:100, Jackson ImmunoResearch).

Finally, the sections were and then incubated in with the nuclear marker bisbenzimide, DNA-fluorochrome (Hoechst 33258, 1:1000, Sigma-Aldrich) in PBS for 5 minutes, washed again three times in PBS and closed with glycerol/PBS 3:1 mounting medium and coverslipped.

For spinal cord, transverse sections (40 μm thick) of L4/L5 spinal cord segment were cut on a cryostat microtome and collected in PBS for free-floating double IF procedures. Ipsilateral and contralateral side of sections were recognized by marking the spinal cord with a notch on the contralateral side before mounting spinal cord trunk on the chuck of the cryostat. Spinal cord sections were first incubated overnight in Triton with different primary antibodies (see table4):
- mouse anti-GFAP (monoclonal, 1:100, Sigma-Aldrich)
- rat anti-CD11b (monoclonal, 1:100, Biorad)
- rabbit anti-p-p38 (polyclonal, 1:100, Santa Cruz).

Afterwards, the sections were washed three times in PBS and were incubated for 2 hours at room temperature in Triton with secondary antibodies:

- donkey anti-mouse fluorescein-conjugated (FITC, 1:100, Jackson ImmunoResearch);
- donkey anti-rat fluorescein-conjugated (FITC, 1:100, Jackson ImmunoResearch);
- goat anti-rabbit rhodamine-conjugated (TRITC, 1:100, Jackson ImmunoResearch).

Finally, the sections were washed three times in PBS, incubated in PBS with the nuclear marker Hoechst 33258, (1:1000), for 5 minutes, washed again and mounted on slides, air dried and cover slipped with glycerol/PBS 3:1 mounting medium.

To exclude non-specific signals of secondary antibodies and to ensure optimal results, control sections have been stained with secondary antibody alone, which did not show appreciable staining (data not shown).

Tissue	Antibody	Target
Nerve	**VAMP2**	Isoform 2 of VAMP protein
	GFAP	Dedifferentiated nonmyelinating Schwann Cells
	PMP22 and P0	Peripheral myelin proteins
	Neurofilament 200	Neurons intermediate filaments
	S100β	Dedifferentiated myelinating Schwann Cells
	Cdc2	Proliferanting Schwann Cells
	CD11b	Polymorphonuclear Cells, monocytes and NK Cells
	CC1	Mast Cells Chymase
Spinal cord	**GFAP**	Astrocytes
	CD11b	Microglia
	p-p38	Activated Cells

Table 4. Tissue and antibodies with their target.

Three animals per group were considered and three sections per animal of sciatic nerve and spinal cord were randomly selected and analyzed by experimenters blinded as to the treatment groups. Low (10x) magnification images of spinal cord sections and High (63x) magnification images of spinal cord and sciatic nerve of different sections were captured, using fixed parameters, by laser scanning confocal microscopy (TCS SP5 microscope, Leica Microsystem) connected to digital camera diagnostic instruments operated by I.A.S. software (Delta Systems, Italy). Since the part of the injured sciatic nerve close to the neural body did not show differences compared to the control sciatic nerve, only the part of nerve including the ligature was used. In sciatic nerve sections, the analysis of IF staining (in term of emitted fluorescence), was performed by using the ImageJ software (version 1.41, National Institutes of Health, USA). The fluorescence was quantified with RGB (red, green, blue) method, which uses brightness values for calculation (Fu et al., 2016). The number and emitted fluorescence of IF cells was automatically analyzed for each one of three nerve sections. Afterwards, the mean for each group of mice was calculated. For production of figures, brightness and contrast of images were adjusted by taking care to leave a light tissue fluorescence background to help visual appreciation of the lowest fluorescence intensity features and comparison among different experimental groups.

In spinal cord sections, to quantify positive glial cells immunoreactivity (IR), images of the ipsilateral side of the dorsal horn (medial portion of laminae I–IV) and ventral horn (dorsolateral column, lamina IX) for each one of three spinal cord sections was examined. Identification of laminae areas was helped by Hoechst staining of cells nuclei. Quantification was performed by using the ImageJ software. Image contrast was adjusted

such that the background level just disappeared and the cell bodies and processes appeared without boundaries; the same cutoff level was used for all images. For analysis of labeling of GFAP or CD11b positive IR-cells and their colocalization with p-p38, the number of positive IR cells was quantified for each one of the three spinal cord sections. Subsequently, the mean for each group of mice was calculated.

Results

Behavioral Effects of BoNT/B on Neuropathic Pain

Effects of BoNT/B on mechanical nociceptive threshold

The unilateral ligature of the sciatic nerve induces mechanical allodynia in the hindpaw ipsilateral to the ligature. To determine if the treatment with BoNT/B is analgesic, we analyzed the effect of the toxin on mechanical nociceptive threshold in neuropathic mice.

The withdrawal thresholds, of both the right and left hindpaw, was expressed as the force (in grams) at which mice withdrew their paw in response to the mechanical stimulus. Figure 7 shows that, after CCI, the mechanical nociceptive threshold decreased in CCI-saline injected mice around 50% in the ipsilateral compared to contralateral hind paw. Animals withdrew their ipsilateral paw after very low stimuli (6 grams), which did not evoke reaction in contralateral paw, therefore, we considered the ipsilateral response as allodynia. In CCI-saline treated mice, a robust ipsilateral allodynia was maintained for at least 50 days and, even if reduced, it was still present after 3 months, being the mechanical thresholds always different from those of contralateral paw. On the contrary, a single i.pl. injection of 5 or 7.5 pg/paw of BoNT/B in CCI-mice clearly antagonizes ipsilateral allodynia, as indicated by a net enhancement of the withdrawal threshold, with a recovery of 30%, corresponding to 80% of the value of the contralateral paw. In the CCI-BoNT/B treated groups the strong antiallodynic effect of the toxin was observed from the day after the injection and persisted for a very long time (see figure 7). Two-way ANOVA for repeated measures showed a

significant main effect for treatment ($F_{2,27}$ =10.941; p<0.0003), days ($F_{17,459}$ =68.739; p<0.0001) and treatment x days interaction ($F_{34,459}$ =7.031; p<0.0001) for mechanical threshold of ipsilateral hindpaws. For contralateral hindpaws only a main effect for days was observed ($F_{17,459}$ =1.7111; p=0.0369). Post*hoc* comparisons between CCI-saline and 5 pg/paw CCI-BoNT/B injected mice showed a significant effect from the day 6 (the day after the injection) till day 31 while the dose of 7.5 pg/paw of BoNT/B resulted significantly different from CCI-saline treated mice till day 41 (Tukey-Kramer, p<0.05).

Additional Two-way ANOVAs for repeated measures between ipsi and contralateral hindpaws indicated a strong significant main effect in the following experimental groups:

- CCI-saline mice: treatment ($F_{1,18}$ =200.301; p<0.0001), days ($F_{17,306}$ =13.299; p<0.0001), treatment x days ($F_{17,306}$ =16.585; p<0.0001);
- CCI-BoNT/B 5 pg/paw mice: treatment ($F_{1,18}$ =106.817; p<0.0001), days ($F_{17,306}$ =15.155; p<0.0001), treatment x days ($F_{17,306}$ =8.933; p<0.0001);
- CCI-BoNT/B 7.5 pg/paw mice: treatment ($F_{1,18}$ =121.787; p<0.0001), days ($F_{17,306}$ =10.893; p<0.0001), treatment x days ($F_{17,306}$ =12.198; p<0.0001).

Post*hoc* comparisons showed that, for all experimental groups, the withdrawal threshold of the ipsilateral hindpaw was always significantly different from the withdrawal threshold of contralateral hindpaw (Tukey-Kramer, p<0.05). Overall these data indicate a strong analgesic effect of the toxin that persists for at least 30 days. The analgesic effect of BoNT/B is similar for both doses, but its action in animals treated with the higher dose is long-lasting. In CCI-BoNT/B injected mice, mechanical allodynia in the ipsilateral hindpaw is reduced compared to

the CCI-saline treated animals, however, as for the control group, any BoNT/B group reaches the value of the uninjured contralateral paw (see figure 7).

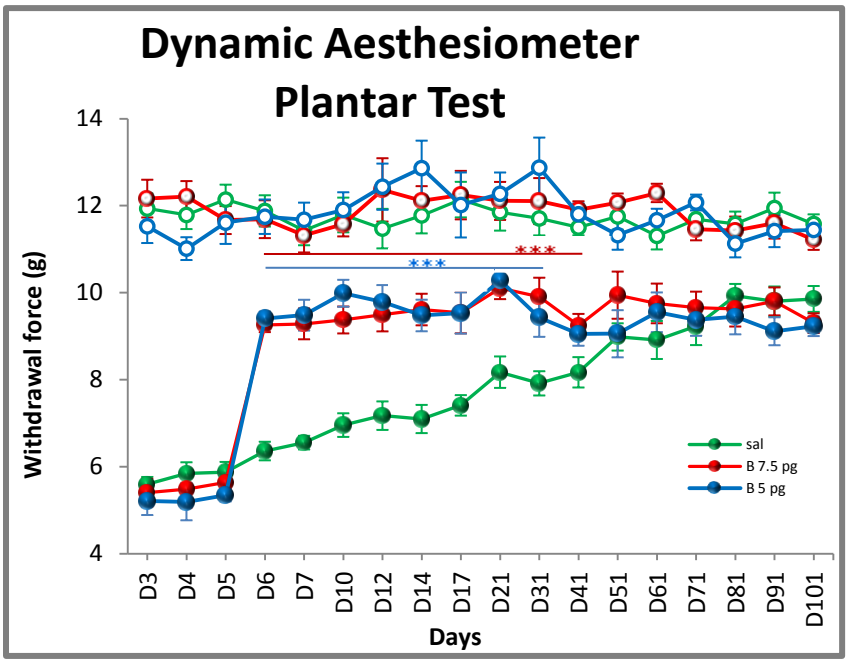


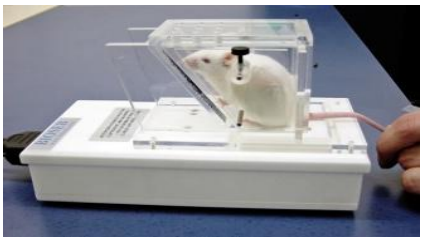
Figure7. Effects of a single injection into hind paw ipsilateral to the injury of saline (● ipsi, ○ contra), BoNT/B 5 pg/paw (.● ipsi, ○ contra) and BoNT/B 7.5 pg/paw (● ipsi, ○ contra) on mechanical allodynia. In CCI-BoNT/B injected groups the strong antiallodynic effect of the toxin is observed starting from the day after the injection and persists for a very long time (***$p<0.0001$).

Effects of BoNT/B on functional recovery

Weight bearing on the two hindlimbs

The weight bearing value on the two hindlimbs, expressed by the ratio: ipsi/(ipsi+contra), was determined by an incapacitance test. The value of 50% indicates an equal weight distribution between ipsilateral and contralateral hindlimbs. As shown in figure 8, just after CCI, in all experimental groups the weight distribution on the two hindlimbs was altered (weight bearing value <50). The graph indicates that in all animal groups there was a slow but not complete functional recovery over time and that the animals injected with both doses of the toxin showed no difference compared to control animals (Two-way ANOVA for repeated measures: treatment $F_{2,27}$ =1.013; p=0.3765; days $F_{17,459}$ =5.341; p<0.0001; treatment x days $F_{34,459}$ =1.226; p=0.1824).

These results indicate that BoNT/B, both at low and high doses, has no effect on functional recovery.



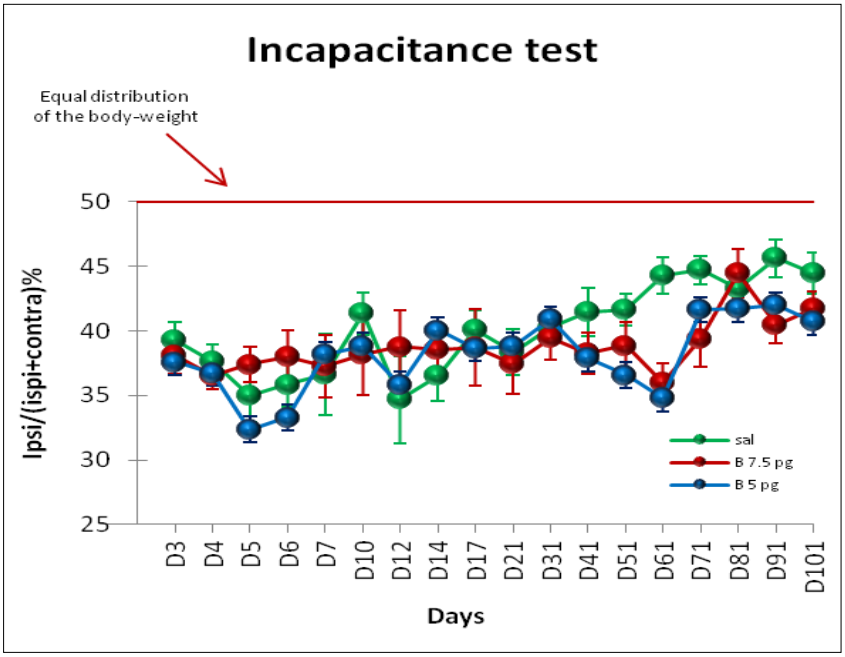


Figure 8. Effects of a single injection into hind paw ipsilateral to the injury of saline (●), BoNT/B 5 pg/paw (●) and BoNT/B 7.5 pg/paw (●) on weight bearing. CCI-animals injected with both doses of the toxin did not show difference compared to CCI-saline treated group. The graph indicates that in all animal groups there was a slow but not complete functional recovery over time.

Walking track analysis

A second **e**valuation of functional recovery was conducted using the walking track analysis by foot print test. Figure 9.1 shows a representative example of hind paws' walking pattern taken from a naive mouse. The gradual functional recovery of the animals was monitored measuring the sciatic static index (SSI) were a value of SSI close to 0 corresponds to normal function while a value of -100 is equivalent to complete functional loss. Figure 10 shows the trend of functional recovery of animals over time. All experimental groups manifested a severe impairment of motor function as revealed by SSI values, around 80, till day 32, then they showed a slow but not complete functional recovery. Moreover, animals with both doses of the toxin did not show improvement compared to CCI-saline injected animals, rather their performance was even reduced. Two-way ANOVA for repeated measures showed a significant main effect for days ($F_{9,243}$ =32.786; $p<0.0001$) and for treatment x days ($F_{9,243}$ =2.867; p=0.0001).

Post*hoc* comparisons between saline and 5 pg/paw BoNT/B treated mice showed a significant main effect from the day 62 to day 82 while the dose of 7.5 pg/paw of BoNT/B resulted significantly different from day 42 to day 82 (Tukey-Kramer, $p<0.05$), probably because the lower variability of the higher dose.

These results indicate that BoNT/B slowed the functional recovery of mice. This slowdown appeared to be dose-dependent, in fact, the animals treated with the higher dose of BoNT/B expressed more negative values respect to CCI-saline injected animals for a longer period. At day 92, when probably the negative effect of the toxin disappeared, animals treated with BoNT/B returned to take SSI values similar to those of CCI-

saline injected animals although at day 101 these still show clear signs of atrophy of the injured paw (Fig. 9.2).

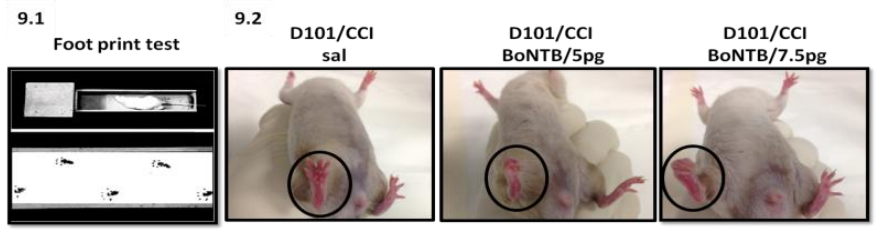


Figure 9.1. Rappresentative examples of foot print test. **9.2**. At day 101 post-CCI BoNT/B injected mice still show clear signs of atrophy of the injured paw compared to CCI-saline treated animals.

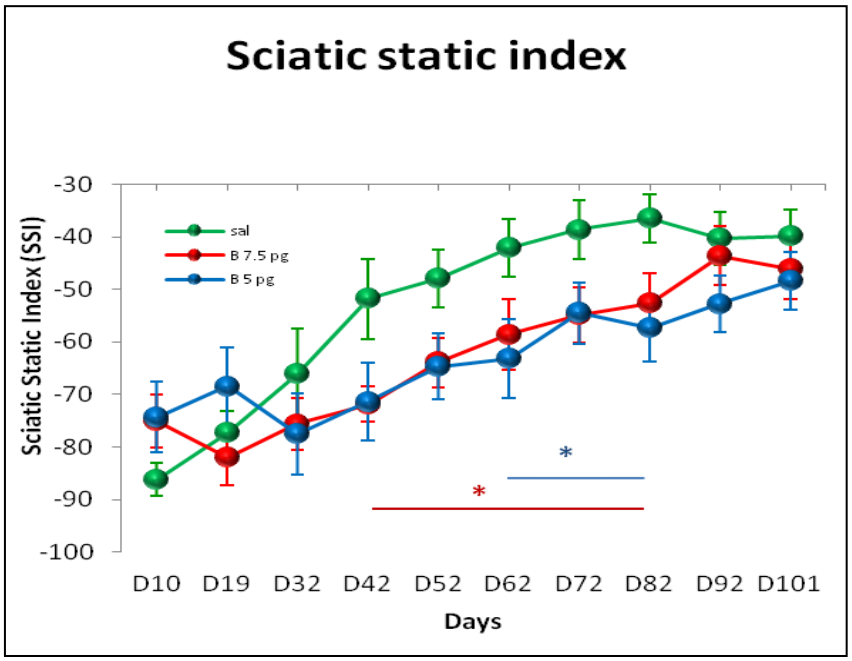


Figure 10. Effects of a single injection into hind paw ipsilateral to the injury of saline (●), BoNT/B 5 pg/paw (●) and BoNT/B 7.5 pg/paw (●) on SSI. BoNT/B slows functional recovery of mice. This slowdown appears to be dose-dependent, animals treated with the higher dose of BoNT/B expressed more negative values respect to the CCI-saline injected animals for a longer period (*****$p<0.05$).

Immunohistochemistry analysis

Effect of BoNT/A and BoNT/B on sciatic nerve after CCI

In the first part of our study we wanted to verify the presence of the target of BoNT/B on the nerve. Figure 11 shows immunofluorescence (IF) images of sciatic nerve section of naive, D7/CCI-saline and D7/CCI-BoNT/B 7.5 pg/paw injected mice after incubation with rabbit polyclonal antibodies to VAMP2. As already demonstrated in rat (Jacobsson et al., 1998), in naive animals VAMP2 was not detectable whereas VAMP2 was present in CCI-mice. It has been suggested that the increased expression of VAMP2 in injured nerve stimulates the autocrine secretion of neurotrophins by motor neurons and increases the release of neuropeptides involved in regenerative processes (Jacobsson et al., 1998).

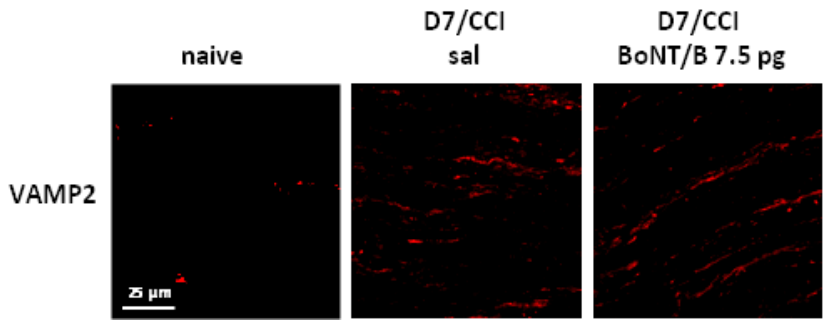


Figure 11. In naive animals no detectable VAMP2 has been seen whereas VAMP2 is present in CCI-mice. Scale bar: 25 µm.

The injury caused by CCI determines structural changes in damaged nerve, induces the recall and activation of immune cells and promotes the synthesis of proteins necessary to support the subsequent axonal regeneration. Changes in the nerve at the site of injury begin almost

immediately. After axonal injury, the distal portion of the nerve undergoes progressive degeneration due to a series of events that induce the degradation of cytoskeletal proteins. The distal segment degeneration process is known as Wallerian Degeneration (WD) (Röyttä et al., 1987). To be able to observe the cytoskeleton of the nerve fibers we incubated sections of naive, D7/CCI-saline, D7/CCI-BoNT/A 15 pg/paw and D7/CCI-BoNT/B 7.5 pg/paw treated mice with rabbit polyclonal antibodies for Neurofilament-200 (NF200), marker of neurons intermediate filaments (figure 12).

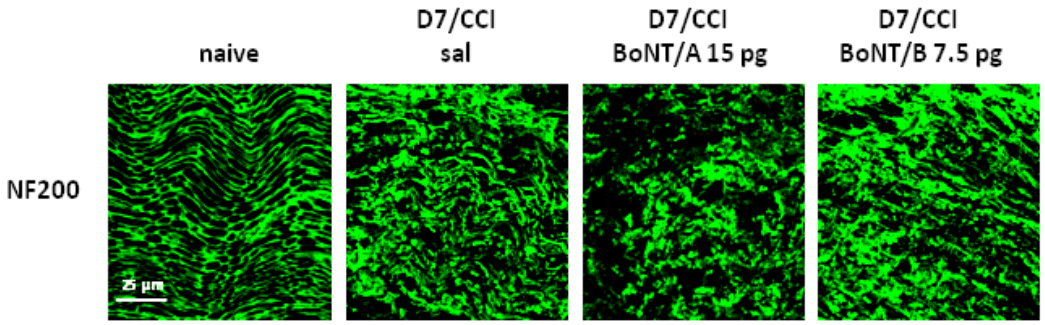


Figure 12. In naive animals the structure of the tissue is more uniform and compact than in CCI-mice. Scale bar: 25 µm.

As shown by figure 12, naive animals present a more uniform and compact structure of the tissue while in CCI-mice, 7 days after injury, the tissue appears disjointed and broken up. As the axon disappears, Schwann Cells (SC), having lost axonal contact, are able to isolate small whorls of myelin and generate characteristic ovoids containing fragmented myelin. SCs play a key role in the breakdown of the myelin sheath and in the clearance of its debris. The loss of axon–SC contact is a signal causing the SCs proliferation, an important event that promotes the

axon regeneration. During proliferation, myelinating and nonmyelinating SCs change their phenotype from differentiated to dedifferentiated cells and this phenotypic switch is associated with down-regulation of the myelin-associated genes (e.g., P0, PMP22) and up-regulation of the regeneration-associated genes (e.g., neurotrophins and adhesion molecules) (Dubový, 2011). Figure 13 and 15 show IF double-staining in sciatic nerve section of naive, D7/CCI-saline, D7/CCI-BoNT/A and D7/CCI-BoNT/B injected mice. In figure 13.A, the double-staining is for GFAP (green), marker of dedifferentiated nonmyelinating SCs, and P0 (red), marker of a major peripheral myelin protein (P0 represents roughly half of the total of the peripheral myelin). Low levels of green fluorescence, emitted from GFAP, were detected in naive mice. After nerve lesion, GFAP expression is upregulated and this upregulation is visible one day after injury, increases at 3-5 days and decreases slightly at 7 days (Berg et al., 2013). According, at day 7 post-surgery, our results showed an increased expression of GFAP in CCI-saline treated mice compared to naive animals. One-way ANOVA showed a significant main effect for treatment ($F_{3,68}$ =16.591; $p<0.0001$). Post*hoc* comparisons showed a significant effect between CCI-saline and naive, CCI-saline and CCI-BoNT/A, CCI-BoNT/A and naive, CCI-BoNT/A and CCI-BoNT/B treated animals (Fisher's PLSD analysis $p<0.05$). Instead, animals treated with BoNT/B did not show differences in comparison either with naive or CCI-saline injected animals (figure 13.B).

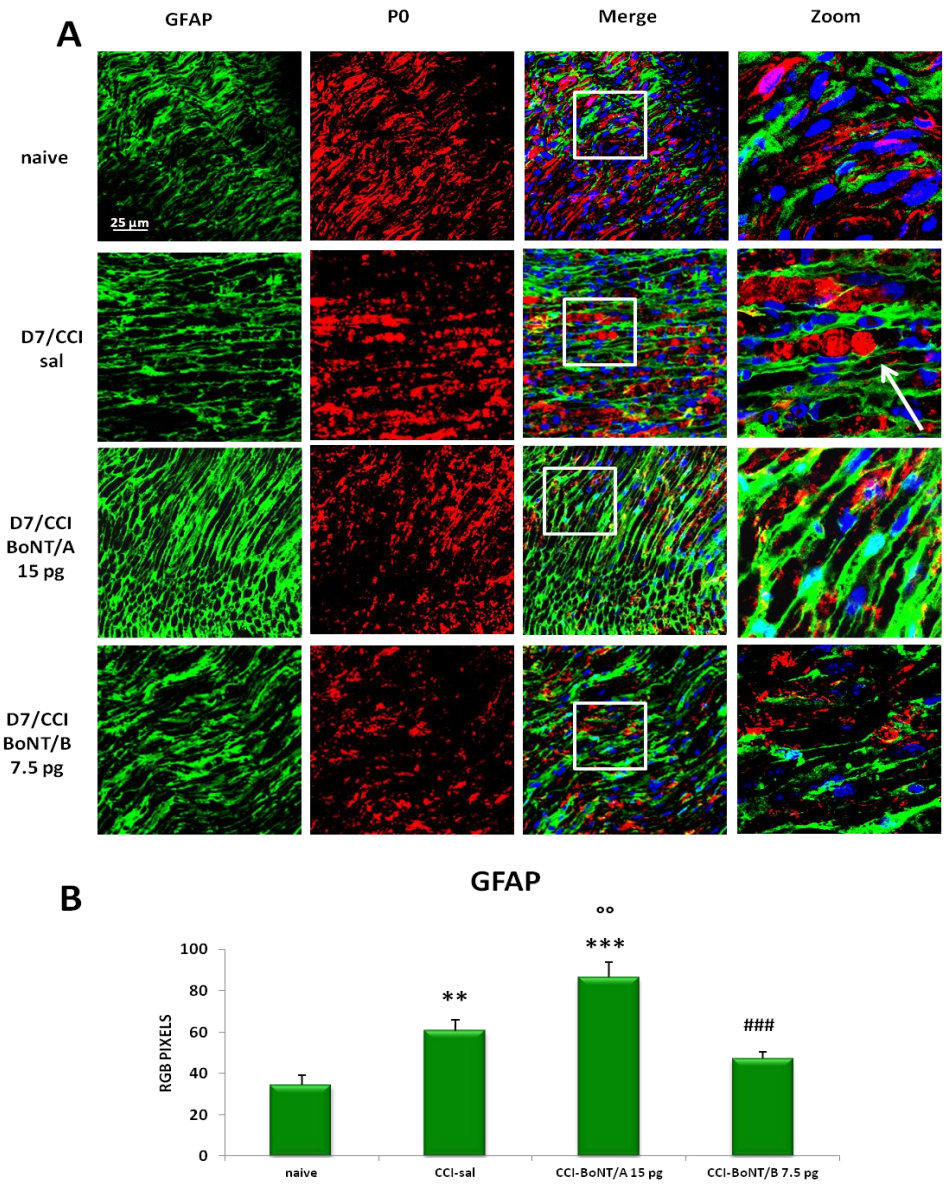


Figure 13.A. Representative examples of IF images showing the expression of GFAP (green) and P0 (red) in sciatic nerve sections taken from naive, CCI-saline, CCI-BoNT/A and CCI-BoNT/B treated mice. **13.B.** Strong increase expression of GFAP is observed in CCI-BoNT/A injected mice. (**p<0.001;***p<0.0001 vs naive;°°p<0.001 vs saline; ###p<0.0001 vs BoNT/A).Scale bar: 25 µm.

Figure 13.A shows, *via* P0 marker (red), the myelin morphology before and after CCI. As expected, in CCI-saline injected mice P0 was found aggregated and accumulated in characteristic ovoids (see white arrow in figure 13.A). A different result was observed in CCI-BoNT/A and CCI-BoNT/B treated mice where the presence of myelin aggregates was lower and the myelin seemed similarly distributed as for naive animals. Images analyzed by means of RGB technique (figure 14) revealed a higher expression of P0 in CCI-saline treated group. One-way ANOVA indicated a strong significant main effect for treatment ($F_{3,32}$ =3.577; p=0.0245) and Post*hoc* comparisons showed a significant effect of CCI-BoNT/A and CCI-BoNT/B vs CCI-saline injected mice (Fisher's PLSD analysis $p<0.05$).

It is important to emphasize that the RGB method for measuring the fluorescence is a semi-quantitative technique. As previously described in other studies (e.g., Marinelli et al., 2014), myelin aggregates in injured nerve, in comparison with uninjured nerves, lead to increased emission of fluorescence that is not derived from an increase of the myelin protein but dependent on a greater brightness issued by the ovoids containing fragmented myelin. In animals treated with the toxins, the absence of ovoids could indicate anticipation on myelin phagocytosis process in comparison with CCI-saline injected mice.

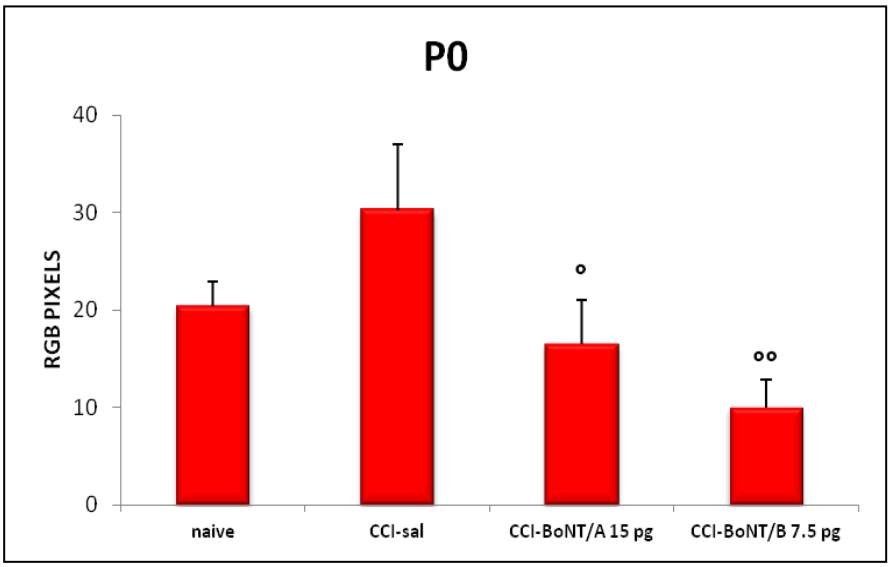


Figure 14. RGB technique reveals a higher expression of P0 in CCI-saline injected mice compared to CCI-BoNT/A and CCI-BoNT/B treated mice. (°$p<0.05$;°°$p<0.001$ vs saline).

Figure 15.A shows IF double-stainings for GFAP (green) and PMP22 (red). PMP22 represents 2–5% of the total amount of the peripheral myelin proteins expressed in SCs (Quarles, 2002). As for P0 protein, images were analyzed by means of RGB technique (figure 15.B).

RGB analysis revealed a higher expression of PMP22 in CCI-saline injected group compared to naive. One-way ANOVA indicated a strong significant main effect for treatment ($F_{3,32}$ =12.458; $p<0.0001$) and Post*hoc* comparisons showed a significant main effect between CCI-saline and naive, between CCI-saline and both CCI-BoNT/A and CCI-BoNT/B and between naive and CCI-BoNT/B injected mice. Animals treated with BoNT/A instead did not show difference in comparison with naive animals (Fisher's PLSD analysis $p<0.05$).

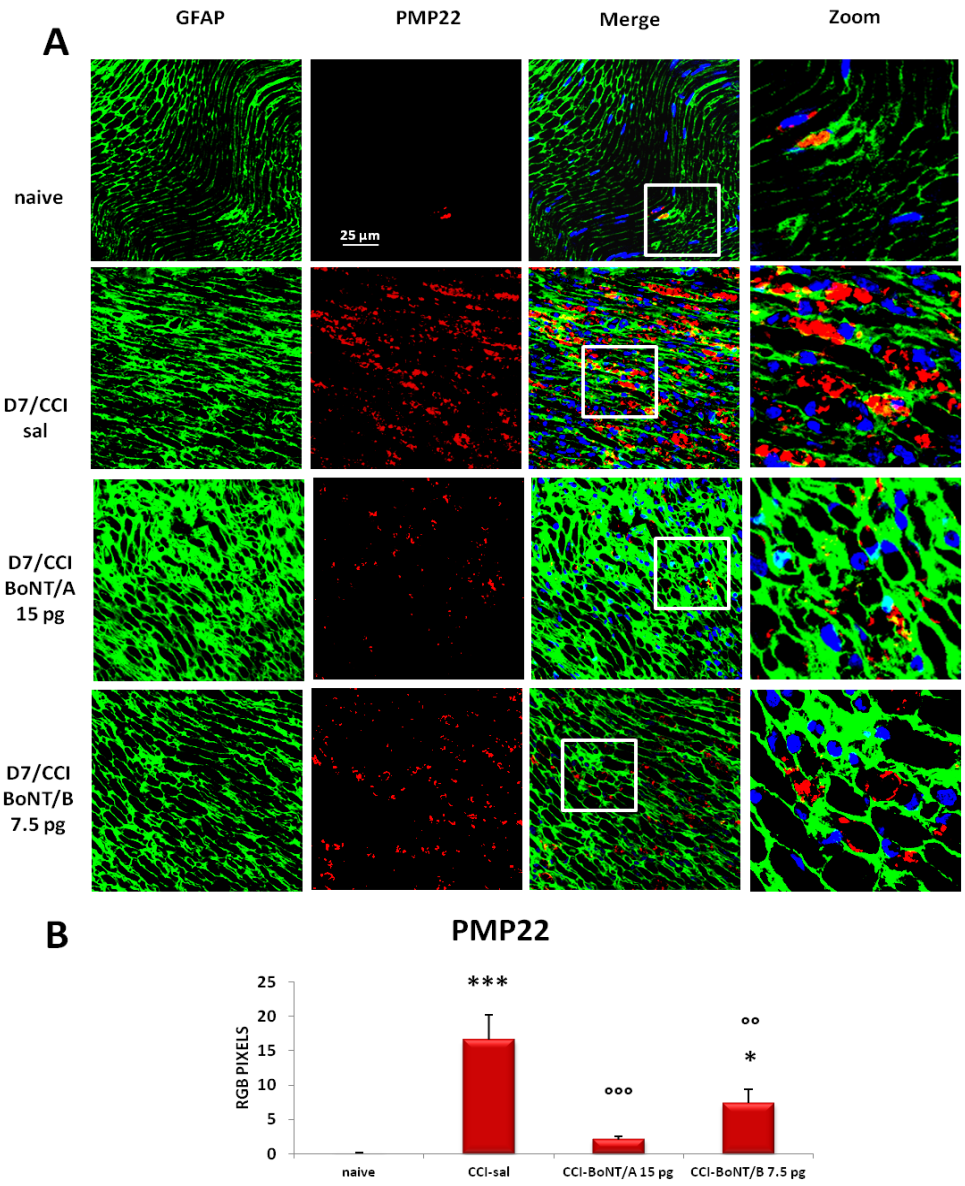


Figure 15.A. Representative examples of IF images showing the expression of GFAP (green) and PMP22 (red) in sciatic nerve sections taken from naive, CCI-saline, CCI-BoNT/A and CCI-BoNT/B treated mice. **15.B.** RGB technique reveals a higher expression of PMP22 in CCI-saline injected mice. (*p<0.05,***P<0.0001 vs naive; °°p<0.001,°°°p<0.0001 vs saline). Scale bar:25 µm.

Also in this case, the reduction of the PMP22 protein in CCI-animals treated with the toxins could indicate anticipation on myelin phagocytosis process in comparison with CCI-saline treated mice. To be able to observe the dedifferentiated myelinating SCs, the second type of proliferating SCs, we incubated the sections with mouse anti-S100β antibodies (figure 16.A). One-way ANOVA for S100β expression indicated a strong significant main effect for treatment ($F_{3,32}$ =3.497; p=0.0266). Post*hoc* comparisons showed a significant main effect between CCI-saline and both naive and CCI-BoNT/A, CCI-BoNT/A and CCI-BoNT/B and between CCI-BoNT/B injected mice and naive animals (Fisher's PLSD analysis $p<0.05$). These results indicated that, in injured nerve, the BoNT/A reduces the level of S100β to those observed in naive mice while BoNT/B does not alter the expression of the protein in CCI-animals in fact, the level of S100β in CCI-BoNT/B treated mice is similar to CCI-saline injected animals (figure 16.B).

Figure 16.A shows the expression of the protein Cdc2 (red), a marker of proliferating cells. In intact nerve the fluorescence emitted from Cdc2 is practically absent. Conversely, in CCI-mice the regenerative process associated to nerve lesion is accompanied by an increased level of Cdc2 expression (figure 16.B). One-way ANOVA for Cdc2 indicated a strong significant main effect for treatment ($F_{3,32}$ =36.339; $p<0.0001$) and Post*hoc* comparisons showed a significant effect between CCI-saline and both naive and CCI-BoNT/A, CCI-BoNT/A and both naive and CCI-BoNT/B and between CCI-BoNT/B injected mice and naive animals (Fisher's PLSD analysis $p<0,05$).

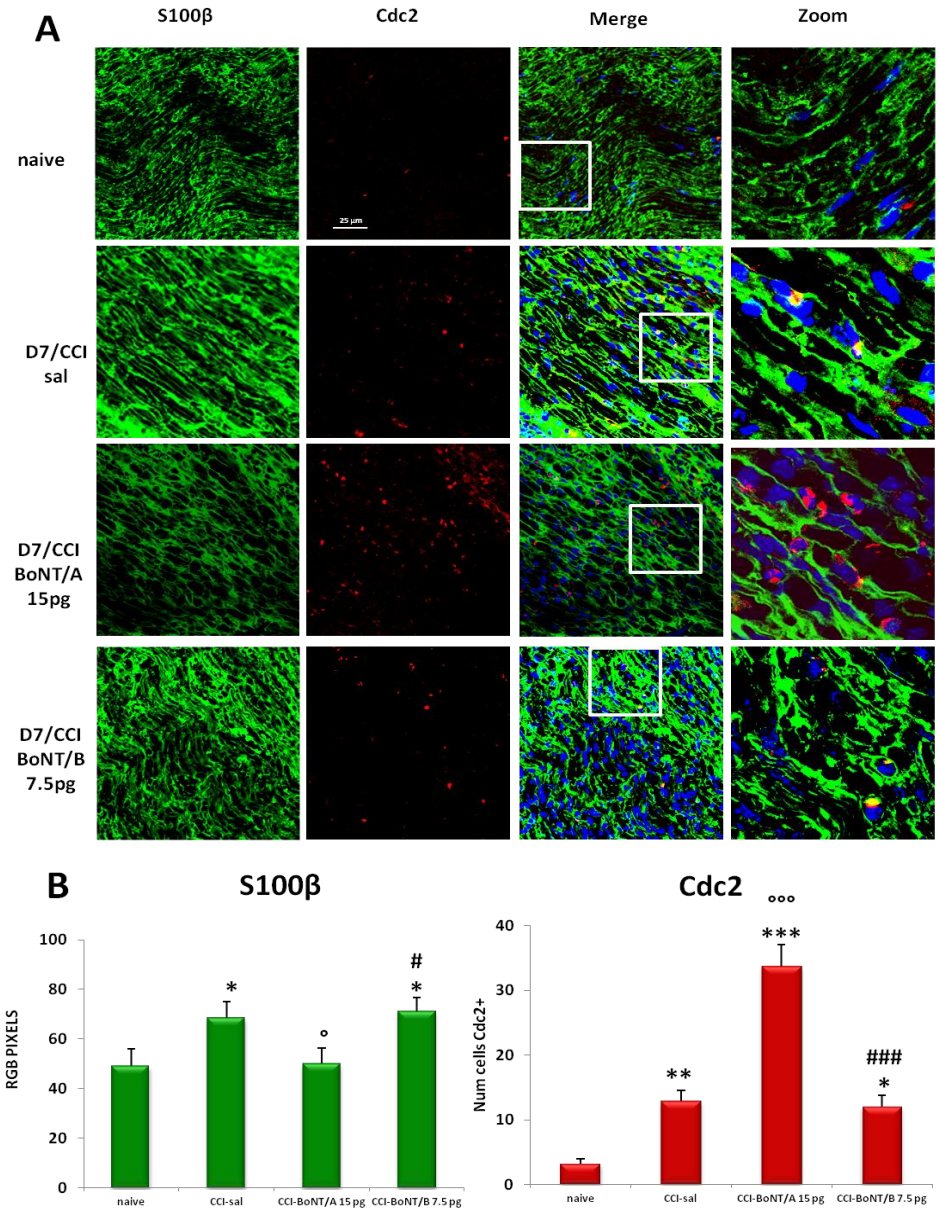


Figure 16.A. Representative example of immunofluorescent (IF) analysis of the dedifferentiated myelinating SCs (S100β, green) and cyclin-dependent kinase 2 (Cdc2, red) in naive and D7/CCI-mice treated with different drugs. **16.B.** RGB evaluation of S100β (green) and Cdc2 (red) expression in naive and D7/CCI-mice. (*p<0.05,**p<0.001,***p<0.0001 vs naive; °p<0.05,°°°P<0.0001 vs saline; #p<0.05,###p<0.0001 vs BoNT/A). Scale bar: 25 µm.

It is interesting to note that, while in CCI-BoNT/A injected mice the Cdc2 level increases about 10 times, both in CCI-saline and CCI-BoNT/B treated animals the level of Cdc2 is only 4 times greater than in the naive mice.

Furthermore, comparing the levels of GFAP and S100β of all experimental groups we noticed that the expression of S100β is higher in D7/CCI-saline and D7/CCI-BoNT/B treated mice and lower in naive and D7/CCI-BoNT/A while, the expression of GFAP is higher in D7/CCI-BoNT/A treated animals and lower in the other experimental groups (figure 17).

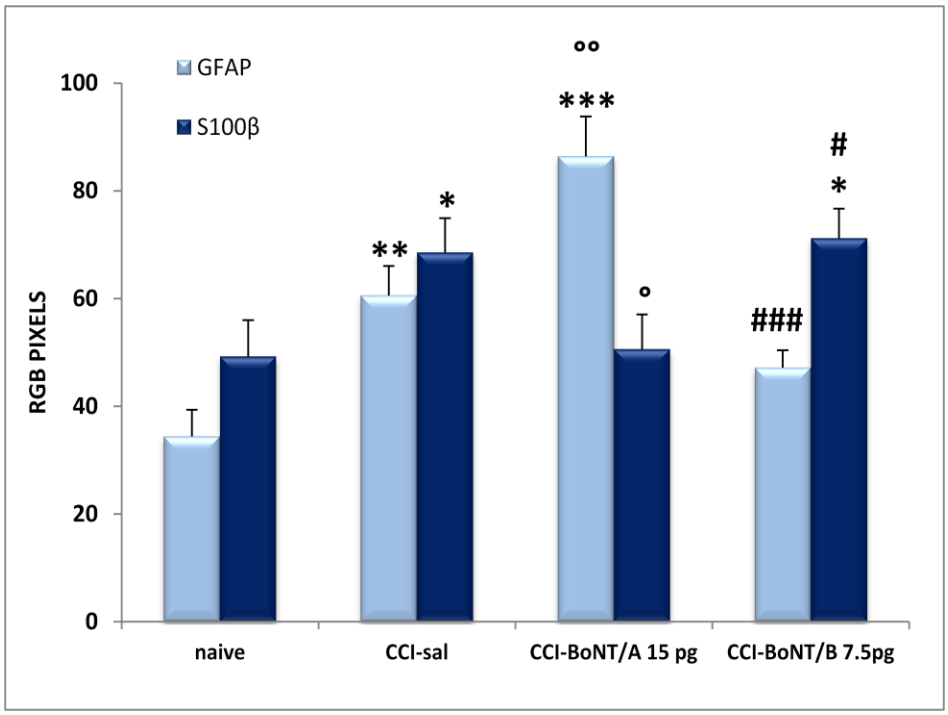


Figure 17. D7/CCI-BoNT/A treated animals show lower expression of S100β and higher expression of GFAP compared to the other experimental groups. (*p<0.05,**p<0.001,***p<0.0001 vs naive; °p<0.05,°°P<0.001 vs saline; #p<0.05,###p<0.0001 vs BoNT/A).

Moreover, the expression of S100β in D7/CCI-BoNT/B animals is similar to that observed in D7/CCI-saline, while in D7/CCI-BoNT/A is similar to that of naive.

After peripheral nerve damage, mast cells are the first immune cells to be activated. Activated mast cells degranulate and release different proinflammatory mediators including histamine, leukotrienes and chemokines which help on recruitment of hematogenous macrophages at the site of injured nerve (Thacker et al., 2007). In order to observe the expression of mast cells in injured nerve we incubated sciatic nerve sections with mouse monoclonal antibodies anti-CC1 (Mast Cells Chymase 1). Figure 18.A shows a strong increase of mast cells in D7/CCI mice compared to naive animals. This increase is particularly evident in CCI-BoNT/A injected mice (figure 18.B). One-way ANOVA for CC1 indicated a strong significant main effect for treatment ($F_{3,32}$ =63.463; $p<0.0001$) and Post*hoc* comparisons showed a significant effect between CCI-saline and naive, CCI-BoNT/A and CCI/BoNT/B, CCI-BoNT/A and both naive and CCI-BoNT/B and between CCI-BoNT/B treated mice and naive animals (Fisher's PLSD analysis $p<0.05$).

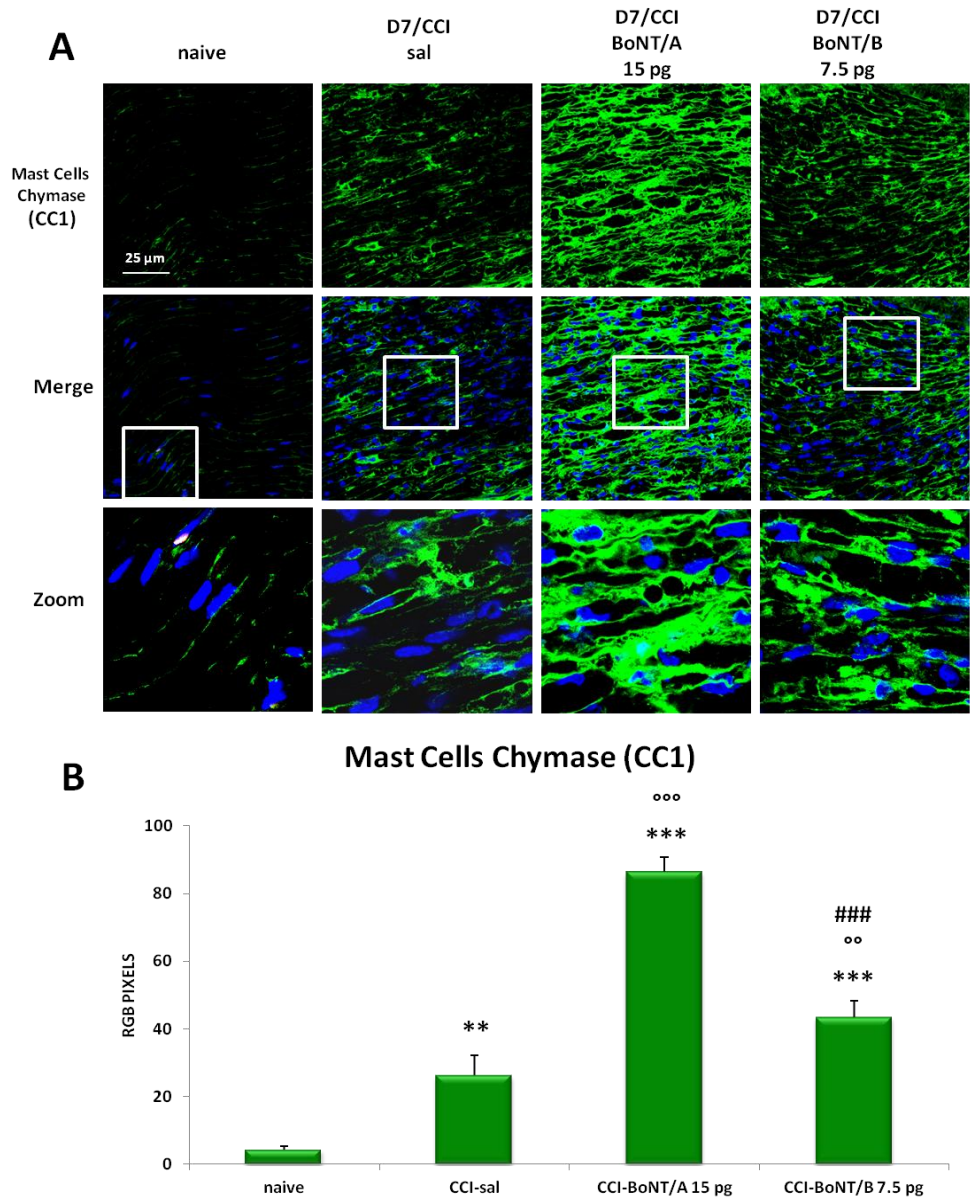


Figure 18.A. Representative examples of IF images showing the expression of CC1 (green) in sciatic nerve sections in naive and D7/CCI-mice treated with different drugs. **18.B.** Strong increase expression of CC1 is observed in D7/CCI-mice compared to naive animals. This increase is particularly evident in CCI-BoNT/A treated mice. (**p<0.001;***p<0.0001 vs naive; °°p<0.001,°°°p<0.0001 vs saline; ###p<0.0001 vs B0NT/A). Scale bar: 25 µm.

From 2 to 3 days after injury there is an important infiltration of haematogenous macrophages into degenerating nerve. Macrophages recruitment leads to a rapid elimination of myelin debris and making it easier nerve regeneration. Macrophages not only remove axon and myelin debris, but also participate in the production of mitogenic factors for SCs (Dubový, 2011; Perry & Brown, 1992). To observe the macrophages we incubated the sections with rat anti-CD11b antibodies (figure 19.A). One-way ANOVA for CD11b expression indicated a strong significant main effect for treatment ($F_{3,32}$ =14.542; $p<0.0001$). However, despite RGB analysis revealed an increase of expression of CD11b in CCI-saline treated group compared to naive animals, Post*hoc* comparisons showed a significant main effect only between CCI-BoNT/A and naive, CCI-saline and CCI-BoNT/B injected animals (Fisher's PLSD analysis $p<0.05$) (figure 19.B). Since macrophages release mitogenic factors for SCs (Dubový, 2011; Perry & Brown, 1992), the increase of the CD11b expression in D7/CCI-BoNT/A injected mice could be related to the increase of Cdc2+ and GFAP+ cells and to the anticipation of phagocytosis that we observed in this experimental group.

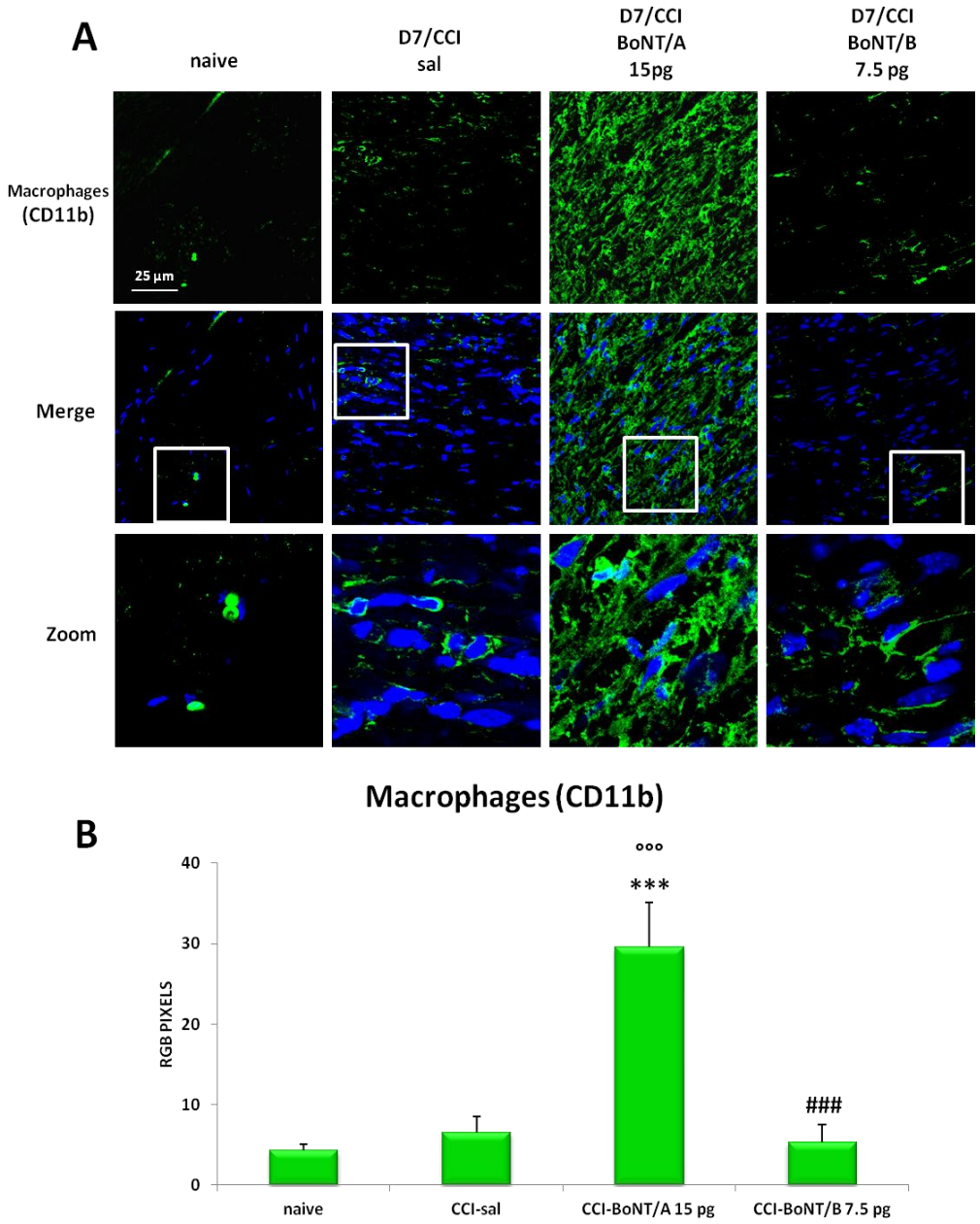


Figure 19.A. Representative examples of IF images showing the expression of CD11b (green) in sciatic nerve sections in naive and D7/CCI-mice treated with different drugs. **19.B.** Strong increase expression of CD11b is observed in D7/CCI-BoNT/A injected mice. (***p<0.0001 vs naive; °°°p<0.0001 vs saline; ###p<0.0001 vs BoNT/A). Scale bar: 25 µm.

Table 3 summarizes the IF results on sciatic nerve of D7/CCI-BoNTs treated animals compared to D7/CCI-saline injected mice.

Sciatic Nerve	CCI-BoNT/A 15pg	CCI-BoNT/B 7.5pg
Dedifferentiated nonmyelinating SCs (GFAP)	↑↑	=
Proliferating SCs (Cdc2)	↑↑↑	=
Mast Cells Chymase (CC1)	↑↑↑	↑↑
Macrophages (CD11b)	↑↑↑	=
Dedifferentiated myelinating SCs (S100β)	↓	=
Perypheral myelin protein (P0)	↓	↓↓
Perypheral myelin protein (PMP22)	↓↓↓	↓↓

Table 3. Summary of IF results of the botulinum neurotoxins types A and B effects on sciatic nerves of D7/CCI-BoNTs treated animals compared to D7/CCI-saline injected mice.

Immunohistochemistry analysis has shown an increase of proliferanting SCs-GFAP+, mast cells and macrophages and a decrease of proliferanting SCs-S100β+ and myelin proteins in CCI-BoNT/A treated mice compared to CCI-saline injected group. On the contrary, CCI-BoNT/B treated group showed only some effects produced by BoNT/A (a decrease of myelin proteins and an increases of mast cells) without modifying the other analyzed markers.

Effect of BoNT/B on spinal cord after CCI

After nerve injury, in the dorsal horn of the spinal cord, where primary afferents of damaged fibers project, dense cluster of microglial activated cells are detected. Massive microglial activation is also observed in the ventral horn, around the cell bodies of motor neurons (Scholz & Woolf, 2007; Colburn et al., 1997). The peak of microglial activation in both dorsal and ventral horns occurs one week after injury and is followed by a slow decline over several weeks. This cell activation, characterized by phosphorylation of p38 MAP kinase (p-p38), induces the production and secretion of proinflammatory cytokines that contribute to the onset and maintenance of pain hypersensitivity (Beggs & Salter, 2007). The block of microglial response with minocycline can prevent nerve injury-induced hypersensitivity in rat (Calvo & Bennett, 2012). To be able to observe microglial cells, members of the monocyte/macrophage family, we incubated transverse sections of L4/L5 spinal cord segment of D7/CCI-saline and D7/CCI-BoNT/B 7.5 pg/paw treated mice with rat monoclonal antibodies anti-CD11b and with rabbit polyclonal antibodies anti-p-p38 to observe the activation of the same cells. Figure 20.A shows immunoreactivity (IR) images of ipsilateral-side of dorsal horn of

D7/CCI-saline and D7/CCI-BoNT/B injected mice. For analysis of labeling of CD11b positive IR-cells, and their colocalization with p-p38, the number of positive IR cells was quantified (figure 20.B). One-way ANOVA for CD11b and CD11b/p-p38 positive cells expression for treatment indicated that the animals injected with the toxin showed no difference compared to control animals (CD11b+: $F_{1,10}$ =0.804; p=0.3911; CD11b+/p-p38+: $F_{1,10}$ =3.586; p=0.0875). Similar results have been obtained from the analysis of ipsilateral-side of ventral horn (figure 21.A and figure 21.B). One-way ANOVA for treatment for CD11b and CD11b/p-p38 positive cells expression in ventral horn indicates no difference between experimental groups (CD11b+: $F_{1,10}$ =0.028; p=0.8716; CD11b+/p-p38+: $F_{1,10}$ =0.000).

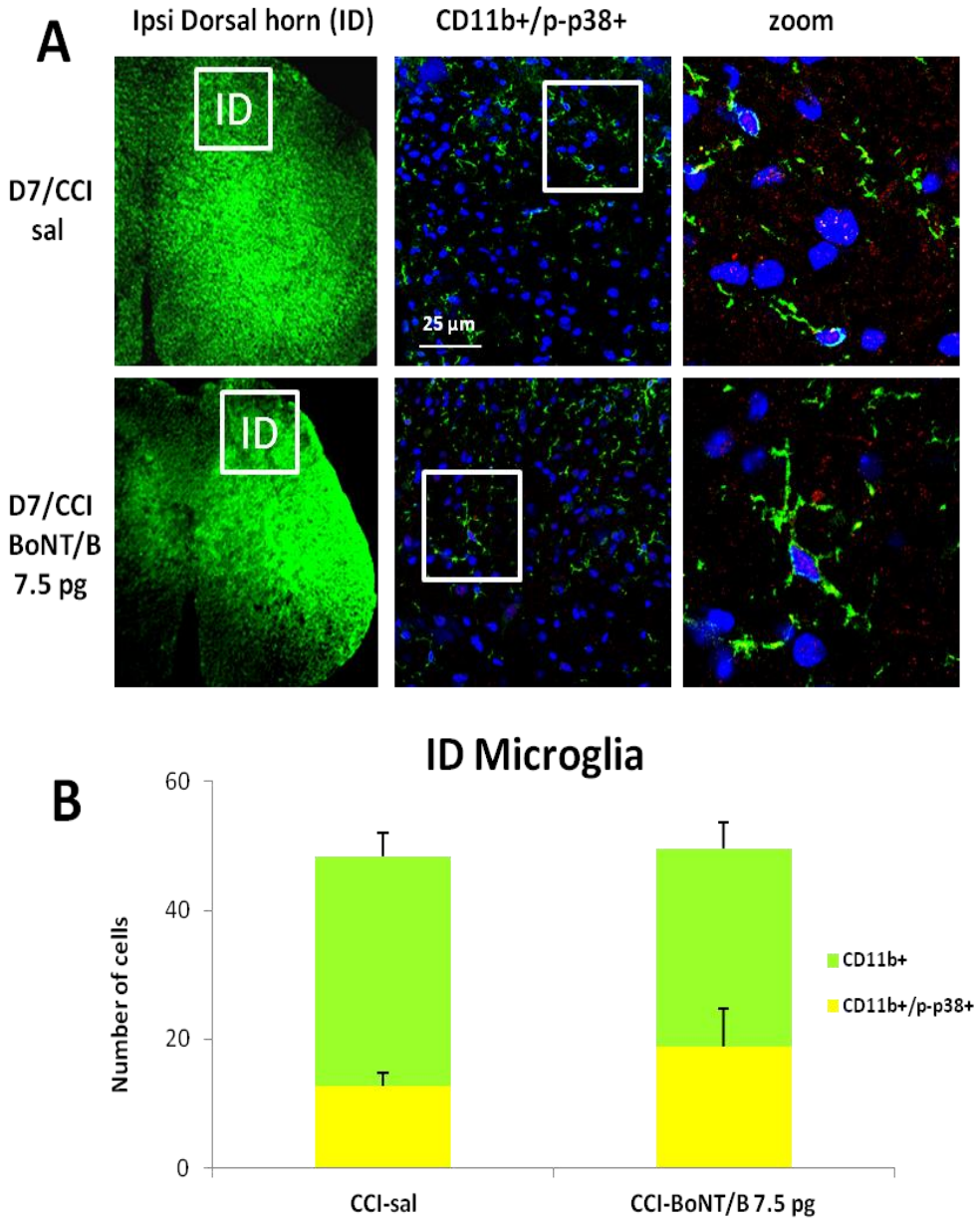


Figure 20.A. Representative examples of IR images showing the expression of CD11b+ (green) and CD11b+/p-p38+ (yellow) cells in dorsal horn of spinal cord sections in D7/CCI-saline and D7/CCI-BoNT/B 7.5 pg injected mice. **20.B.** Analysis of CD11b and CD11b/p-p38 positive cells expression in dorsal horn indicates no difference between experimental groups. Scale bar: 25 µm.

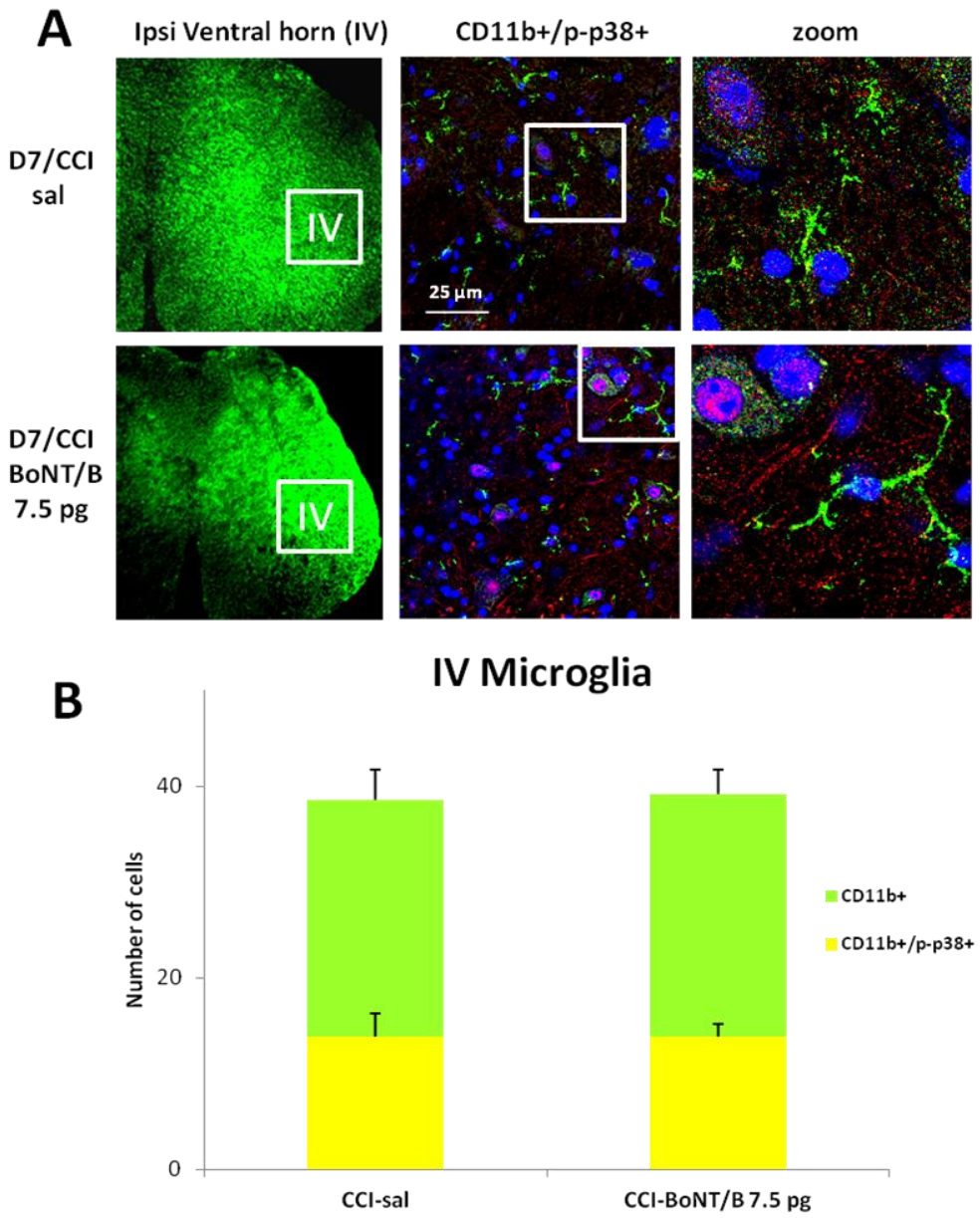


Figure 21.A. Representative examples of IR images showing the expression of CD11b+ (green) and CD11b+/p-p38+ (yellow) cells in ventral horn of spinal cord sections in D7/CCI-saline and D7/CCI-BoNT/B 7.5 pg injected mice. **21.B.** Analysis of CD11b and CD11b/p-p38 positive cells expression in ventral horn indicates no difference between experimental groups. Scale bar: 25 µm.

In case of damage to the PNS, the density of spinal cord microglia increases through the migration of these cells from other sites and through local proliferation. In response to traumatic nerve injury microglia exhibit variable alterations in functions and morphologies (from a resting state, where the cell body is small and there are long and thin processes, into an effector state in which the cells present an amoeboid form) (Calvo & Bennett, 2012). These changes are associated with different activation states of microglia (Ji et al., 2013). From images obtained with IR technique we also observed that, differently from CCI-saline injected mice, in CCI-BoNT/B treated animals the microglial morphology did not show the typical amoeboid form of the strong activated state. Microglial recruitment and activation in dorsal and ventral horn is accompanied by proliferation and activation of astrocytes in the ipsilateral-side of spinal cord. Compared with the microglial response, astrocyte proliferation begins relatively late, progresses slowly and is sustained for a longer period (Scholz & Woolf, 2007). Together with microglia, astrocytes play a critical role in chronic pain sensitization and their activation is correlated with chronic pain behaviors (Wang et al., 2009; Liu et al., 2012). In spinal cord astrocytes, damage to peripheral nerves induces hypertrophy, hyperplasia, proliferation, increased of glial fibrillary acidic protein (GFAP) and, in severe cases, scar formation (Tsuda et al., 2011). Previous studies have shown that suppression of astrocyte proliferation, and activation, produces a recovery from tactile allodynia (Tsuda et al., 2011).

Figures 22 and 23 show IR images for astrocytes (GFAP, green) and their colocalization with p-p38 (yellow) on ipsilateral-side of dorsal and ventral horn (figure 22.A and 23.A. respectively) of D7/CCI-saline and D7/CCI-BoNT/B treated mice.

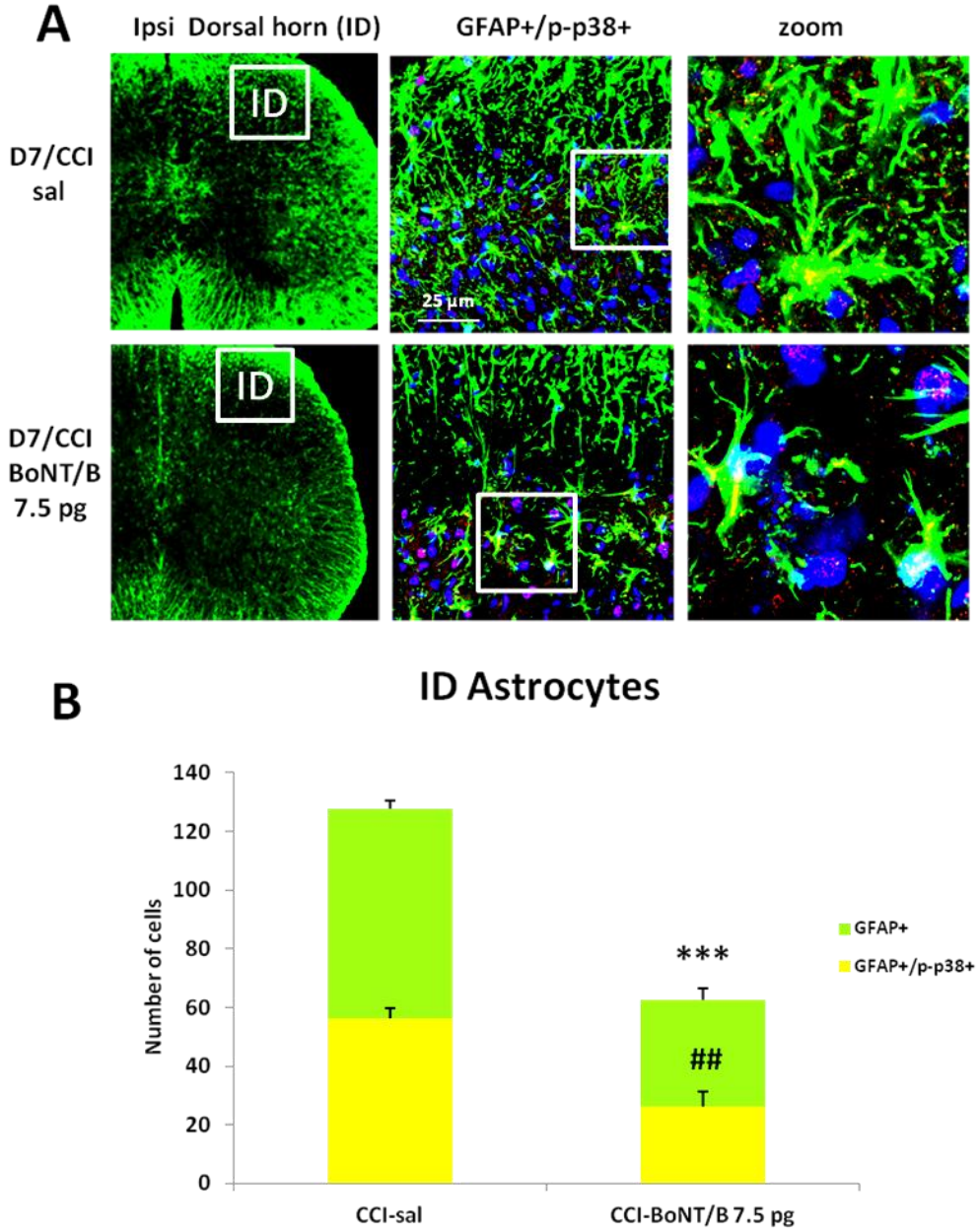


Figure 22.A. Representative examples of IR images showing the expression of GFAP+ (green) and GFAP+/p-p38+ (yellow) cells in dorsal horn of spinal cord sections in D7/CCI-saline and D7/CCI-BoNT/B 7.5 pg injected mice. **22.B.** Analysis of GFAP and GFAP+/p-p38 positive cells expression indicates a strong significant main effect for treatment (***$p<0.0001$, for GFAP+;##$p<0.001$, for GFAP+/p-p38+). Scale bar: 25µm.

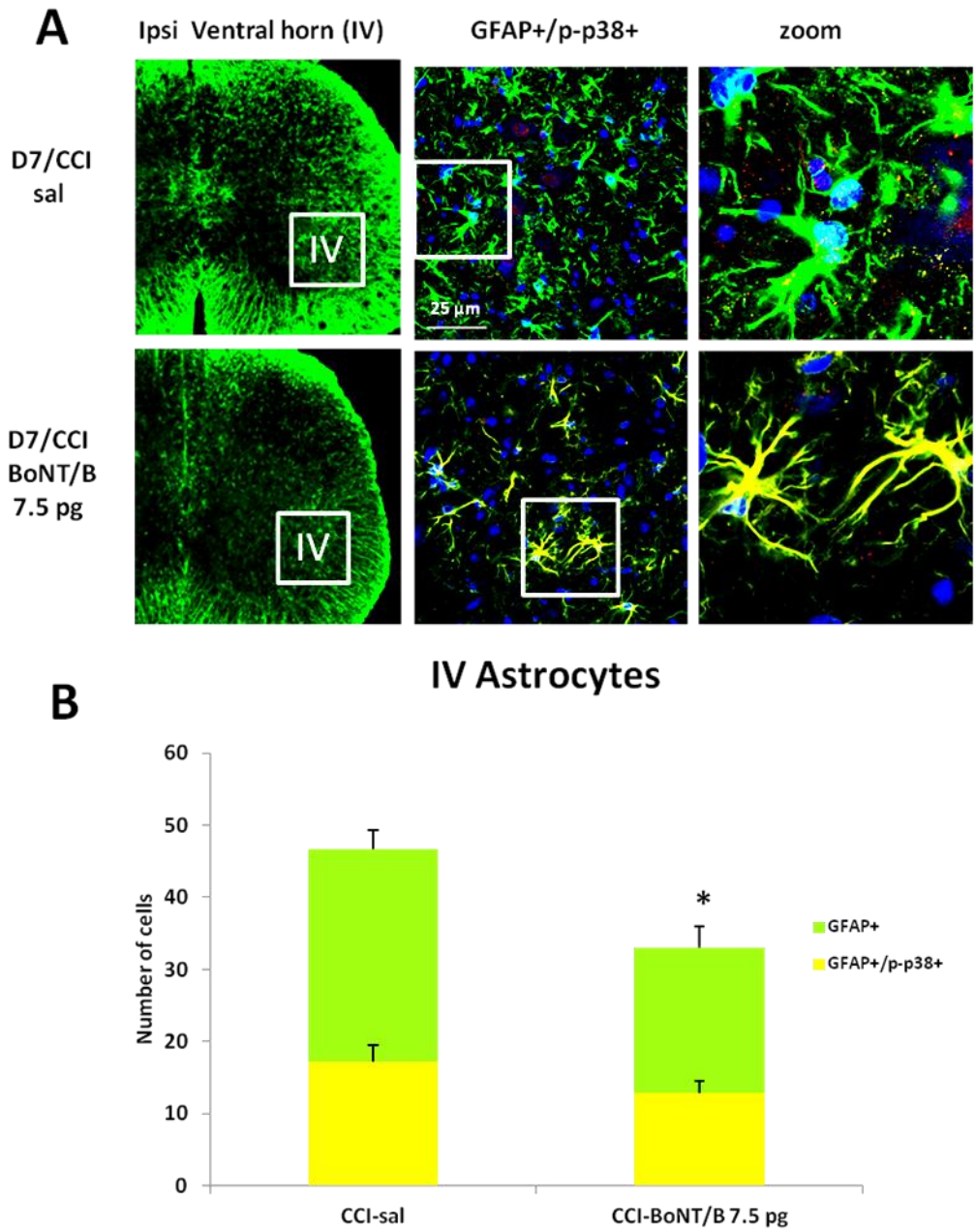


Figure 23.A. Representative examples of IR images showing the expression of GFAP+ (green) and GFAP+/p-p38+ (yellow) cells in ventral horn of spinal cord sections in D7/CCI-saline and D7/CCI-BoNT/B 7.5 pg treated mice. **23.B.** Analysis of GFAP and GFAP/p-p38 positive cells expression indicates a strong significant main effect for treatment only for GFAP (*$p<0.05$). Scale bar: 25 µm.

One-way ANOVA for GFAP and GFAP/p-p38 positive cells expression on ipsilateral-dorsal horn (figure 22.B) indicated a strong significant main effect for treatment for both markers (GFAP+: $F_{1,10}$ =59.387, $p<0.001$; GFAP+/p-p38+: $F_{1,10}$ =22.510, p=0.0008; Fisher's PLSD analysis $p<0.05$). Unlike dorsal horn, in ipsilateral-side of ventral horn (figure 23.B) One-way ANOVA showed only a strong significant GFAP positive cells expression (GFAP+: $F_{1,10}$ =5.604, p=0.0395; Fisher's PLSD analysis $p<0.05$; GFAP+/p-p38+: $F_{1,10}$ =2.169, p=0.1715).

As already mentioned, microglia and astrocyte proliferation and activation are correlated with chronic pain behaviors. The resting state of the microglia and the reduced number of activated astrocytes, observed in dorsal horn of ipsilateral-side of spinal cord, may explain the analgesic effect of BoNT/B in mice noted during the behavioral tests. In ventral horn of both experimental groups we have not observed differences in the number of activated cells, however, in CCI-BoNT/B injected mice, IR images have highlighted a mild/moderate reactive astrogliosis. Reactive astrogliosis is a spectrum of changes in astrocytes that occur in response to all forms of injury and disease in CNS. In its mild and moderate forms, reactive astrogliosis can be resolved and cells can reacquire a morphology similar to that in healthy tissue (Sofroniew, 2010). The presence of mild/moderate reactive astrogliosis in the ventral horn of CCI-BoNT/B treated animals could be related to the reduced performance observed during behavioral testing in this experimental group.

Discussion

The results reported in this thesis indicate that a single intraplantar administration of BoNT/B (5 or 7.5 pg/paw) induces antiallodynic effect in mice subjected to Chronic Constriction Injury (CCI) of sciatic nerve. This effect was long-lasting and almost immediate, starting from the day after the injection, and lasted for at least 30 days.

However, despite the clear evidence of a strong analgesic effect of the neurotoxin, behavioral tests on functional recovery showed that BoNT/B interferes, if not even compromises, the injured limb functional rehabilitation. Results of immunofluorescence (IF) experiments associated to functional data revealed that BoNT/B, acting directly or indirectly on neuronal and non-neuronal cells of both peripheral and central nervous system: i) decreases the expression of peripheral myelin proteins (P0 and PMP22) suggesting an increase of the degradation and elimination processes, ii) changes the expression of mast cells (CC1) in sciatic nerve and in dorsal and ventral horns of spinal cord, iii) alters the activation of macroglia and microglia cells (GFAP and CD11b) leading to the development of reactive astrogliosis, *id est* an inflammatory process of the CNS. Previous experiments have already shown the action of BoNT/A and BoNT/B as analgesics in several pain models (Cui et al., 2004; Luvisetto et al., 2006; Huang et al., 2011; Marinelli et al., 2010, 2012; Park et al., 2015; Ramachandran et al., 2015; Huang et al., 2011). In particular, in animals subjected to peripheral nerve injury (CCI of sciatic nerve or L5 nerve ligation) it was demonstrated that a single non-toxic dose of BoNT/A or BoNT/B into plantar surface of injured paws was sufficient to induce antiallodynic effects and counteract pain symptoms (Marinelli et al., 2010; Park et al., 2015; Luvisetto et al.,

2007). The results obtained in our experiment confirm the antiallodynic effect of BoNT/B on mechanical nociceptive threshold. It has been reported that BoNT/A, in addition to having analgesic effects, also accelerates functional recovery in CCI-mice restoring the normal weight bearing value on the two hindlimbs in incapacitance test and improving SSI scores of the foot print test (Marinelli et al., 2010). Differently from this positive effect of BoNT/A on functional recovery, the results of our study showed that BoNT/B, independently of the dose administered, slows down functional rehabilitation of mice. This effect is observed for most of the period of monitoring of animals and, except in the last days of observation, the animals treated with the neurotoxin showed no differences in performance compared to CCI-saline injected animals. CCI-BoNT/B treated mice still showed clear signs of atrophy of the injured paw even at 101 days post- surgery. With the aim to better define the effect of BoNT/B on neuropathic pain of CCI-mice, IF experiments were associated to behavioral tests. After axonal injury, the distal portion of the nerve undergoes progressive degeneration due to a series of events that induce the degradation of cytoskeletal proteins. In order to evidence structural changes in damaged nerve, we have analyzed with IF technique the expression of NF200, marker of neurons intermediate filaments. By observation of the IF images we found a tissue disjointed and broken up in all CCI-animals to indicate that both BoNT/A and BoNT/B do not affect cytoskeletal proteins. Further changes in the structure of the nerve can be detected by analyzing the distribution and expression of the peripheral myelin protein P0 and PMP22. It is important to consider that, as the axon disappears, Schwann Cells (SC), having lost axonal contact, become able to isolate small whorls of myelin and generate ovoids containing fragmented myelin (Röyttä et al., 1987;

Dubový, 2011). SCs play a key role in the breakdown of the myelin sheath and in the clearance of its debris. SCs clear myelin debris in three distinct ways: they degrade their own myelin, phagocyte extracellular myelin debris, and present myelin to macrophages (Vargas & Barres, 2007). Rapid clearance of myelin appears to be the most important precondition for axonal regeneration after peripheral nerve injury due to the presence of axon growth inhibitors in peripheral nerve myelin (Barrette et al., 2008). Based on this evidence, by means of IF analysis we observed that in CCI-saline injected mice P0 and PMP22 peripheral myelin proteins are aggregated and accumulated in characteristic ovoids while in CCI-BoNT/A and CCI-BoNT/B treated mice myelin seems similarly distributed as for naive animals and the ovoids containing fragmented myelin are reduced. We suggest that, in animals injected with the neurotoxins in comparison with saline-treated mice, the absence of ovoids, the naive-like distribution of myelin and the low expression of P0 and PMP22 could indicate anticipation of myelin phagocytosis process. As previously reported, SC have an important role in regenerative processes (Vargas & Barres, 2007; Barrette et al., 2008). In injured nerves, SCs regain capacity to proliferate and their migration facilitates peripheral nerve regeneration (Han et al., 2007). After injury, myelinating and nonmyelinating SCs change their phenotype from differentiated to immature cells, acquire the expression of molecules characteristic of embryonic development and up-regulate cytoskeletal constituents such as GFAP (marker of dedifferentiated nonmyelinating SCs) and S100β (marker of dedifferentiated myelinating SCs) (Berg et al., 2013; Bhatheja & Field, 2006; Hayashi et al., 2007). In accord with these data, our IF analysis show increased expression of GFAP, S100β and Cdc2 (marker of proliferating cells) in CCI-saline injected mice

compared to naive animals. Animals treated with BoNT/B did not show differences in the expression of these markers in comparison with CCI-saline injected group, indicating that BoNT/B does not modify the activity of SCs after nerve injury. On the contrary, we found a great increase of GFAP and Cdc2 and a clear reduction of S100β expression in CCI-BoNT/A injected mice in comparison with CCI-saline group. As described in previous studies, BoNT/A is able to accelerate the process of nerve regeneration stimulating the SCs proliferation (Marinelli et al., 2010, 2012). The increased expression of GFAP and Cdc2 in BoNT/A treated group observed in our experiment confirms this BoNT/A ability. Previous studies also demonstrated that SC-mediated S100β secretion is required for peripheral nerve regeneration (Perrone et al., 2008). In the nervous system S100β is expressed in Schwann Cells, astrocytes and in certain neuronal population. It has been demonstrated that S100β, released by the same cells that produce it, can act in autocrine, paracrine or endocrine manner with concentration-dependent effects (Michetti et al., 2012). In particular, it has been reported that the maintenance of physiological concentrations of S100β contributes to promote neurite extension, it protects neuron survival and helps to regulate the muscle development and regeneration (Businaro et al., 2006; Michetti et al., 2012; Sorci et al., 2003). On the contrary, higher concentrations of S100β increase the production of reactive oxygen species in neurons and, up-regulating inducible NOS, induce NO-dependent death of neurons. Furthermore, several researches have shown a correlation between high S100β level in biological fluids (blood, urine, saliva, CSF) and different pathological conditions, such as neuroinflammatory/neurodegenerative disorders, brain trauma/injury and cardiac events (Michetti et al., 2012; Donato et al., 2013). By means of IF analysis we observed: i) a strong

reduction of S100β expression in CCI-BoNT/A injected mice in comparison with CCI-saline treated group and, ii) similar expression of S100β in CCI-BoNT/A treated group to that observed in naive animals. We hypothesize that the expression of S100β observed in CCI-BoNT/A injected animals could be associated to an extracellular release of the protein by SCs similar to that in physiological conditions, and explain the positive effect of BoNT/A observed on nerve regeneration. Conversely, a higher expression of S100β, such as that in CCI-saline and CCI-BoNT/B treated animals, could explain the functional impairment observed in these two experimental groups and could be ascribed to an up-regulation of inducible NOS and to the consequent death of neurons. To confirm this hypothesis further experiments should be carried out, investigating the extracellular concentrations of S100β in naive and CCI-mice treated with the neurotoxins, and evaluating a possible correlation between S100β release and S100β expression in SCs.

The activation of immune cells that occurs after the nerve damage was examined by IF technique. The analysis showed an increase of mast cells in CCI-mice compared to naive animals. This enhancement has been particularly evident in CCI-BoNT/A injected mice. Furthermore, in CCI-BoNT/A treated group, but not in the other CCI-group, was also observed a great increase of macrophages. Activated mast cells release different pro-inflammatory mediators and chemokines which help the recruitment of hematogenous macrophages at the site of injured nerve (Thacker et al., 2007). Instead, macrophages remove axon and myelin debris and participate in the production of mitogenic factors for Schwann Cells (Dubový, 2011; Perry & Brown, 1992). The increase of mast cells and macrophages observed in CCI-BoNT/A injected group, compared to other CCI-groups, once again confirms a role of this neurotoxin on

facilitation of nerve regeneration. On the contrary, the increase of mast cells, not accompanied by an enhancement of macrophages and proliferative SCs, that we observed in CCI-BoNT/B treated animals, could indicate a pro-inflammatory effect of this serotypes rather than a pro-regenerative effect.

Overall, these results indicate that, at peripheral level, BoNT/A and BoNT/B act in different manner. Particularly, as concerns the structural alterations associated to nerve injury, BoNT/B seems to produce only some effects induced by BoNT/A, as the anticipation of myelin phagocytosis processes, but, unlike BoNT/A, does not modify the expression of markers related to the nerve regeneration such as GFAP, S100β, Cdc2 and CD11b. Rather, BoNT/B appears to promote the expression of mast cells which, releasing a greater amount of pro-inflammatory molecules could have also an effect on functional recovery of animals.

Previous studies suggested that BoNTs can act not only peripherally but also at the central level (Cui et al., 2004; Luvisetto et al., 2006; Marino et al., 2014; Park et al,. 2015; Mika et al., 2011; Marinelli et al., 2012). In order to observe the effects of BoNT/B on spinal cord we carried out IF analysis on ipsilateral-side of dorsal and ventral horns. Activation of microglial (Cd11+/p-p38+) and astrocytes (GFAP+/p-p38+) in both dorsal and ventral horns was investigated in CCI-saline and CCI-BoNT/B 7.5 pg/paw treated mice. Microglia and astrocytes play a critical role in chronic pain sensitization and their proliferation and activation are correlated with chronic pain behaviors (Wang et al., 2009; Liu et al., 2012). By IF analysis we revealed that CCI-BoNT/B injected group showed no difference compared to CCI-saline treated animals for CD11b and CD11b/ p-p38 positive cells expression in both dorsal and ventral

horn. However, we also observed that, differently from CCI-saline injected mice, in CCI-BoNT/B treated group the microglial morphology did not show the typical amoeboid form of the strong activated state as in CCI-saline injected group, rather, the form was similar to that observed in the resting state, where the cell body is small with long and thin processes. Different results were obtained from the analysis of astrocytes. In particular, IF analysis showed a strong effect of the BoNT/B in dorsal horn, where the astrocytes appear to be less numerous and less activated compared to the CCI-saline treated group. Unlike dorsal horn, in ipsilateral-side of ventral horn the number of astrocytes decreased in CCI-BoNT/B injected animals and, although the number of activated cells was similar between the two experimental groups, IF images highlight a mild/moderate reactive astrogliosis induced by BoNT/B, as indicated by the intense expression of p-p38 in GFAP+ cells and by its hypertrophic morphology. Previous studies have already showed that, after CCI of sciatic nerve, microglia activation in spinal cord can be reduced by peripheral administration of BoNT/A in rats (Mika et al., 2011). Vacca et al. (2013) have also shown that treatment with BoNT/A, 3 days post-CCI, induces strongly reduction of activated and not activated astrocytes and microglial cells in dorsal and ventral horns of mice. Marinelli and colleagues (2012) showed that BoNT/A, administrated into plantar surface of injured paws of CCI-mice, can be carried by the peripheral nerve endings to the spinal cord where it can be transcytosed by nociceptive fibers to astroglial cells. Our results showed that, as BoNT/A, also BoNT/B acts at central level but with different mechanisms. In spinal cord we found that BoNT/B reduces the activation state but not the expression of microglial cells while the astrocytes appears reduced and, in the dorsal horn also less activated. The resting

state of microglia and the reduced number of activated astrocytes observed in dorsal horn of spinal cord, may explain the analgesic effect of BoNT/B observed during the behavioral tests in mice. In particular, BoNT/B, acting on astrocytes, probably by transcytosis, could inhibit the glutamate release from astroglial cells and, consequently, contributes to the reduction of pain. Effectively, the possibility that BoNTs can be transcytosed to astrocytes has been already suggested (Marinelli et al., 2012), as it has been also demonstrated the ability of BoNT/B to inhibit the release of glutamate by astrocytes cultures (Jeftinija & Stefanovic, 1997). Conversely, the presence of reactive astrogliosis in the ipsilateral-side of ventral horn in CCI-BoNT/B treated animals could explain the reduced performance observed during behavioral testing and the failure of functional recovery, testified by marked atrophy of the injured paw of CCI-BoNT/B mice at 101 days post-surgery.

The results of our study confirm BoNT/B as a powerful biological molecule that can reduce neuropathic pain symptoms for a long period of time. However, we provide the first evidence that BoNT/B, although it alleviates neuropathic pain-related behavior, increases the expression of cells that release pro-inflammatory molecules in injured nerve and, in ventral horns of spinal cord, induces reactive astrogliosis development that could lead to glial scar formation and irreversibly compromise the functional recovery.

Conclusion

Based on our results, we believe that the use of BoNT/B as therapeutic alternative to BoNT/A for the treatment of pain syndromes must be avoided. The development of astrogliosis observed in ventral horn of spinal cord and the clear signs of atrophy of the injured paw of mice at 101 days post-CCI are compatible with the possibility that BoNT/B can induce formation of a glial scar, compromising the functional rehabilitation.

Therefore, on the basis of this evidence, we do not recommend the use of BoNT/B in pain conditions associated with nerve injury as long as the molecular pharmacology and the mechanism of action of neurotoxin will not fully understood and more information becomes available.

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