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From poison to clinical application: Is Botulinum Toxin A a treatment option for pain arising after spinal cord injury?

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP	Adenosine triphosphate
BSCB	Blood Spinal Cord barrier
BONTs	Botulinum toxins
BTX-A	Botulinum Toxin A
CCL21	Cysteine-Cysteine Chemokine Ligand 21
DSD	Detrusor-Sphincter Dyssynergia
CGRP	Calcitonin gene-related peptide
CNS	Central Nervous System
CSPGs	Chondroitin Sulphate Proteoglycans
cVLM	Caudal Ventrolateral medulla
DRG	Dorsal Root Ganglia
DRt	Dorsal reticular nucleus
GABA	Gamma-aminotyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial Fibrillary Acidic Protein
LC	Locus Coeruleus
MAI	Myelin associated inhibitor
NK1	Neurokinin 1
OMgp	Oligodendrocyte Myelin Glycoprotein
P2X	Purinergic receptors
PAG	Periaqueductal grey area
PEAP	Place escape/Avoidance Paradigm test
PNS	Peripheral Nervous System
PWT	Paw Withdrawal Threshold
RVM	Rostroventral medulla
SCI	Spinal Cord Injury
SGCs	Satellite cells
SNAP-25	Synaptosomal-associated protein 25
SNARE	Soluble NSF attachment receptor
SV2	Synaptic vesicle protein 2

TrK-B	Tropomyosin receptor kinase B
TRPV1	Transient Receptor Potential Vanilloid-1
VAS	Visual Analogue Scale
WDR	Wide-dynamic range neurons

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Sumário

Introdução: As lesões vertebro-medulares são consequência de eventos catastróficos tais como acidentes rodoviários e/ou quedas relacionadas com desportos radicais. As suas consequências são devastadoras. A mais reportada é a perda da função motora, que pode ser recuperável dependendo da severidade da lesão. Além disso, a dor neuropática e a perda do controlo da atividade vesical são os sintomas mais prevalentes após a lesão.

Objetivos e métodos: Diversos estudos envolvem o uso da toxina botulínica do tipo A no tratamento da disfunção do sistema urinário e da dor neuropática em diversos modelos animais com recurso à sua administração ao nível do Sistema Nervoso Periférico. De forma a verificar a sua influência no Sistema Nervoso Central através de uma injeção intratecal, os animais foram submetidos a uma lesão vertebro-medular incompleta a nível T8-T9. Durante 4 semanas o comportamento dos animais foi monitorizado. Após injeção, os animais foram novamente avaliados a nível comportamental e vesical. A expressão dos recetores TRPV1, GFAP e SNAP-25 foi determinada e a zona lesionada da medula espinhal fora recolhida para posterior análise histoquímica. A função vesical fora também avaliada.

Resultados: A maioria dos animais lesionados manifestou alodínia mecânica, sensibilidade térmica e reduzida atividade exploratória uma semana após a cirurgia. Por sua vez, a atividade vesical dos animais lesionados revelou-se mais intensa e a amplitude das contrações da bexiga mostrou-se aumentada. Os recetores avaliados através da análise do tecido não mostraram alterações significativas. Após injeção, a ação da toxina potenciou o aumento do limiar de ação dos neurónios nociceptores, revertendo a alodínia mecânica. A nível vesical, a amplitude das contrações da bexiga diminuiu significativamente nos animais lesionados que receberam a injeção intratecal da toxina.

Conclusão: O modelo experimental de lesão vertebro-medular incompleta desenvolveu alodínia mecânica e sensibilidade térmica. O aumento da amplitude e frequência das contrações

da bexiga estão presentes neste modelo. A injeção botulínica do tipo A manifesta-se como uma possível terapia para o tratamento da dor neuropática e respetiva disfunção vesical, ambos sintomas das lesões vertebro-medulares.

Abstract

Introduction: Spinal cord injury results from catastrophic events such as motor vehicle crashes, sports-related injuries and/or falls. Its respective consequences are devastating. Motor dysfunction is the most reported symptom after SCI. Along with paralysis, neuropathic pain and bladder dysfunction are considered bothersome symptoms arising from this condition. Locomotor function might be recovered depending on the severity of the lesion.

Aims and methods: Several studies described Botulinum toxin A as a good therapeutic approach for the treatment of neurogenic detrusor overactivity and for neuropathic pain. Its known route of administration is commonly performed in the Peripheral Nervous System. In order to verify its influence in the Central Nervous System, an intrathecal Botulinum Toxin A injection was performed. Animals underwent a T8-T9 laminectomy and an incomplete lesion was performed by cutting 2 mm perpendicularly to the spinal cord. For four weeks, behavioural data was collected. After injection, animals were tested for another week of behavioural analysis. TRPV1, SNAP-25 and GFAP relative expression was determined and immunohistochemical reactions of the spinal lesion were performed. Cystometries to assess bladder function were also performed.

Results: Most of the animals developed mechanical allodynia and thermal sensitivity from week 1 onwards. Lesioned animals showed less burrowing behaviour. Injured animals revealed higher bladder frequency and amplitude of contractions. After injection, mechanical allodynia was reversed and bladder amplitude decreased in injured animals which received the toxin.

Conclusions: The animal model of incomplete spinal cord injury was established and developed mechanical allodynia and thermal sensitivity. Bladder dysfunction was also present in the model. Intrathecal injection of Botulinum toxin A might be a possible therapy for the treatment of SCI-related neuropathic pain and respective bladder dysfunction.

I. Introduction

Spinal Cord Injury

Spinal cord injury (SCI) is one of the most catastrophic events that affects individuals in their peak productive phase of life. With a change in the epidemiology and causes, currently most SCI cases result from falls, sports-related injuries and motor vehicle crashes (Lee et al., 2014) resulting in sectioning, contusion or ischemic damage of the spinal cord. Typically, cervical and high thoracic segments are the most affected due to abrupt flexion or rotation of the head, neck or back. With the evolution of medical care, better quality of emergency services and upgrading of security policies, life expectancy for SCI patients has greatly increased in recent years and is expected to further rise.

Clinical manifestations of SCI are highly variable and depend on the level and severity of injury. With the improvement of travelling conditions, complete cord transections occur less frequently while contusion and incomplete sectioning of spinal tissue became more common. In the latter case, considerable functional recovery may occur both in patients and animal models. These functional improvements reflect plastic adaptions occurring within the Central Nervous System (CNS) involving molecular and cellular changes as well as reorganization of neuronal circuits (Zorner et al., 2010). (Filli et al., 2014).

Following SCI, patients show impairment of motor, sensory and autonomic functions resulting in a decline on a person's health, social participation and quality of life. Common complications include neuropathic pain, spasticity, hypotension, autonomic dysreflexia and urinary incontinence due to Neurogenic Detrusor Overactivity (NDO) (Haisma et al., 2007). In fact, chronic pain and lower urinary tract dysfunction are among the most frequent symptoms described by SCI patients (Finnerup, 2017; Yang et al., 2017), two major concerns for SCI patients that cause a severe impact in their quality of life (Finnerup et al., 2015; Finnerup et al., 2016; Park et al., 2017).

SCI pathophysiology

SCI-induced cord changes at the injury site occur in four steps. The **initial step** refers to direct tissue damage (compression, laceration, shearing of the cord) that results in profound histological changes at the site of injury (Ramer et al., 2005). Tissue damage initiates a series of destructive events during the **second stage** of SCI. These events include vascular changes like damage of the blood spinal cord barrier (BSCB) (Tran et al., 2018), production of free radicals, lipid peroxidation, altered ATP production, invasion by neutrophils (facilitated if there is rupture of blood vessels), activation of resident glial cells due to the release of alarmins (Tran et al., 2018) and neuronal and glial apoptosis (Silver and Miller, 2004; Ramer et al., 2005). Primary injury leads to a prolonged secondary injury until the wound seals and the glial scar matures. After BSCB damage and subsequent neutrophil infiltration, the **third step** is marked by leukocytes invading the injured area. Monocytes differentiate into macrophages and are recruited to the core and margins of lesion (Kigerl et al., 2009). In response to alarmins, microglia migrate to the injury site and release several pro-inflammatory factors (Gadani et al., 2015). At the lesion, microglia clears debris (Greenhalgh and David, 2014) and helps to seal and block the spread of the lesion (Hines et al., 2009).The **fourth and final step** consists in the formation of a glial scar, composed by chondroitin sulphate proteoglycans (CSPs), myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Bradbury and McMahon, 2006; Bradbury and Carter, 2010). Glial scar formation is accompanied by axonal demyelination and Wallerian degeneration of surviving spinal neurons (George and Griffin, 1994) (Figure 1). Once a stable scar is formed and tissue remodelling has terminated, the molecular environment at the lesion site has become hostile and highly inhibitory for surviving cells. Thus, full recovery of function is not always possible and maladaptive plasticity will occur and lead to chronic pain and neurogenic detrusor overactivity (NDO).

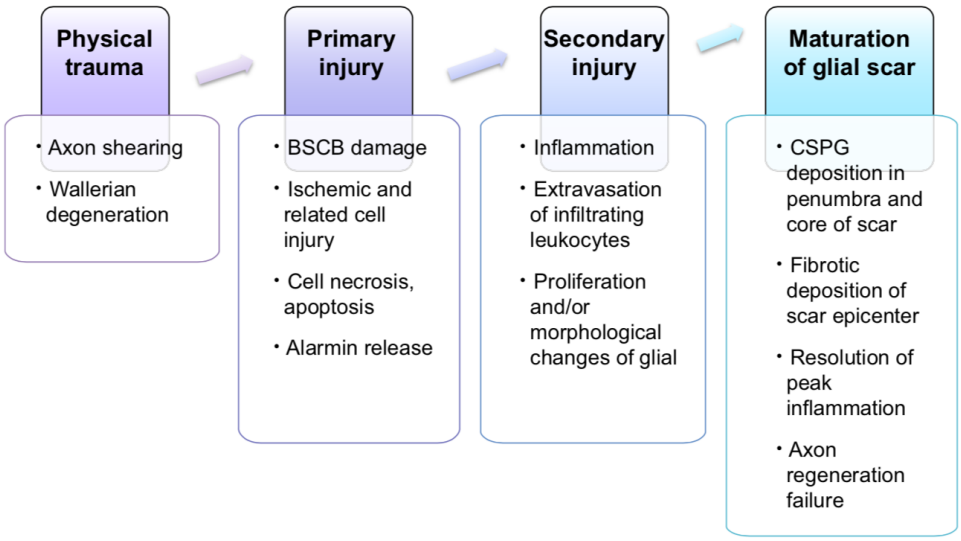

Figure 1: **Representation of SCI pathophysiology.** Spinal cord injury occurs in four different stages: physical trauma, primary injury, secondary injury ending in the maturation of the glial scar (Tran et al., 2018)**.**

Pain

Pain perception and ascending pathways

Pain is defined by the International Association of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage". Pain perception is initiated by the activation of specific peripheral neurons, called nociceptors. Nociceptors can be activated by a variety of noxious mechanical, thermal and chemical stimuli and are be differentiated according to their neurochemical profile and diameter of the cell body (Nickel et al., 2012). While Aδ nociceptors have medium-sized cell bodies and thin myelin sheaths covering their processes, C-type nociceptors have small-sized cell bodies and unmyelinated fibres, with slower transmission velocities (Basbaum et al., 2009).

Activation of the nociceptive somatosensory pathway is initiated by nociceptors which convey noxious stimulus from damaged tissues and project to the spinal cord or brainstem. Primary afferent nociceptive neurons synapse with second-order neurons such as wide-dynamic (WDR) or interneurons present in the superficial layers of the dorsal horn. These spinal neurons are part of the spinothalamic pathway and project to thalamic neurons which, in term, project to several cortical areas and some brainstem nuclei (Boadas-Vaello et al., 2016) (Figure 2).

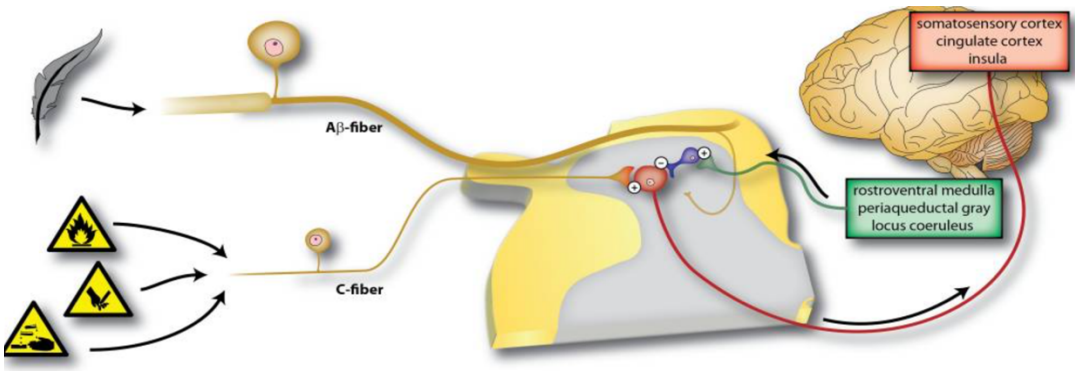

Figure 2: **The nociceptive pain circuit (von Hehn et al., 2012).**

Alterations in ascending pain pathways caused by nerve injury

Tissue injury promotes the generation of algogenic mediators and enhances nociceptors sensitivity to them (Amaya et al., 2013) (Figure 3). These mediators bind to ion channels or metabotropic receptors present in nociceptors and activate intracellular signalling pathways

that generally lead to changes in gene expression, resulting in increased expression of receptors and other phenotypic changes ultimately leading to sensitization of nociceptors (Taylor, 2001, 2009). Hyperexcitability of injured nociceptors results in increased electric activity potentiated by a decreasing in afferent threshold.

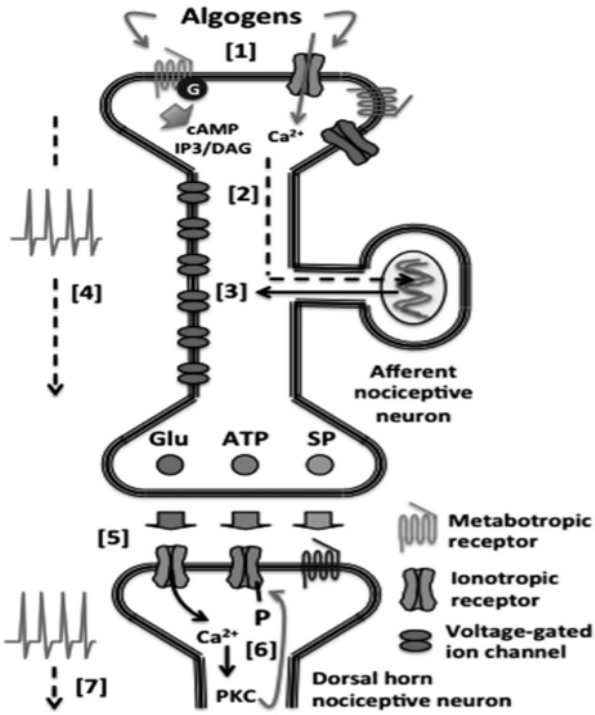

Figure 3: **Representation of the alterations provoked by injury in nociceptive ascending pathways (Boadas-Vaello et al., 2016)**

Algogens (1) bind to membrane receptors and facilitate the transcription of genes (2;3) thereby resulting in an overexpression of receptors leading to the hyperexcitability of afferent neurons (4). More neurotransmitters will be released (5) in the synaptic cleft resulting in sensitization of neurons distant to the injury site.

Glutamate released by afferent nociceptive neurons binds to N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, present in spinal interneurons and WDR cells (Boadas-Vaello et al., 2016), resulting in increased activation of the spinothalamic pathway.

In addition, activation of this circuit can be enhanced by spinal release of neuropeptides, such as substance P or calcitonin gene-related peptide (CGRP) , present in many C type nociceptors. In addition, spinal transmission of noxious input can be modulated and can be exacerbated by microglia and astrocyte, which can release several factors such as IL-1β, TNF-α and IL-6 and contribute to chronic pain, particularly of the neuropathic type (Kawasaki et al., 2008; Zhang et al., 2008).

Inhibitory descending pain pathways

Ascending pain transmission is counteracted by descending inhibitory pain pathways. Several supra-spinal nuclei have been associated with descending inhibition, with the periaqueductal grey-rostral ventromedial medulla (PAG-RVM) system being one of the most well-studied. In the PAG-RVM, the dorsal reticular nucleus (DRt) and the caudal ventrolateral medulla (cVLM) are crucial as they are part of a spinal-supraspinal-spinal feedback loop that modulate pain (Boadas-Vaello et al., 2016). (Le Bars, 2002; Monconduit et al., 2002). The RVM is in contact with afferent fibres and interneurons, receiving inputs from the PAG and projecting outputs to the spinal cord through the dorsolateral funiculus. The major inhibitory inputs to the spinal cord are made by serotonergic RVM neurons and noradrenergic neurons present in Locus Coerulus (LC) (Millan, 2002).

RVM neurons can be divided into two groups: 'ON'-RVM neurons and the 'OFF'-RVM neurons. The 'ON' cells show pro-nociceptive properties and express receptors like NMDA/AMPA, the high affinity receptor for brain derived neurotrophic factor (Trk-B) and the transient receptor potential vanilloid 1 (TRPV1) (Fields, 2000; Guo et al., 2006; Da Silva et al., 2010). These neurons are inhibited by opioids and facilitate nociceptive pain by exciting dorsal horn neurons (Bourne et al., 2014). The 'OFF' cells display an anti-nociceptive effect (Ossipov et al., 2010), expressing mainly TRPV1 and NMDA/AMPA receptors (Heinricher et al. 2009; (Palazzo et al., 2008). In physiological conditions, 'ON' cells are inhibited by GABAergic interneurons and the 'OFF' neurons remain activated and the fire response is decreased. There is a balance between the activation and inhibition of both types of RVM-neurons.

Alterations in descending pain pathways caused by neuronal injury

When injury occurs, the balance between 'ON' and 'OFF'-neurons is disrupted. The hyperexcitability of the nociceptive ascending neurons leads to an exacerbated release of neurotransmitters, contributing to a switch in 'ON' cells sensitivity. The 'ON' neurons block from

PAG and become excited by inputs from the spinothalamic tract. In this case, 'OFF' neurons become insensitive to excitatory projections from the spinothalamic pathway. Altogether, these alterations lead to an amplification of the descending excitatory pathway drived by RVM-'ON' neurons while there is an inhibition of the descending inhibitory pathway of pain transmission mediated by RVM-'OFF' neurons. (Boadas-Vaello et al., 2016) (Figure 4).

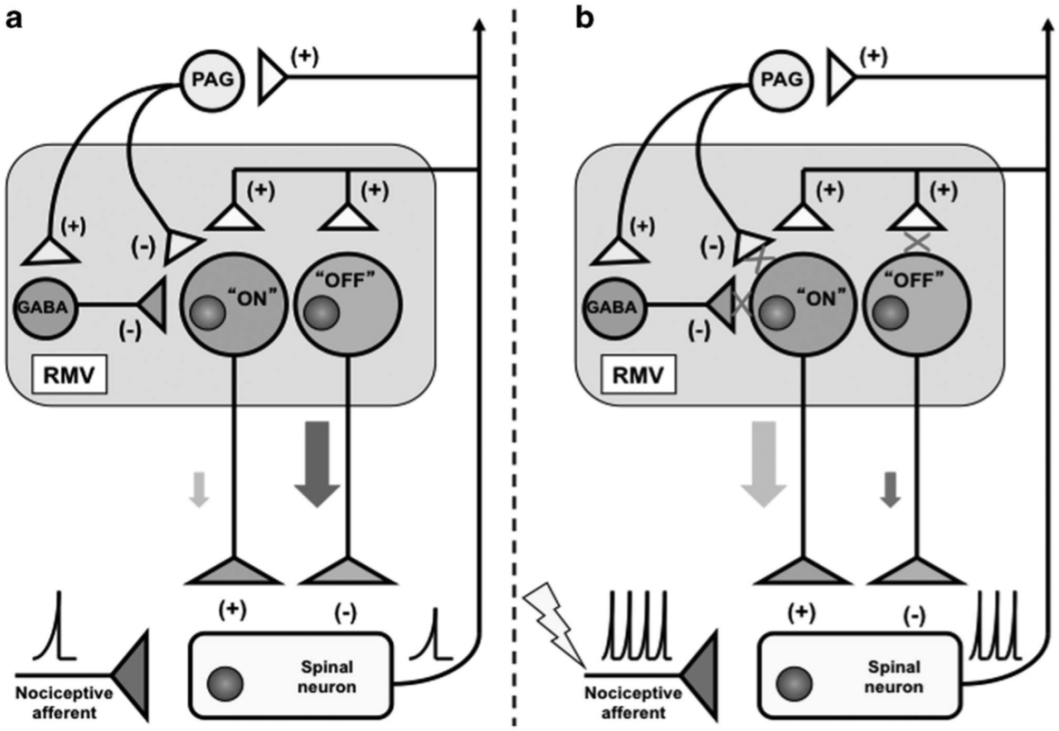

Figure 4: **Representation of 'ON' and 'OFF' RVM-neurons action in physiological conditions and in neuropathic pain (Boadas-Vaello et al., 2016)**

a) In normal conditions, the nociceptive afferent neuron projects inputs to the spinal neuron and pain sensation goes through the spinothalamic tract. Along with 'ON' and 'OFF' neurons in the RVM, PAG neurons become activated thereby exciting GABAergic interneurons and these will inhibit 'ON' neurons. The 'OFF' remains activated and the fire response is decreased.
b) Neuropathic pain arising from tissue injury or spinal cord injury alters the expression of receptors and ion channels, contributing to a switch in the sensitivity of 'ON' neurons. The PAG neurons will excite GABAergic interneurons and due to the receptors' overexpression and neurons' hyperexcitability, 'ON' neurons deny inputs from the PAG and become excited due to inputs from the spinothalamic tract. The 'OFF' neurons are also, in these conditions, insensitive to excitatory projections. The fire response is increased and there is a descending facilitation of pain from the RVM.

Pain arising after SCI

Pain described by SCI patients is ongoing, burning, stabbing, shooting, or shocking and often arises with no stimulus. Less frequently, people with SCI also experience pain out of context with the stimulus (e.g., light touch can be painful) (Kramer et al., 2017). A major cause for chronic pain after SCI is the abolishment of the endogenous descending inhibitory circuits that modulate pain perception (Heinricher et al., 2009; Martins et al., 2015). In addition, following spinal injury, a series of inflammatory pro-nociceptive modulators are released in the spinal cord by surviving and activated neurons, astrocytes and microglial cells (Cregg et al., 2014), in an attempt to protect non-injured tissue, induce regrowth and reconnection of neuronal processes and produce a glial scar that seals the injury site (Ramer et al., 2005; Ramer et al., 2014). Examples of these molecules include neurotrophic factors (Krenz and Weaver, 2000; Brown et al., 2004; Cameron et al., 2006), reactive oxygen species (Chen et al., 2013), and cytokines as TNF-alpha, IL-1β and IL-6 (Park, 2013; da Silva et al., 2015; Pellett et al., 2015), all of which have been linked to the establishment of chronic pain (Ji et al., 2014). While in an initial stage of disease, these modulators are upregulated in the vicinity of the injury site, with time they also increase in more distant locations (Andrews et al., 2012). To make matters more complicated, it should be recalled that nociceptive sensory neurons also undergo morphological and functional changes, including neurite extension and increased expression of neuropeptides (Krenz and Weaver, 1996; Cameron et al., 2006; Ackery et al., 2007), which are also involved in the development of chronic pain after SCI. Current treatment for chronic pain after SCI includes gabapentin, opioids and pregabalin (Widerstrom-Noga, 2017) but these drugs are not always effective and do not prevent pain worsening.

Chronic pain reported by SCI patients is neuropathic in nature and reflects maladaptive plasticity of the CNS, resulting from collateral sprouting and changes in expression of ion channels, neurotransmitters and receptors (Deumens et al., 2008). Neuropathic pain is characterized by spontaneous and stimulus-evoked painful sensations. This kind of pain is

described as being episodic or continuous, superficial or deep and reflect neurons' ectopic activity. Neuropathic pain arising after spinal cord injury can occur either at- or below the level of the injury (Shiao and Lee-Kubli, 2018). Although several changes occur in injured neurons, neighbouring neurons can initiate non-evoked afferent input and thereby giving rise to painful sensations (Wu et al., 2002). These changes may occur due to the expression, distribution and phosphorylation of many ion channels, such as ionotropic and metabotropic glutamate receptors (Meacham et al., 2017) resulting in rhythmic firing bursts in the absence of a stimulus. Impairment between the descending facilitation and inhibition is also a major contributor to ongoing pain (Apkarian et al., 2004).

Peripheral SCI-pain related mechanisms

Several mechanisms can be accounted for SCI pain not only at the spinal cord, where the lesion occurs, but also at the peripheral nervous system, with reports documenting changes in nociceptors' function (Yang et al., 2014). In fact, it has been argued that central changes are important, but only happen due to a peripheral drive.

After SCI, an increased excitability of dorsal root ganglion (DRG) neurons is observed (Bedi et al., 2010). DRG neurons widely communicate with satellite cells that ensheath them. After spinal cord injury, the activation of satellite cells (SGCs) results in the overexpression of connexin-43, a gap junction protein that facilitates communication between adjacent SGCs (Shiao and Lee-Kubli, 2018). The overexpression of connexin-43 is increased in DRGs after severe SCI and it is believed to play an important role in at-level pain (Jasmin et al., 2010), suggesting peripheral sensitization. Campana and its co-workers showed that intrathecal administration of connexin-43 antagonist, carbenoxolone, reduced at-level pain withdrawal responses (Lee-Kubli et al., 2016). These observations do not exclude an effect of the drug at the CNS level as connexin-43 is also expressed by astrocytes (Chen et al., 2012) and overexpressed in spinal cord injury (Abram et al., 2006), making it conceivable that intrathecal drug delivery simultaneously affected the DRG and spinal cord.

Central SCI-pain related mechanisms in the spinal cord

Spinal mechanisms can also be accountable for SCI pain such as reactive gliosis, spinal disinhibition and spinal hyperexcitability (Shiao and Lee-Kubli, 2018). Indeed, SCI animals show an increase in glial fibrillary acidic protein (GFAP) when compared with intact animals, demonstrating the presence of activated astrocytes following spinal lesion (Carlton et al., 2009). Astrocytes release several cytokines such as IL-6, IL-1β and TNF-α, important mediators for the development of neuropathic pain (Obata et al., 2010). Microglia also releases inflammatory factors contributing to microgliosis in an autocrine manner and may be important for the development of hypersensitivity and to stimulate the transition from acute stages to chronic pain (Peng et al., 2016). Spinal microglial cell activation and consequent release of cytokines, chemokines and prostaglandins are a direct cause of spinal cord injury as they affect sensory afferents either caudally or rostrally to the lesion site, causing aberrant spike bursts and pain neurotransmission (Rider, 2014). In addition, the severity of the lesion determines the necrotic death of astrocytes and neurons, leading to the release of glutamate which also interacts with nociceptive projection neurons. These sensory inputs will travel through the spinothalamic tract and RVM-'ON' cells will become activated, facilitating pain transmission.

Spinal GABAergic neurons also play a role in gating sensory stimuli and a reduced GABA inhibition can lead to hypersensitivity to innocuous stimuli. In SCI, this phenomenon can occur due to the loss of GABAergic neurons or, when surviving, reduced activity of these neurons, contributing to spinal disinhibition (Shiao and Lee-Kubli, 2018). (Huang and Grau, 2018).

Loss of descending fibre tracts, due to tissue damage, also contributes to the development of neuropathic pain. For example, it is known that serotonin fibres sprout widely rostrally to the spinal cord lesion whereas this sprout is not present caudally to the injury (Hains et al., 2002).

Central SCI-pain related mechanisms in the brain

SCI-induced neuropathic pain may develop may also reflect the magnitude of reorganization occurring in the primary somatosensory cortex (Jutzeler et al., 2015). The thalamus, that relays information to the somatosensory cortex, also undergoes several changes due to SCI-related pain. Thalamic neurons develop spontaneous and hyperresponsive firing due to reduced activity present in the *zona incerta* (inhibitory control over thalamic neurons), as seen in studies following unilateral lesion of the spinothalamic tract (Gerke et al., 2003; Liang and Mendell, 2013). SCI also causes the suppression of activity in GABAergic nucleus of the *zona incerta*, leading to an increase of the activity of the posterior nucleus of the thalamus (PO) (Park et al., 2018). The posterior nucleus of the thalamus, a higher order nucleus involved in somatosensory and pain processing, thus becomes hyperexcitable due to reduction of inhibitory inputs, sensitization and hyperexcitability of thalamic neurons, glial reactivation and over release of inflammatory mediators contributing to neuropathic pain (Boadas-Vaello et al., 2017).

Neuronal control of bladder function

The lower urinary tract (LUT), composed of a reservoir (the urinary bladder) and an outlet unit (the bladder neck, urethra and striated muscles of the external urethral sphincter), stores urine as it is produced and eliminates urine when bladder capacity is overcome. A strict coordination between the bladder contraction and sphincteric structures relaxation is necessary for efficient micturition. This coordination requires the participation of complex neuronal circuits involving peripheral and centrally located neurons. In addition, unlike other visceral organs, LUT function is under voluntary control. In fact, LUT function is regulated by behaviour learned during maturation of the CNS (Fowler et al., 2008).

The LUT operates in a switch-like manner. While the bladder fills, bladder sensory afferents, arranged in two plexuses, one around detrusor smooth muscle cells and the other with a suburothelial location, are silent. Sympathetic fibers are active, promoting continence by relaxing the detrusor, contracting the outlet and inhibiting parasympathetic neurotransmission in parasympathetic ganglia. As the bladder distends with the accumulation of urine, sensory afferents become active and the information of bladder fullness is conveyed to the dorsal horns of the lumbosacral spinal cord where ascending neurons will transmit the fullness information to the periaqueductal gray area (PAG) and, from there, to the cerebral cortex (Fowler et al., 2008; Drake et al., 2010). If a decision to void is made, PAG will change the pontine micturition center mode, located in the brainstem, from storage mode to voiding. Information will then be relayed from the pontine micturition center to spinal motoneurons. Sympathetic output to the bladder will be interrupted, preventing further detrusor relaxation and promoting the opening of the bladder neck. At the same time, motoneurons of the Onuf Nucleus are inhibited, further relaxing the urethral sphincter. Finally, local parasympathetic bladder innervation will be activated. As an overall result, the detrusor contracts while the urethra relaxes, allowing the expulsion of urine (de Groat and Yoshimura, 2001; Fowler et al., 2008). The considerable complexity of neuronal mechanisms regulating LUT function shows its high sensitivity to a

variety of injuries and diseases, particularly those affecting the nervous system, such is the case of spinal cord injury (SCI) (Figure 5).

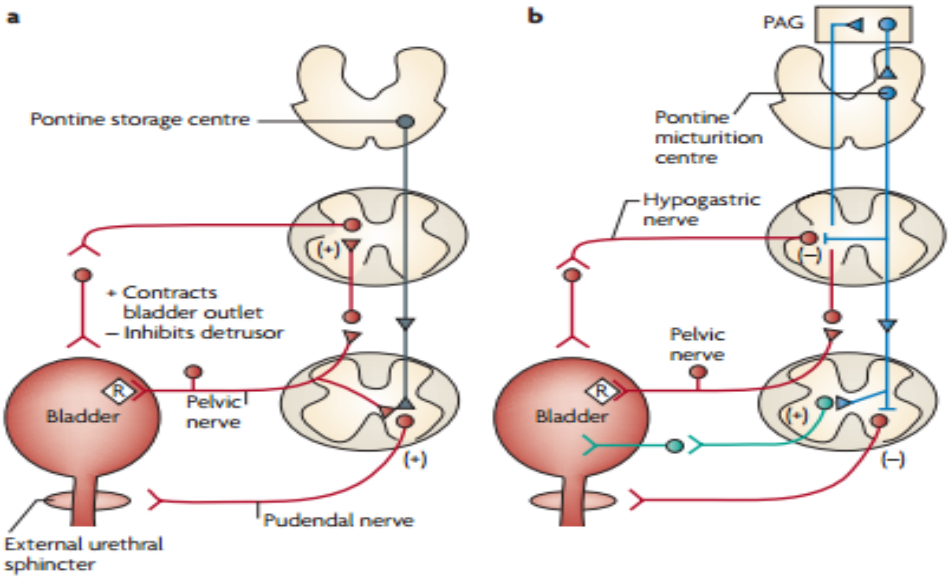

Figure 5: **Urinary control of micturition.**

a| During the storage of urine, distention of the bladder produces low-level vesical afferent firing. Consequently, there is a stimulation of the sympathetic outflow in the hypogastric nerve to the bladder outlet and the pudendal outflow to the external urethral sphincter. This occurs by spinal reflex pathways and represent guarding reflexes, which promote continence. Sympathetic firing also inhibits contraction of the detrusor muscle. Pontine storage center might increase striated urethral sphincter activity. **b|** During the elimination of urine, intense bladder-afferent firing in the pelvic nerve activates spinobulbospinal reflex pathways that pass through the pontine micturition center. The parasympathetic outflow to the bladder and to the urethral smooth muscle and inhibits the sympathetic and pudendal outflow to the urethral outlet. Ascending afferent input from the spinal cord might pass through relay neurons in the periaqueductal grey (PAG) before reaching the pontine micturition center. (Fowler et al., 2008)

Neurogenic detrusor overactivity after SCI

SCI is followed by the spinal shock period, characterized by bladder arreflexia and urinary retention, the duration of which varies with species. In the rat, it last between 10 to 14 days whereas in Humans many months are necessary to overcome the spinal shock. Spinal shock is outweighed by the emergence of a new micturition reflex, totally located at the lumbosacral spinal cord. This reflex is out of voluntary control. During bladder filling, involuntary detrusor

contractions are common, named as neurogenic detrusor overactivity (NDO) in urodynamic testing, eventually generating urgency to void and urinary incontinence. Another major consequence of SCI is detrusor-sphincter-dyssynergia (DSD), which can cause severe obstruction to bladder emptying leading to high intravesical pressures. Ultimately, these high pressures may result in life-threatening deterioration of the upper urinary tract. Therefore, the treatment of DSD is typically the first measure to be taken when initiating the management of SCI patients. Once this aspect is under control, quality of life emerges as the critical point to patients. Quality of life surveys clearly indicate that, after improving motor function, regaining bladder control is the highest priority for SCI patients (Ku, 2006; French et al., 2010; Simpson et al., 2012). Loss of bladder control is the second most important aspect referred by SCI patients, due to its impact on social engagement. In some cases, embarrassment is such that patients will abandon their physiotherapy sessions, skip medical appointments and avoid social arrangements. Therefore, dealing with NDO assumes a foremost importance in the management of SCI patients.

Current NDO management aims to facilitate urine storage and to reduce periods of high intravesical pressures which are harmful to the upper urinary tract. NDO therapy is initiated by antimuscarinic drugs, usually in doses above those approved by authorities. It is, therefore, not surprising that adverse side-effects among SCI patients are common, including dry mouth and constipation (Ku, 2006). More recently, the use of detrusor injections of BTX-A has been approved by authorities for the treatment of NDO refractory to anti-muscarinic drugs. (Cruz et al., 2011). Recent studies, however, have shown that intrathecal administration of this neurotoxin is also effective to treat urinary dysfunction following SCI (Coelho et al., 2016b). In this case, effects of BTX-A were restricted to sensory afferents. In addition, the same approach has also been demonstrated to reduce pain and improve bladder reflex activity in an animal model of chronic bladder inflammation (Coelho et al., 2014).

Botulinum Toxin A – a therapeutic toxin?

Botulinum neurotoxins, known as BoNTs, are neurotoxins produced by bacteria belonging to the *Clostridium* genus. Six serotypes are known and share a similar molecular structure, despite differences in the aminoacid sequence. One of the most well-known neurotoxins is botulinum toxin A, clinically used to treat pain and NDO (Matak and Lackovic, 2014; Park and Chung, 2018)). BTX-A is composed by a light chain (50 kDa) and a heavy chain (100 kDa) linked by a disulphide bond (Pirazzini et al., 2017) (Figure 6). Specific binding to neurons is regulated the by the heavy chain, that bind to polysialogangliosides present on the cell surface of neuronal cells (Simpson and Rapport, 1971). BTX-A is known to exclusively interact with the GT1b type of gangliosides (Rummel et al., 2004). The complex toxin-ganglioside forms a net that accumulates the toxin on the plasma membrane surface. Once the axonal terminal is depolarized and neurotransmitter-containing vesicle bind to the axonal membrane, the high-affinity receptor for BTX-A synaptic vesicle protein 2 (SV2) (Dong et al., 2006) is exposed. The accumulated toxin will then interact with SV2 and, upon vesicle recycling, will become internalized (Ahnert-Hilger et al., 2013) (Evans et al., 1986).

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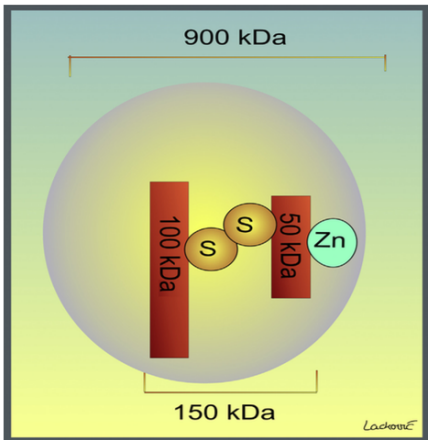


Figure 6: **Representation of BTX-A neurotoxin with respective light and heavy chain (Matak and Lackovic, 2014).**

After internalization of the toxin, pH decreases inside the endosome and a structural rearrangement of the toxin occurs as the disulphide bridge is broken (Koriazova and Montal, 2003). Once released in the cytosol, BTX light chain, which have zinc-dependent endopeptidase activity, specifically target one of the SNARE proteins (Keller and Neale, 2001) inhibiting vesicle exocytosis and neurotransmitters release. Several studies have confirmed that cleaved SNAP-25 is a product of BTX-A action and can serve as a marker of the toxin's activity to identify the site of action of BTX-A (Coelho et al., 2012; Oliveira et al., 2017).

BTX-A to treat pain

While the ipsilateral and local effect of BTX-A in pain reduction is consensual, there is also evidence of translocation of the toxin. Several studies indicate that peripheral BTX-A injection reduces mechanical and thermal hypersensitivity ipsi- and contralaterally (Bach-Rojecky et al., 2010).In fact, cleaved SNAP 25 can be found in several regions of the PNS and in the CNS (in the facial nucleus in the brainstem, superior colliculus and motor region of the spinal cord) following injection of the toxin into rat whisker muscles, rats' eye and unilateral intramuscular injection respectively, in what concerns cleaved SNAP-25 expression in the CNS. (Antonucci et al., 2008; Matak et al., 2011; Restani et al., 2011; Matak et al., 2012; Koizumi et al., 2014)). Matak and his co-workers showed that cleaved SNAP-25 was absent from the spinal cord in animals receiving sciatic nerve injections with BTX-A that have been pre-treated with colchicine (Matak et al., 2012), demonstrating that the CNS effects of peripheral BTX-A administration reflect microtubule-dependent retrograde axonal transport (Matak et al., 2011; Restani et al., 2012).

Two functional anti-nociceptive effects are currently attributed to BTX A: effects on the motor nervous system (by blocking cholinergic terminals) and effects on sensory fibres (generating an analgesic effect) (Park and Chung, 2018). Several studies support the anti-nociceptive effect of this toxin. In guinea pig formalin-induced pain model, BTX-A reduces the slow phase of KCl-evoked glutamate release (McMahon et al., 1992). CGRP release is decreased by BTX-A administration in cultured rat trigeminal ganglion neurons (Durham et al., 2004).

Subcutaneous injection of BTX-A reduces TRPV1 expression in a transection of the lumbar 5 ventral root animal model and as a similar effect in P2X3 receptors, one of the purinergic receptors associated with pain perception (Xiao et al., 2011; Xiao et al., 2013). These findings suggest that BTX-A reduces pain by inhibiting neurotransmitters release from peripheral nerve endings.

In what concerns the application of BTX -A for central neuropathic pain in clinical studies, two clinical reports were performed, albeit with very small sample sizes. In both cases, after receiving a subcutaneous BTX-A injection, patients showed improvements in pain severity and reduced number of painful events (Jabbari et al., 2003; Han et al., 2014). More recently, in 2016, Han and his co-workers performed a double-blind, randomized controlled study in SCI-neuropathic pain, with 40 patients that received a subcutaneous injection of BTX-A (200U) or normal saline . The VAS score, the Korean version of the McGill Pain Questionnaire and a test to assess quality of life were performed. Results showed that pain was reduced in the BTX-A treated group (Han et al., 2016).

BTX-A in NDO management

The efficacy of BTX-A treatment for LUT disorders is currently well documented and safety policies are good. Intravesical BTX-A injection for treating SCI patients with NDO have been reported since 2000 in which, for the first time, complete continence was observed (17 out of 19 cases) following treatment (Schurch et al., 2000). Currently, BTX-A is recommended as the gold-standard second-line treatment for NDO (Nambiar and Lucas, 2014).

When SCI occurs, voluntary control of micturition is interrupted due to interruption of the inhibitory pathway (composed by GABAergic neurons) and to the sacral spinal reflex (Andersson and Pehrson, 2003), generating urgency to void and urinary incontinence. When injected in the bladder, BTX-A injection blocks acetylcholine release from parasympathetic fibers (Coelho et al., 2016b), contributing to the inhibition of the detrusor muscle contraction (Jhang and Kuo, 2018),

thereby facilitating urine storage and reducing periods of high intravesical pressure. Other studies revealed that BTX-A injection could reduce CGRP and ATP expression with improvement in sensory symptoms (Moore et al., 2016). In fact, intrathecal administration of the toxin reduces bladder activity and amplitude by cleavage of SNAP-25, decrease in CGRP expression and impairment of several cellular processes (Coelho et al., 2016b).

II. Research Goals

Research Goals

Chronic pain and NDO are major issues for SCI patients. While options for NDO treatment have been developed and are in current use with good long-term results, there is a generalized lack of new treatments for pain. Ideally, a new effective treatment would simultaneously reduce pain and improve bladder function. Recent studies suggest that intrathecal administration of BTX-A can be used to alleviate pain and, at the same time, improve bladder reflex activity (Coelho et al., 2014; Coelho et al., 2016b). Thus, in this study we chose the incomplete spinal cord transection model, which has a stronger translational value than the complete transection model, to test if intrathecal administration of BTX-A would reduce pain and NDO. The main goals of the present thesis were:

- To establish and characterize a model of incomplete spinal cord injury in terms of pain development and urinary dysfunction;

- To investigate if the intrathecal administration (4 weeks after the surgery) of BTX-A in incomplete SCI animals could attenuate pain and improve bladder function;

III. Material & Methods

Material & Methods

Animals

All experiments were performed in Female Wistar-Han rats weighing 200-250g (obtained from the vivarium of the Faculty of Medicine of Porto and originally derived from the Charles River strain). Animals were maintained under a 12h light/dark schedule and controlled temperature and air humidity, with *ad libitum* access to food and water. Animals were housed in groups of three per cage. All efforts were made to reduce the number of animals used and their suffering and the ethical guidelines for investigation of experimental pain in animals (Zimmermann, 1983) and the European Commission Directive of 22 September 2010 (2010/63/EU) were carefully followed in all procedures included in this study. The study has been approved by the local authorities and internal regulations of the Faculty of Medicine of Porto were carefully followed in all procedures included in this study.

All animals were subjected to a habituation period of two weeks prior to any experimental procedure. During this period, rats were handled five times a week and habituated to the room and to the experimenter to avoid any stress and fear motivated behaviours. During the last week of handling, animals were also habituated to the devices used in each behavioural test (Von Frey test, hot/cold plate test, burrowing test). Behavioural tests were performed twice a week during the experimental period. All behavioural tests were performed by an experimenter blinded to the treatment groups (Figure 7).

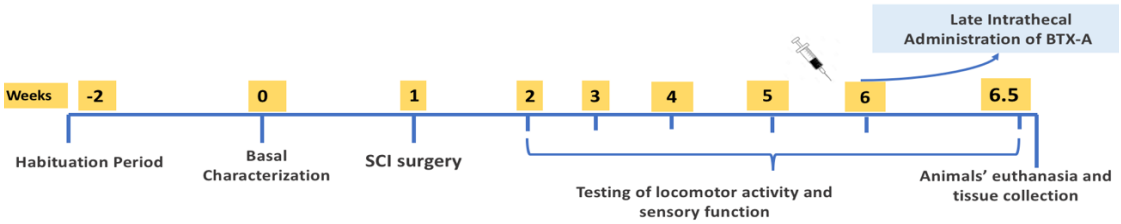


Figure 7: **Experimental plan, depicting the number of weeks the animals were followed and major interventions.**

Surgical spinal lesion and placement of intrathecal catheter

After the habituation period (Figure 7), animals were anaesthetized intraperitoneally with a mixture of ketamine (60 mg/Kg) and medetomidine (0.25mg/kg). A linear incision was made in the dorsal area of the animal. A laminectomy was performed at T8-T9 level. Paravertebral muscles and fascia from T7 to T10 were partially removed to expose the lamina of the vertebral arch. The meninges were pierced to allow the insertion of a sterile silicone catheter, according to previous studies (Cruz et al., 2005; Cruz et al., 2008; Coelho et al., 2014; Coelho et al., 2016b). One tip of the catheter was pushed until L5-L6 level whereas the other tip was placed subcutaneously close to the neck and externalized for later drug administration. The spinal cord was then partially sectioned. A 2-mm cut was performed at T8-T9 level in the dorsal part of the spinal cord to avoid performing a complete transection. Analgesics (Tramadol, 0.4 mg/kg) were given to the animals for 24 hours after surgery while antibiotics (Enrofloxacin, 1 mg/kg) daily for 2 weeks to avoid urinary infections due to vesical dysfunction. During this 2 week-period, bladders were emptied manually by abdominal compression twice a day. Control animals were submitted to a similar procedure but without cord sectioning (sham surgery).

Behavioural assessment

Cutaneous mechanical sensitivity

The Von Frey test was used to assess cutaneous sensitivity at baseline, following spinal injury and after intrathecal administration of botulinum toxin A. Animals were placed in individual chambers (23x17x14 cm) on top of a wire mesh floor and allowed to acclimatize for 15 min or until cage exploration stopped (Frias et al., 2013) . Cutaneous sensitivity was performed in the hind paws using Von Frey monofilaments (rated at 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 15.0, 26.0 and 60.0 g). Monofilaments were applied perpendicularly to the plantar surface in the left hind paw and tested five times. The response was considered positive if there was visible paw withdrawal in three out of the five times. The test was executed at day 0 before any procedure

and after spinal cord injury, twice a week during the phase of behavioural testing (5 weeks). Since SCI animals develop motor impairment after the surgery, the response was also considered positive if the animals tried to escape from the application of the filament.

Cutaneous thermal sensitivity

Thermal sensitivity to hot and cold temperatures at hind paws was assessed with the hot/cold plate (Bioseb, Chaville, France). For that, animals were placed in a transparent plastic box (22x28). Five minutes after placement or after cage exploration stopped, the temperature of the plate was increased from 32°C to 52°C (heat sensitivity) or decreased from 22°C to 0° C (cold sensitivity). In both cases, the temperature changed in a similar rate (5ºC/min), controlled by a dedicated computer. The temperature, in the hot or cold range, at which the animal showed the following behaviours was recorded (Thibault et al., 2011):

Awareness: The animals stopped exploring or grooming and turned his head towards his hind paws. This behaviour was observed for temperatures between 13-18°C for cold temperatures and between 36-41°C for hot temperatures and interpreted as an awareness of changes in cold or heat perception.

Discomfort: The animal stopped moving and started placing his body weight on his toes, rather than on the full surface of his hind paws. This behaviour was considered as signal of discomfort but not yet as a painful response. This was observed at 6.5-13 °C for cold temperatures and between 41-46 °C in case of hot temperatures. In SCI animals, with motor impairment, discomfort was also considered when the forepaw frequency of steps increased significantly.

Nociception: The animal exhibits one of the following: withdrawing, licking, shacking one of his hind paws, jumping or animals' vocalization. This behaviour was noticed at 1-8°C for cold temperatures and in case of hot temperatures at 45-49 °C. For SCI animals, with motor impairment, signs of nociception also included forepaw licking.

Spontaneous behaviour

To assess spontaneous signs of anxiety and non-induced pain behaviour, animals were submitted to the burrowing test. Animals were placed individually in cages with an empty plastic tube with 32 cm length and 10 cm in diameter. One end of the tube was elevated 10 cm above the cage floor and left open. The other end was closed with a plastic lid. To avoid celling effects, (e.g. both groups burrow all the gravel outside the tube and no group difference can be seen), 2-4 mm burrow gravel-like substract was used. For habituation, rats were placed in empty cages with burrowing tubes filled with 1.5kg of gravel. On days 1 and 2, animals were placed in pairs for social facilitation. On days 3, 4 and 5 animals were placed alone. For testing, animals were placed alone in each cage-burrow setup for 30 minutes. After burrowing, the amount of substract left in tube was weighed (Rutten et al., 2014; Bryden et al., 2015) .

Locomotor assessment

To evaluate the motor activity during the experimental period, the catwalk gait analysis was performed. For that, animals were placed on top of a glass platform equipped with a LED light placed underneath the platform. This setup was located in a dark compartment. Animals were allowed to walk freely while a bright image with the points of contact between the paw and surface was obtained. Images were captured by a video camera placed under the platform. This camera was connected to a computer equipped with video acquisition software (Ulead Video Studio, Freemont, CA). Signal intensity was analysed after conclusion of behavioural testing and it depended on the area of the paw in contact with the platform and paw pressure.

Intrathecal BTX-A administration

Four weeks after SCI, the catheter tip placed close to the neck either from the SCI and sham animals was externalized and saline (vehicle solution, final volume of 50 µL) or BTX-A (5U diluted in 50 µL of saline) according to previous studies (Coelho et al., 2014; Coelho et al., 2016b) was delivered under isoflurane anaesthesia (5% for induction, 2% for maintenance). Animals were allowed to recover after the injection for a period of two days and then submitted to behavioural assessment.

Cystometries

When all behavioural tests had been concluded following intrathecal saline or BTX-A administration, animals were deeply anaesthetized with urethane (1,2 g/Kg, subcutaneous injection) and maintained at 37ºC using a heating pad. A low abdominal middle incision was executed to expose the bladder. In order to evaluate bladder reflex activity, a 21-gauge needle connected to an infusion pump and to a pressure transducer was inserted in the bladder dome (Coelho et al., 2016a). A continuous infusion of saline was started at a physiological rate of 6 ml/h 15-30 minutes after needle insertion. Intraluminal pressure was recorded for 1h. Cystometrograms obtained were analysed using the LabScribe software (World Precision Instruments, Hertfordshire, UK).

Tissue collection and processing

After cystometry, animals were terminally sedated and euthanised. The lesion site (T8-T9 level) and the spinal cord segments L5 to S1 were collected. While the spinal lesion site was immersion-fixed in cold paraformaldehyde 4% overnight and stored in 30% sucrose (in 0.1 M phosphate buffer), the lumbosacral segments were immediately frozen at -80ºC and stored until further processing.

To detect morphologic changes of tissue in the lesion site, serial longitudinal sections of the lesion site were cut in a cryostat (12 µm), collected on Superfrost slides and stored at -20ºC. When all tissue had been collected, slides were thawed and sections of lesion site (T8-T9 level) were stained with formol-thionin (Donovick, 1974). Sections were afterwards washed and mounted with Histomount mounting medium. Representative images were collected in a Zeiss Axioscope 40 microscope using Leica LAS EZ v3.1.0 software (Leica Microsystems, Switzerland). For immunofluorescence, slides with sections of the lesion site were thawed, washed with phosphate buffer containing 0.3%Triton X-100 (PBST) and blocked with 10% normal horse serum (NHS) in PBST for 1h. Primary antibody raised in rabbit (anti-5-HT 1:10000, Immunostar) was diluted in 2% NHS in PBST and incubated for 2 days at 4ºC. After several washes with PBST, the immunoreactivity was detected using a species specific secondary antibody Alexa Flour® 568 Goat anti-rabbit Ig (H+L) (1:1000; Life Technologies). Slides were mounted using Slowfade® Gold antifade (Life Technologies). Representative images were collected in a Zeiss microscope (Axioimager Z1, Zeiss Z1 from Zeiss,) using the AxioVision 4.6 software. Representative images were taken.

Western Blot analysis

Spinal cord L5-S1 segments were homogenized in lysis buffer (20 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, pH 7.4 and proteinase inhibitors). Homogenates were sonicated in a water bath at 4ºC for 10 minutes and subsequently centrifuged at 14000 rpm for 15 minutes at 4ºC. The supernatant was removed and used for Western blot analysis. The protein concentration was measured using the Bradford dye assay (Bio-rad Laboratories). The extracted protein was mixed with loading buffer and boiled at 65°C for 30 minutes, followed by electrophoresis in a 12% polyacrylamide gel (30 µg/well) at 50 V. The separated protein was transferred into a polyvinylidene fluoride (PVDF) membrane at 60 mA for 90 minutes. Subsequently, the membrane was blocked using 5%milk for 1 hour, followed by incubation with correspond

primary antibodies. The membranes were incubated at 4ºC overnight with a primary antibody specific for TRPV1 (rabbit polyclonal anti-TRPV1 antibody, 1:1000, Alomone), GFAP (mouse monoclonal anti-GFAP antibody, 1:2000, Cell Signalling), SNAP25 (rabbit polyclonal anti-SNAP25 antibody, 1:2000, Synaptic Systems) or GAPDH (mouse monoclonal anti-GAPDH, 1:10000, Abcam). Afterwards, membranes were incubated for 1h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (1:10000, Abcam). Bands were visualized with enhanced chemiluminescence. The positive pixel area of specific bands was measured using ImageJ software (ImageJ 1.51s; National Institutes of Health, USA) and normalized against the corresponding GAPDH loading control bands.

Statistical analysis

Data generated by behavioural tests was analysed using the two-way analysis of variance (ANOVA) with Tukey's multiple comparison tests using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data was presented as mean ± SD, and $p \leq 0.05$ was considered statistically significant.

To access statistical difference between pre- and post-intrathecal BTX-A injection, statistical analysis was performed using the t-test, following Wilcoxon matched-pairs signed rank test using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data was presented as mean± SD, and $p \leq 0.05$ was considered statistically significant.

In what concerns data from Western Blot assays, statistical analysis was performed by running one-way ANOVA. Kruskal-Wallis test followed by Dunns' post hoc test analysis using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data was presented as mean± SD, and $p \leq 0.05$ was considered statistically significant.

IV. Results

Results

Incomplete SCI model characterization

Behaviour analysis: Cutaneous sensitivity to mechanical stimuli

Before SCI surgery, the paw withdrawal threshold (PWT) of SCI animals was 23.7±2.9g, not significantly different from naïve (non-manipulated) and sham animals (submitted to sham surgery) (28.8±6.1g and 28.9±4.6g, respectively). In naïve animals, PWT did not change during the experimental period (4 weeks) (Figure 8).

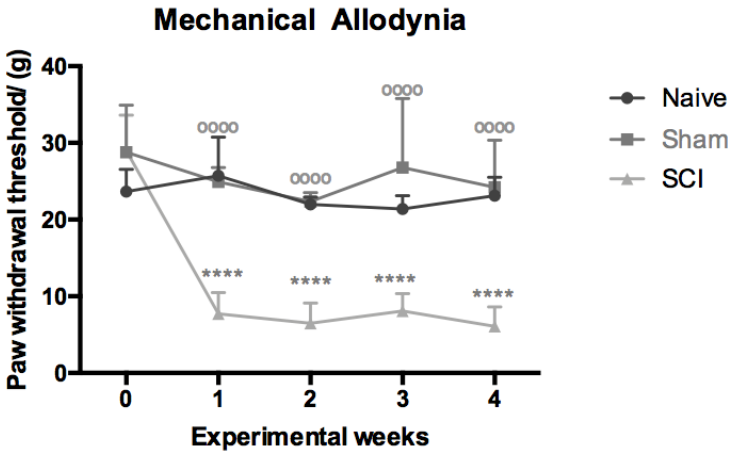


Figure 8: **Changes in cutaneous sensibility to mechanical stimulation of both hind paws of naïve, sham and SCI rats.**

Paw withdrawal threshold decreased considerably one week after SCI surgery. The threshold remained lower than that observed in naïve and sham animals. Results are presented as mean ± SD (n=4 in naïve group and n=8 in SCI and sham groups, respectively) Repeated measures two-way ANOVA, followed by Tukey's post hoc t-test analysis. 'SCI vs naïve' ****p<0.0001; 'SCI vs Sham' ººººp<0.0001.

In contrast, one week after surgery, sham and SCI animals presented a decrease in PWT, much more exacerbated in SCI animals. While the PWT increased in sham animals as animals recovered from surgical manipulation, the PWT of SCI animals remained low until the end of the experimental period. Indeed, the PWT of SCI animals was 7.7±2.78g 1 week after surgery and further decreased, reaching its lowest value 4 weeks post- SCI (6.07±2.5g). At that time point, the PWT of naïve and sham animals respectively was, respectively, 23.1±2.4g and 24.2±6.2g ($p<0.0001$ versus SCI).

Behaviour analysis: Cutaneous sensitivity to thermal stimuli

Sensitivity to thermal stimuli was also assessed, both in the hot and cold range. Three responses were analysed (awareness, discomfort and nociception). At baseline (before spinal lesion), naïve animals (37.5±0.8 °C) showed signs of awareness at a lower temperature than sham and SCI rats (respectively 40.6±1.7 °C and 40.3±1.1 °C; $p<0.01$) possibly due to stress. During the remainder of testing, awareness signs became similar between animals, except at the two weeks post-SCI, when SCI animals presented awareness signs at a significantly lower temperature (36.7±2.0 °C) when compared to naïve and sham animals (39.7±1.3 °C and 38.9±1.0 °C, respectively) (Figure 9A). At the same time point, SCI animals also presented significantly heightened discomfort and nociceptive responses at lower temperatures. Indeed, discomfort (Figure 9B) occurred at 40.9±2.4 °C and nociceptive responses (Figure 9C) were observed at 45.8±1.3 °C in SCI animals. In contrast, sham and naïve animals discomfort signs were respectively observed at 45.4±1.5 °C and 44.8±1.4 °C. In the same experimental groups, nociceptive responses were observed at 48.2±0.7 °C and 47.9±0.6 °C, respectively.

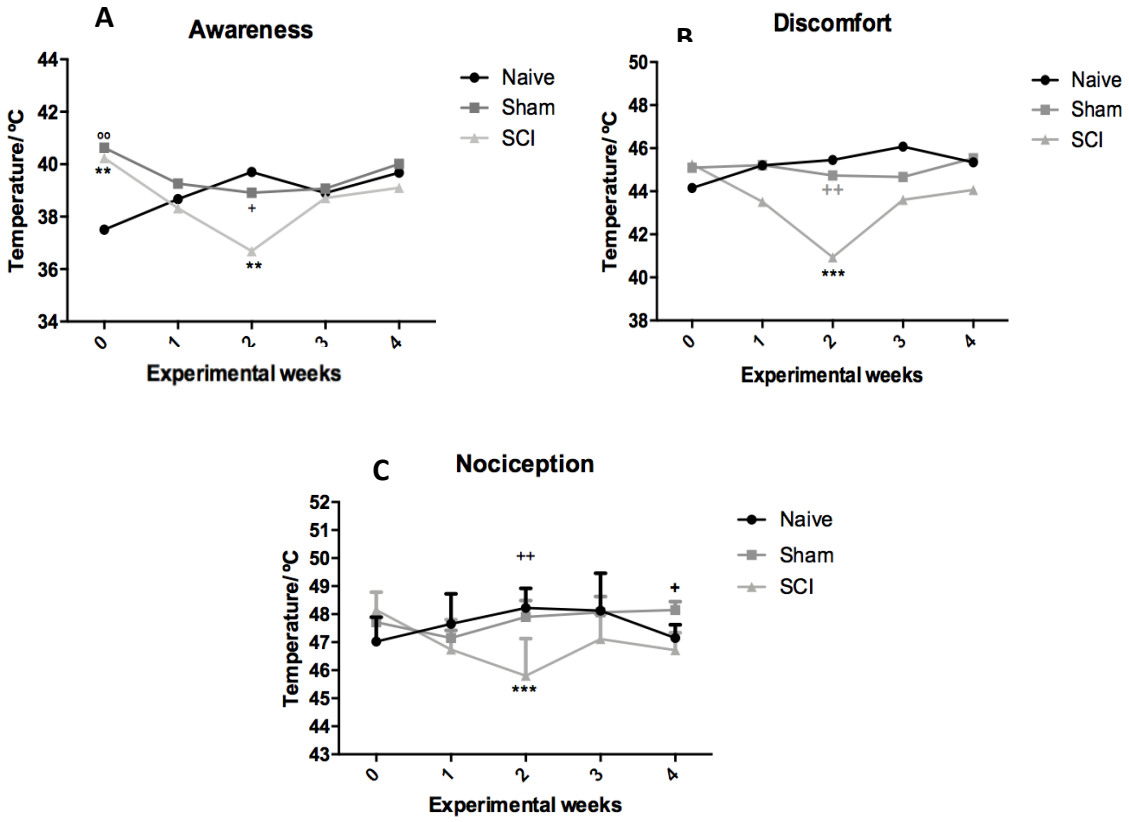


Figure 9: **Changes in cutaneous sensitivity to heat stimulus (Hot Plate test).**

Awareness, discomfort and nociceptive responses recorded during the hot plate tests (temperatures increasing from 32ºC to 52ºC at 5ºC/min). During the 4 weeks of testing, no changes were observed in the awareness (A), discomfort (B) and nociceptive (C) responses of naïve and sham animals to temperature increasing. In contrast, SCI animals showed heightened responses at lower temperatures in all parameters analyzed (A, B and C) two weeks after spinal lesion. Results are presented as mean ± SD (n=4 in naïve group and n=8 in SCI and sham groups); Repeated measures two-way ANOVA followed by Tukey's post hoc t-test analysis. **p<0.01 'SCI vs naïve'; ***p<0.0001 'SCI vs naïve'; ººp<0.01 'Sham vs naive', $^{++}$p≤0.01 'SCI vs Sham'.

Cutaneous sensitivity to thermal stimuli in cold range was evaluated using the cold-plate test. No statistical differences were found between groups in what respects the temperatures at which awareness responses were observed (Figure 10A). However, SCI animals displayed heightened discomfort (Figure 10B) and nociceptive responses (Figure 10C) in comparison with naïve and sham animals. Thus, two weeks after spinal lesion SCI animals showed discomfort at

10.9±2.28 °C and nociceptive responses at 5.7±1.7 °C. These values are significantly higher than those observed in naïve (7.3±1.6 °C and 2.3±2.6 °C, for discomfort and nociceptive responses respectively) and sham control rats (7.8±1.2 °C and 3.1±0.6 °C, respectively for discomfort and nociceptive behaviour). While there was some improvement in signs of discomfort, nociceptive responses of SCI animals remained present at higher temperatures throughout the entire testing period.

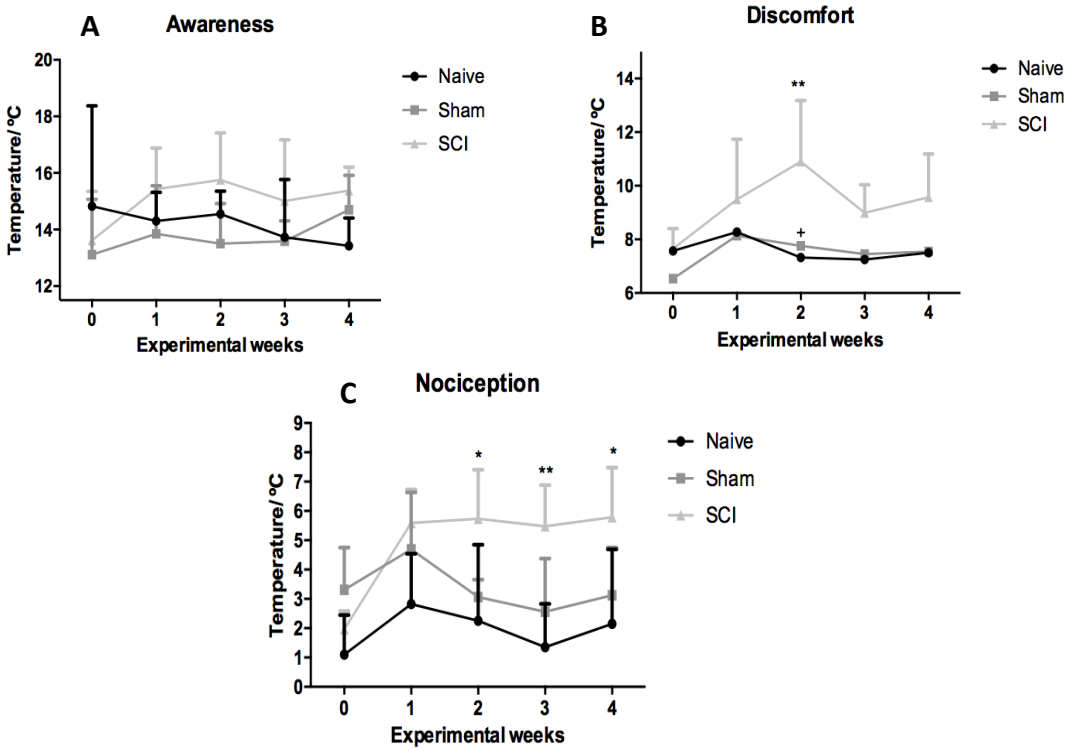


Figure 10: **Changes in cutaneous sensitivity to cold stimulus (Cold Plate test).**

Thresholds of awareness, discomfort and nociceptive reactions were recorded during the cold plate test (temperatures decreasing from 22ºC to 0ºC at 5ºC/min). During the 4 weeks of testing, no significant changes were observed in the awareness (A), discomfort (B) and nociceptive (C) responses of naïve and sham animals to temperature decreasing. In contrast, SCI animals showed signs of discomfort and nociception at higher temperatures than naïve and sham animals (B, C) two weeks after lesion. Results are presented as mean ± SD (n=4 in naïve group and n=8 in SCI and sham groups,). Repeated measures two-way ANOVA, followed by Tukey's post hoc t-test analysis. *p<0.05 'SCI vs naïve'; **p<0.01 'SCI vs naïve'; $^{+}$p<0.05 'SCI vs Sham'

Assessment of spontaneous behaviour

To assess spontaneous behaviour and correlate with mechanical and thermal stimulus-induced responses, animals were submitted to the burrowing test. As naïve animals did not burrow a minimum of 500 g, they were excluded from analysis (Deacon, 2006). Thus, while at baseline, sham-manipulated and SCI animals removed similar amounts of gravel from the cylinder (1080±299.0g and 841.0±317.0g, respectively), this value was markedly reduced for SCI animals at week 1 post-lesion (992.5±421.3g and 140.4±219.9g respectively). The amount of sand moved by SCI animals remained at similar values following SCI surgery until the forth experimental week (260.0±244.2g in week 2, 286.6±346.7g in week 3 and 233.5±256.3g in week 4) (Figure 11).

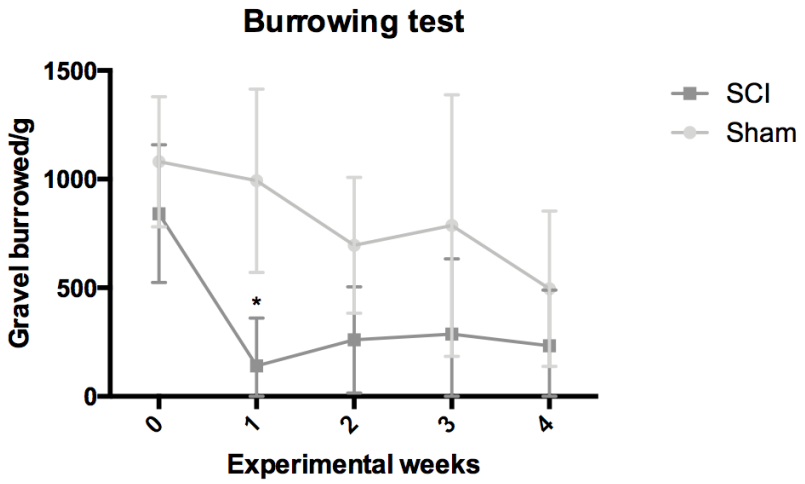
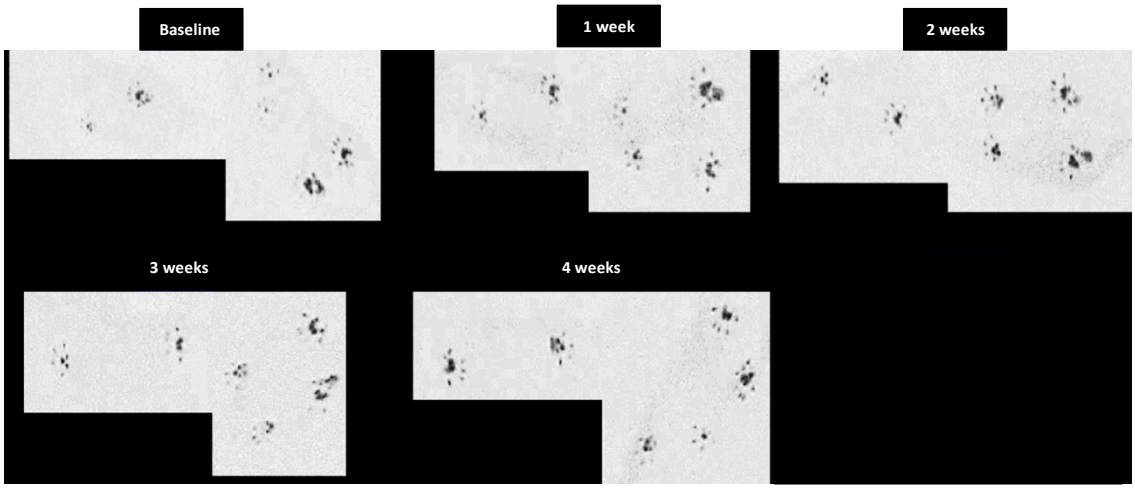


Figure 11: **Gravel burrowed by sham and SCI animals**.
Sham animals removed more gravel from the cylinder than SCI animals, at all time points. N=4 per group; Shapiro-Wilk normality test (alpha=0.05) followed by a t-test. Nonparametric test: Mann-Whitney test matched-unpairs signed rank test. Statistical difference between 'SCI vs Sham' *p<0.05;

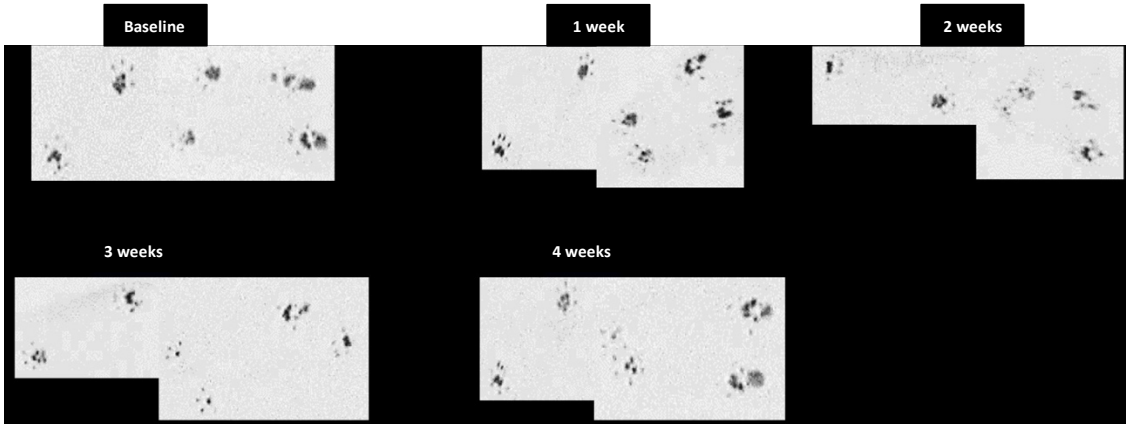
Assessment of Locomotor Function

Evaluation of locomotor activity was also performed during the experimental period to assess the animals' recovery following surgery. Lesions in the spinal cord at thoracic level lead to paralysis of both hind paws immediately after surgery. All experimental groups displayed normal pattern of footprints before the surgery (Figure 12). One week after the surgery, sham animals showed similar footprint pattern as observed at baseline Figure 12B). In contrast, SCI animals developed motor dysfunction after the surgery as observable in Figure 12C. Two weeks following SCI surgery, SCI animals revelead partial motor recovery which was maintained for two weeks onwards. Naïve and sham animals showed standard locomotor function throughout the entire experimental period (Figure 12A-12B).

A Naïve



B Sham



C SCI

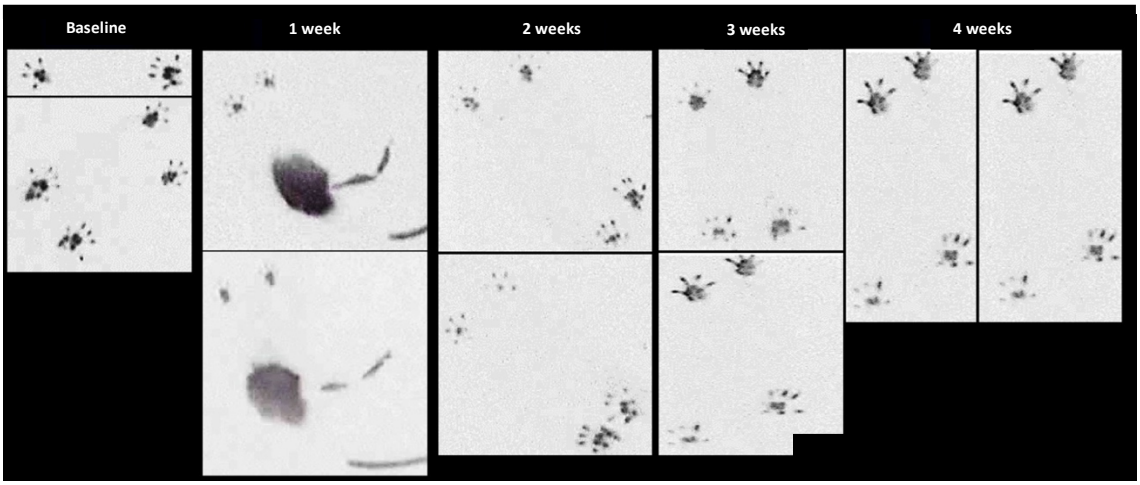


Figure 12: **SCI animals recovered partial motor function two weeks after surgery.**

Representative images of Catwalk gait analysis. (A, B) Naïve and sham animals showed normal motor function throughout the four weeks. (C) SCI animals development motor impairment one week following SCI and recovered partial locomotor two weeks after surgery.

Bladder Function

Five weeks after the surgery, cystometries were performed under urethane anaesthesia to evaluate bladder function. Frequency of bladder contractions in naïve animals was 0.5±0.1 contractions/minute with an amplitude of 12.6±5.9 cm H_2O. These values were similar to those observed in sham manipulated rats. In this case, the frequency was 1.4±0.8 contractions/minute and the amplitude 19.3±13.8 cm H_2O (Figures 13). Following spinal lesion, frequency (1.9±0.8 contractions/minute) and amplitude of bladder reflex contractions (36.5±8.6 cm H_2O) ($p<0.05$) in SCI rats was increased when compared to naïve animals.

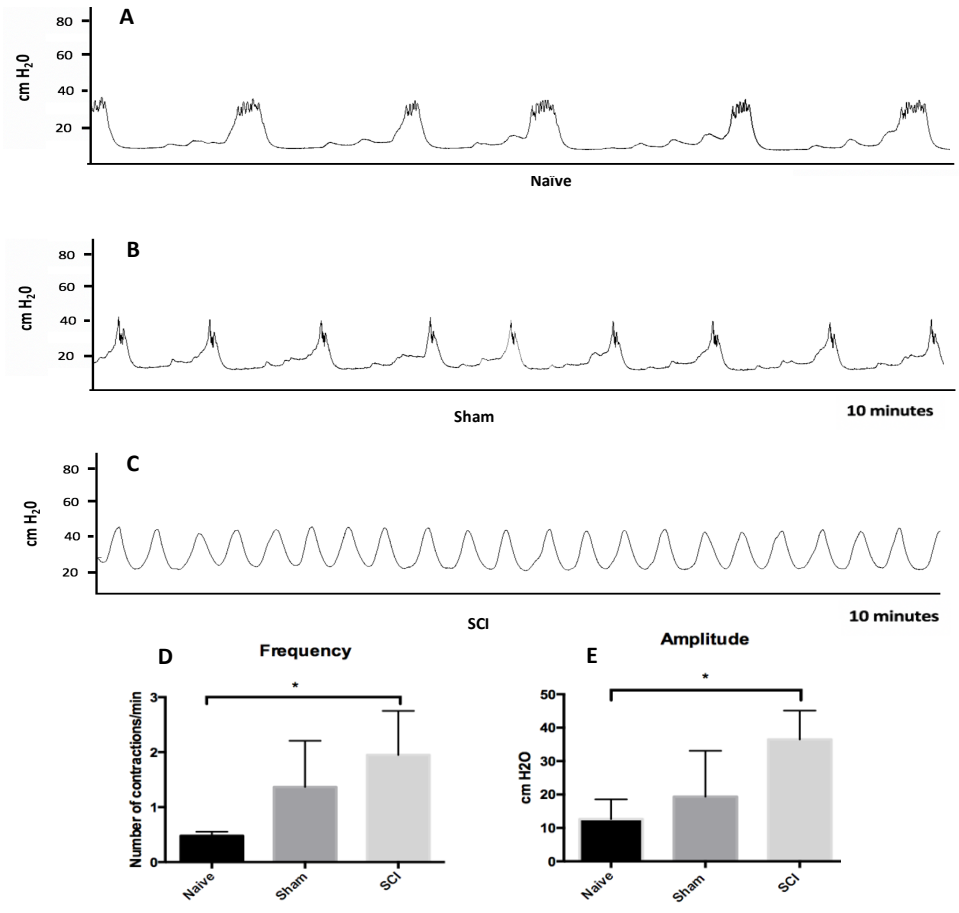


Figure 13: **Representative cystometrograms of naïve, sham and SCI animals.**

(A) In naïve animals, frequency and amplitude of bladder contractions was below 1 contraction per minute and lower than 20 cm H_2O, respectively. (C) In contrast, the amplitude and frequency of bladder contractions of SCI animals were highly increased. **Graph of bars depicting the frequency (D) and amplitude (E) of bladder contractions of naïve (black bars), sham (dark grey bars) and SCI (grey bars).**

Results are expressed as mean± SD (n=3-4). Only contractions above 5 cm H2O were considered. Kruskal-Wallis nonparametric test followed by Dunn's post hoc test analysis**.** 'SCI vs naïve animals' *p< 0.05

SCI leads to morphological changes

In cases of incomplete spinal cord injury, axons located away from the lesion site do not degenerate and remain preserved. Growth ceases and Wallerian degeneration occurs when axons meet the inhibitory environment of the glial scar. To confirm the degree of injury, a serotonin immunofluorescence was performed (Figure 14).

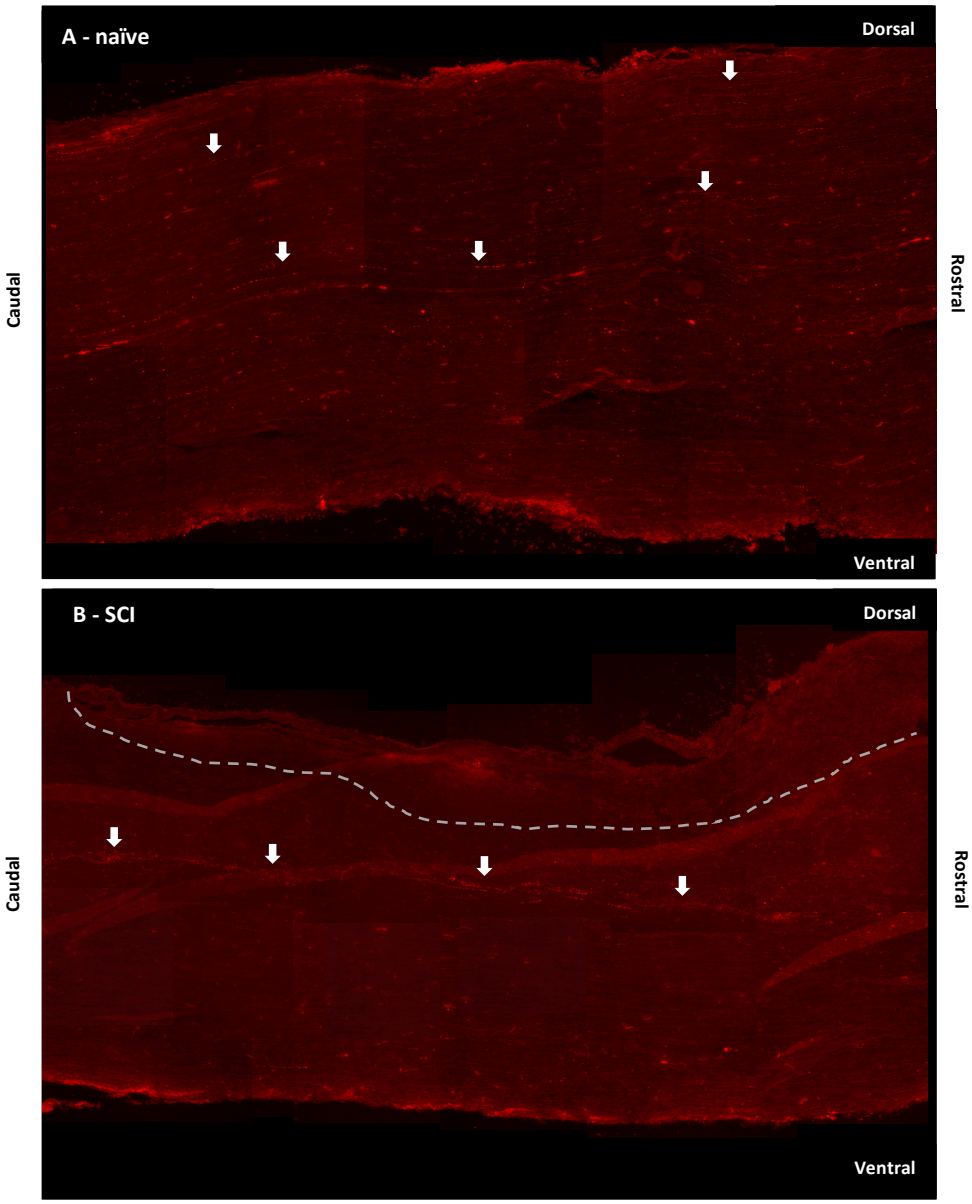


Figure 14: **Representative images of experimental spinal cord injury.**
(A) Several serotonergic projections (white arrows) in naïve animals**. (B)** Absence of serotonergic inputs located next to the lesion site. Below the lesion epicentre (located above grey dotted lines) serotonergic projections are connecting nucleus located rostrally and caudally to the injury site (white arrows). Horizontal section through the lesion site are oriented caudal to rostral from left to right and oriented dorsal to ventral from up and down.

In naïve animals, serotonin projections (pointed by white arrows), known to be of supraspinal origin, are present as continuous immunoreactive fibres coursing the entire length of the cord (Figure 14A). In SCI animals (Figure 14B), serotonergic projections are absent in the lesion epicentre (limited by the slashed line). Below the injury site, serotonergic fibres were present and continuous.

Morphological changes were also assessed with a formol-thionin staining. After lesion, spinal cord tissue is disorganised, and white matter is spared. Inflammatory cell infiltrates are present close to the injury site (blue staining) (Figure 15).

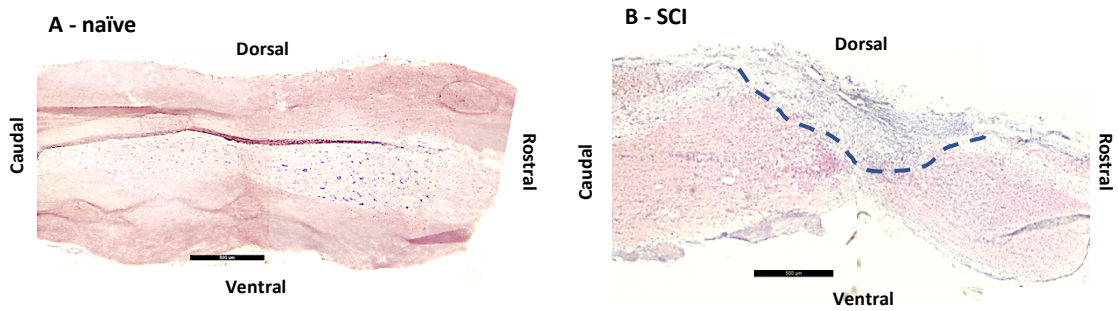


Figure 15**: Representative images of experimental incomplete spinal cord injury.**
Terminal lesion histology shows the lesion site surrounded by inflammatory cell infiltrates (between blue dotted lines). Horizontal section through the lesion site are oriented caudal to rostral from left to right and oriented dorsal to ventral from up and down. Scale bar is 500 μm.

Effects of intrathecal delivery of Botulinum Toxin A

BTX-A intrathecal injection improved cutaneous sensitivity to mechanical stimuli in SCI animals

Response to mechanical stimulation was assessed using the Von Frey test 3 days post-injection of either vehicle or neurotoxin to sham and SCI rats. In sham animals, PWT was not changed by administration of either vehicle or BTX-A. Thus, before injection PWT was 28.6±9.0g for those which received vehicle solution and 20.6±10.3g for those with BTX-A solution delivery. After intrathecal injection of saline and toxin solution, the values respectively were 23.1±6.2g and 18.4±14.1g.

In SCI animals, the PWT was 4.8±3.1g and 6.1±3.9g before intrathecal delivery either of vehicle or BTX-A solution. While intrathecal administration of saline solution (vehicle) to SCI rats did not produce any effect (3.6±2.3g), injection of BTX-A significantly increased PWT to 13.6±7.6g (Figure 16).

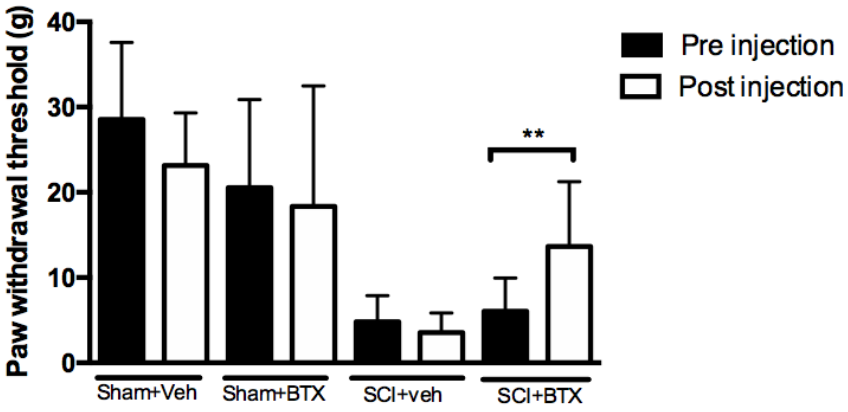


Figure 16: **Intrathecal BTX-A injection improved cutaneous sensitivity to mechanical stimuli in SCI animals.**

While PWT was not changed in sham animals following intrathecal administration of saline (vehicle) or BTX-A, signs of improvement of cutaneous sensitivity were found in SCI animals that received intrathecal BTX-A. Results are presented as mean ± SD (n=4 in all experimental groups); Shapiro-Wilk normality test (alpha=0.05) followed by paired t-test: Statistical difference between **p<0.01 'SCI+BTX-A pre-injection vs SCI+BTX-A post-injection'.

BTX-A intrathecal injection does not affect thermal sensitivity

Thermal sensitivity was also assessed by performing the hot plate and cold plate tests, 4 and 5 days after intrathecal injection of BTX-A, respectively. No changes were found between groups, with awareness, discomfort and nociceptive responses remaining similar before and post-injection of either saline or neurotoxin solution (Figure 17 and 18).

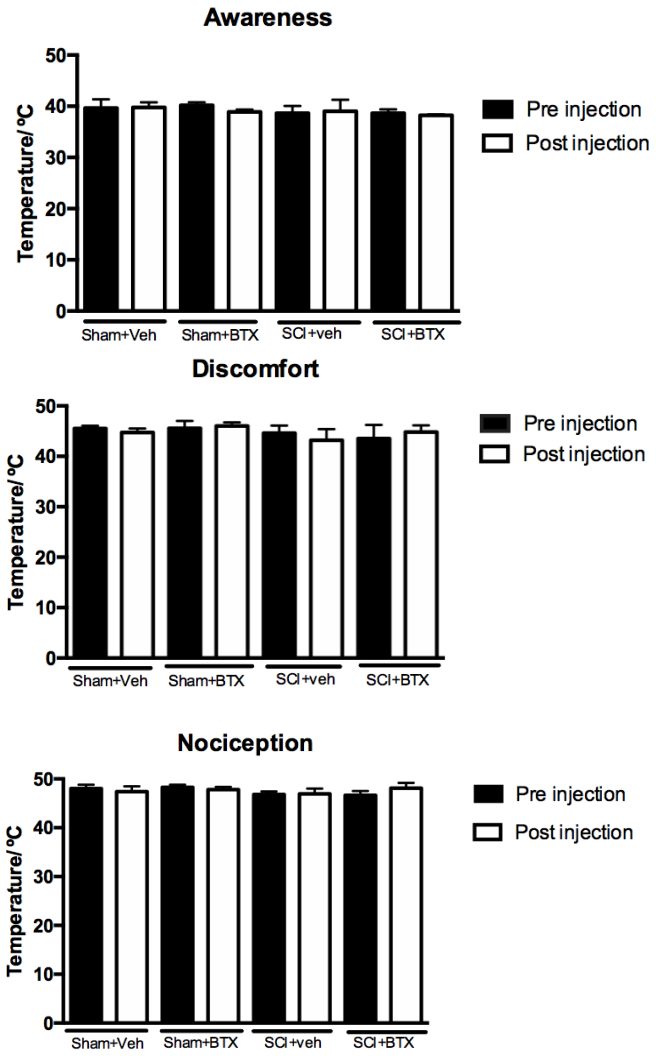


Figure 17: **Intrathecal BTX-A injection does not affect heat sensitivity.**
Signs of improvement of heat sensitivity were not found either in sham or SCI animals that received intrathecal vehicle or BTX-A solution in awareness, discomfort and nociceptive responses. Results are presented as mean ± SD (n=4 in all experimental groups); Shapiro-Wilk normality test (alpha=0.05) followed by t Nonparametric test: Wilcoxon matched-pairs signed rank test.

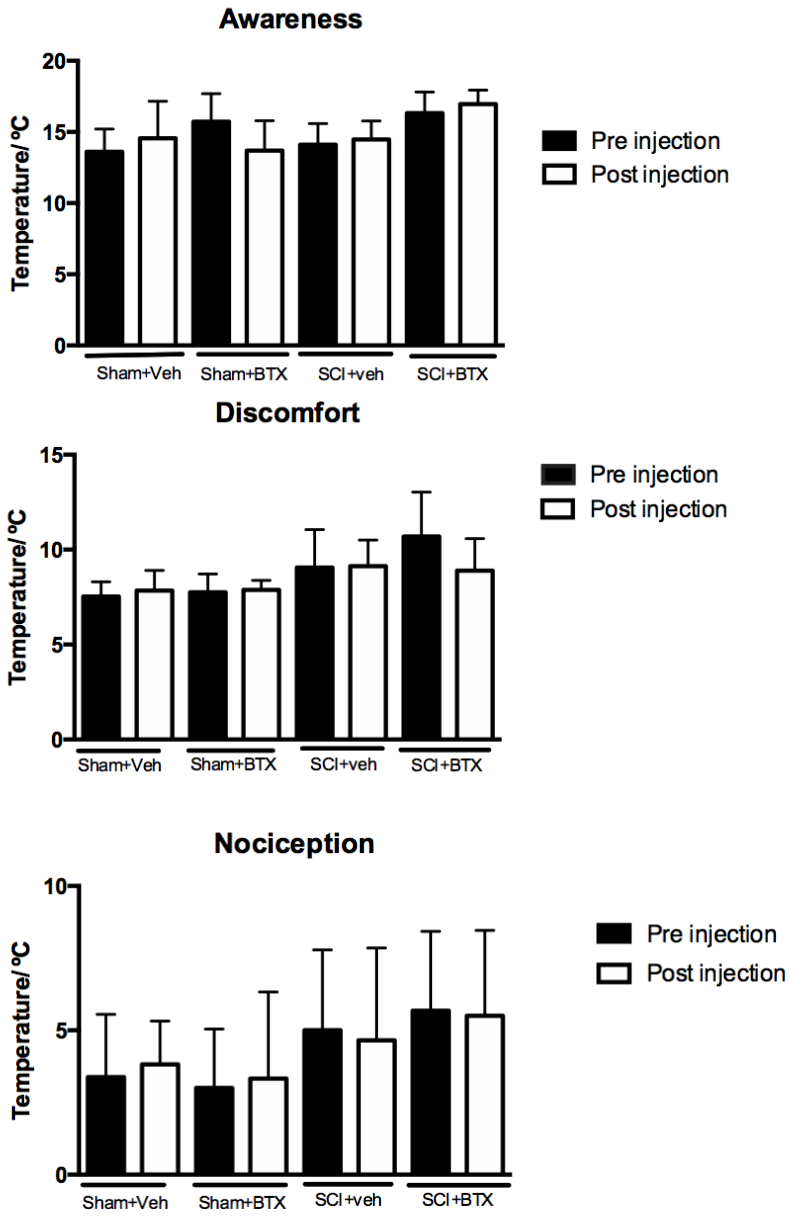


Figure 18: **Intrathecal BTX-A injection does not affect cold sensitivity.**

Signs of improvement of cold sensitivity were not found either in sham or SCI animals that received intrathecal vehicle or BTX-A solution in awareness, discomfort and nociception responses. Results are presented as mean ± SD (n=4 in all experimental groups); Shapiro-Wilk normality test (alpha=0.05) followed by t Nonparametric test: Wilcoxon matched-pairs signed rank test.

BTX-A intrathecal injection does not affect burrowing behaviour

Following BTX-A injection, spontaneous burrowing behaviour was also assessed. Like with thermal sensitivity, intrathecal administration of either saline or BTX-A did not produce any significant effect on the amount of gravel moved by sham and SCI animals (Figure 19).

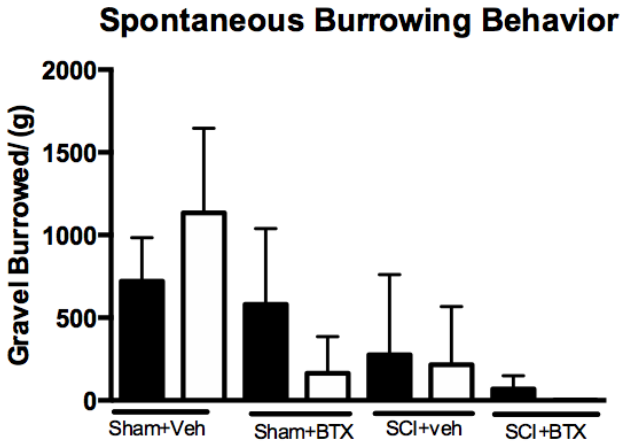
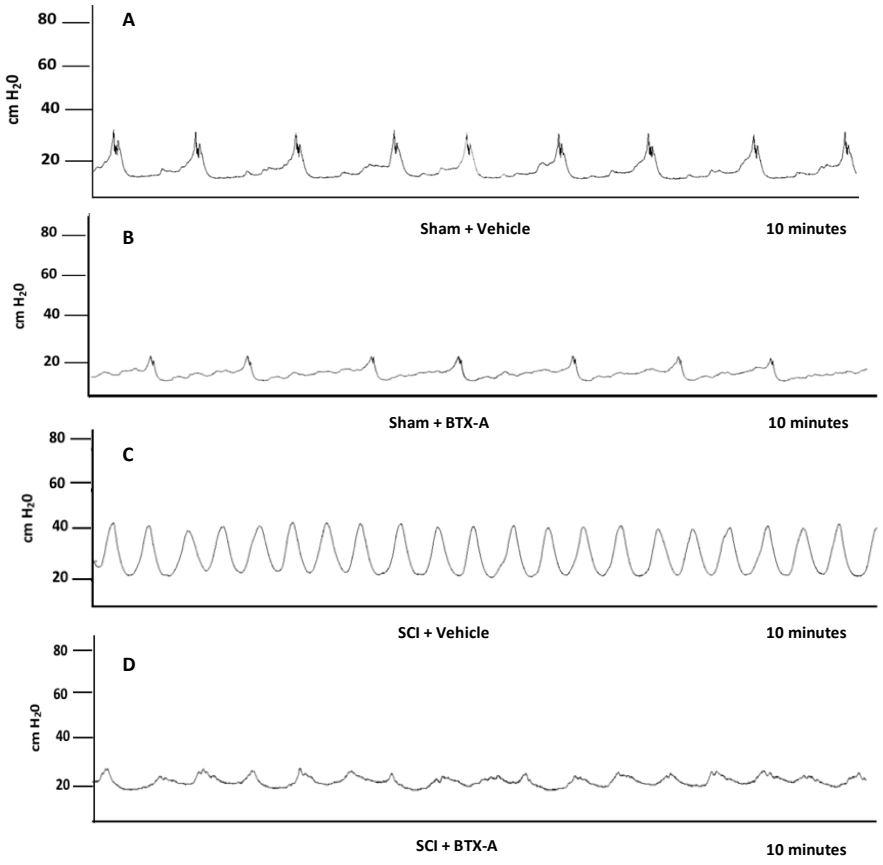


Figure 19: **Intrathecal BTX-A injection does not affect burrowing behaviour.**

The amount of gravel removed from the cylinder by sham or SCI was not changed following intrathecal injection of either saline or BTX-A. Results are presented as mean ± SD (N= from 2 to 5); Shapiro-Wilk normality test (alpha=0.05) followed by t-test analysis. Nonparametric test: Wilcoxon matched-pairs signed rank test.

BTX-A intrathecal injection reduces bladder amplitude

Five weeks after the surgery and 7 days after intrathecal administration of vehicle (saline) or BTX-A solution, cystometries were performed to evaluate the bladder function. In both sham and SCI animals, bladder function was affected by the neurotoxin. Indeed, in sham animals receiving saline, the frequency of bladder contractions was 1.4±0.8 contractions/minute with an amplitude of 19.34±13.75 cm H_2O (Figure 20A, 20E). In sham animals receiving BTX-A, the frequency recorded was 1.4±0.7 contractions/minute and the amplitude was 17.09±6.10 cm H_2O (Figure 20B, 20E). In SCI animals that had been treated with intrathecal saline, the frequency of bladder reflex contractions was 1.7±0.8 contractions/minute with an average amplitude of 36.49±8.63 cm H_2O (Figure 20C, 20E). In SCI animals receiving intrathecal BTX-A, the frequency was 1.3±0.4 contractions/minute and the amplitude of bladder reflex contractions was 14.55±7.28 cm H_2O (Figure 20D, 20E). Thus, as observed, intrathecal injection of BTX-A reduced the amplitude of bladder contractions in SCI animals improving bladder function (Figure 20F).



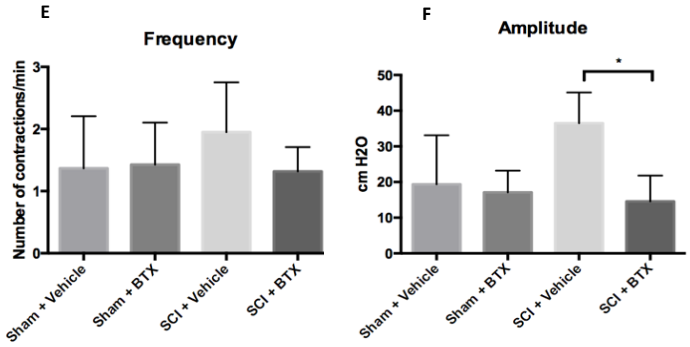


Figure 20: **Representative cystometrograms of sham and SCI animals either receiving intrathecal BTX-A or vehicle (saline) injection.**

(A,B) Sham animals which received vehicle solution (A) show similar number of contractions per minute comparing to sham animals that received BTX-A injection (B). (C) SCI animals show higher frequency of contractions and an increased contractions amplitude. (D) SCI animals which received the injection present lower bladder contractions amplitude. **Graph of bars depicting the frequency (E) and amplitude (F) of bladder contractions of naïve (black bars), sham (dark grey bars) and SCI (grey bars).** Results are expressed as mean± SD (n=3-4). Only contractions above 5 cm H2O were considered. Kruskal-Wallis nonparametric test followed by Dunn's post hoc test analysis**.** 'SCI vs naïve animals' *p< 0.05

Neurochemical changes at the spinal cord following intrathecal BTX-A injection

To investigate the underlying mechanisms of action of BTX-A, samples of the lumbosacral spinal cord were resolved by western blotting to determine changes in SNAP25 (the molecular target of the neurotoxin), TRPV1 (known to decrease following administration of BTX-A (Park and Chung, 2018) and GFAP (a marker of astrocytes, putative targets of BTX-A; (Vacca et al., 2012). We found no significant differences between animals receiving vehicle (saline solution) or BTX-A injection, irrespective of the integrity of the spinal cord, albeit there was a non-significant trend to a decrease in spinal levels of TRPV1 and SNAP25 (Figure 21).

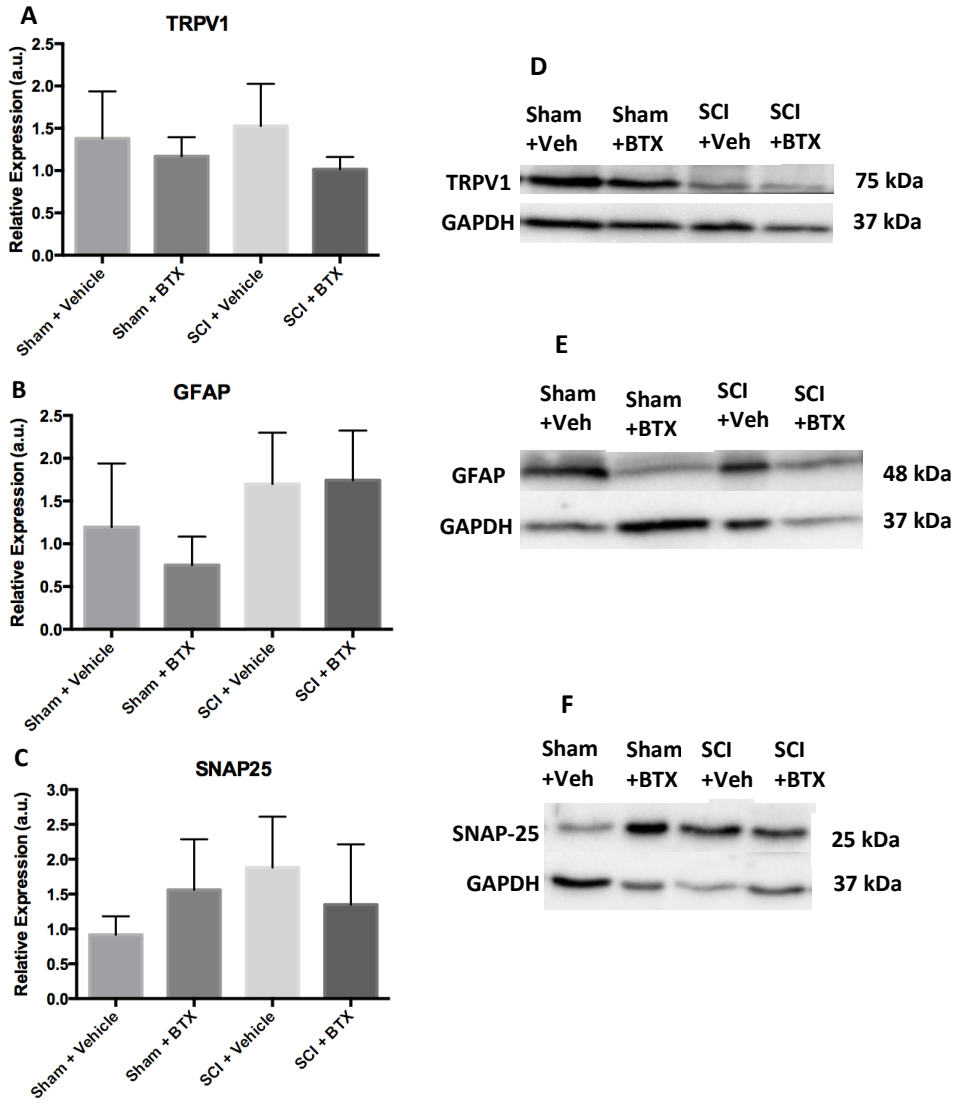


Figure 21: **Western Blot analysis of the amount of TRPV1, GFAP and SNAP25 protein in L5-S1 segments.**

Levels of TRPV1 (A), GFAP (B) and SNAP25 (C) protein were identical in all experimental groups in L5-S1 segments. Results are expressed as mean± SD (n=2 to 5). Kruskal-Wallis nonparametric test followed by Dunn's post hoc test analysis**.**

V. Discussion

Discussion

In the present work we established an incomplete spinal cord injury model to study two of the most common issues that bother SCI patients: neuropathic pain and NDO. For that, cutaneous sensitivity to mechanical and thermal stimuli was investigated, as well as locomotor dysfunction, spontaneous behaviour and bladder function. We found unequivocal signs of pain development as well as NDO in SCI animals. In a second step, animals received intrathecal BTX-A 5 weeks after spinal lesion and effects on cutaneous sensitivity and bladder reflex activity were assessed. This treatment improved pain and SCI-induced bladder dysfunction, suggesting this approach might be useful to tackle two significant problems in these patients.

Spinal Cord Injury model – choice and establishment

Spinal cord injury (SCI) can lead to severe and permanent deficits in motor, sensory and autonomic functions. The most commonly used model is the contusion of the spinal cord, in which a weight drop is released from a specific height and crashes against an exposed spinal cord, immediately creating a lesion (Nakae et al., 2011). Even though the weight drop has the same weight in all the contusions performed, the lesions are highly variable between animals. In the present study, to increase reproducibility between animals, we chose the incomplete transection of the cord, in which a lesion was created by doing a cut of 2mm perpendicularly to the spinal cord.

Spinal Cord Injury model characterization

Here, we observed that SCI animals developed signs of chronic neuropathic pain as shown by the von Frey, hot plate and cold plate tests. Nociceptive behaviour was identified as early as 7 days after spinal surgery. As previously reported, reduced tactile withdrawal thresholds have been associated with neuropathic pain arising after spinal cord injury (Detloff et al., 2013). To

better characterize thermal sensitivity and establish a correlation with clinical trials, we dissected behavioural responses to increases and decreases of the intensity of thermal stimulation and measured awareness, discomfort and nociception responses (Thibault et al., 2011); Cavaleiro et al, unpublished observation). Our SCI animals showed increased heat sensitivity, in agreement with other studies (Kim et al., 2014; Kramer et al., 2017). Our SCI animals also presented signs of cold hypersensitivity with heightened discomfort and nociceptive responses (Baastrup et al., 2010; Gao et al., 2013; M'Dahoma et al., 2014). Likewise, most SCI patients also report cold allodynia (Finnerup et al., 2003) which is also present in several animal models of SCI.

Although responses to mechanical and thermal stimulation showed clear signs of pain in SCI animals, these were stimulus-induced responses. As most SCI animals showed clear signs of pain, such as rounded back posture, piloerection and closed eyes, this suggested the presence of stimulus-independent continuous pain. Thus, spontaneous behaviour was evaluated in SCI animals by performing the Burrowing test. Animals which did not burrow more than 500g were excluded from the study to ensure stable baseline values (Deacon, 2006). Although spontaneous continuous pain has been reported by many SCI patients (Finnerup et al., 2016), results with our SCI animals were somehow inconclusive, most likely due to locomotor impairment as evidenced by the catwalk test in the initial stages after SCI induction. We conclude that the burrowing test might not be the most adequate way to assess spontaneous pain, particularly regarding ongoing pain in SCI animals.

In what refers to bladder function, as with complete spinal cord transection (Cruz et al., 2008; Coelho et al., 2016b), rats submitted to incomplete lesion also developed urinary dysfunction, with increased frequency and amplitude of bladder reflex contractions. Although not directly tested here, it is likely the NDO emergence is also a C-fibre-driven event (Cruz and Cruz, 2011).

Effects of intrathecal administration of BTX-A on pain

BTX-A injection was administered 5 weeks after the surgery. The tip of catheter was placed subcutaneously close to the neck and externalized for drug administration, which was done under deep isoflurane anaesthesia as in other studies (Frias et al., 2015). One animal was not considered in the study due to mispositioning of the intrathecal catheter. A dose of 5U/50μL of BTX-A was administered according to previous studies (Coelho et al., 2014) and lead to a marked improvement of cutaneous sensitivity to mechanical stimulation, also in agreement with previous studies (Coelho et al., 2014) but not to thermal stimuli. Likewise, in a study from Paterson et. al, BTX-A selectively reduced patients' sensitivity to noxious mechanical stimuli, but no change was visible in response to thermal stimuli (Paterson et al., 2014). In addition, it should be noted that, at the time of BTX-A administration, thermal sensitivity had already returned to basal levels.

The interpretation of results obtained in the burrowing test, which assesses spontaneous behaviour, are difficult to interpret given the small sample size and the difficulty in adapting it to motor-impaired animals. No statistical differences were found but there was a trend for BTX-A treated SCI rats burrowing less gravel from the plastic tube. This may suggest the presence of ongoing pain but a recent study from Rutten et. al. showed that burrowing performance cannot be always associated as pain-related readout across pain models and largely depends on the model used (Rutten et al., 2018). In fact, it should be noted that most of the SCI animals did not enter the tube completely after SCI surgery, suggesting that the Burrowing test might not be the best test to assess spontaneous behaviour in these animals.

Effects of intrathecal administration of BTX-A on bladder activity

The development of the new micturition reflex located below the injury site is totally dependent on unmyelinated C-fibers. Along with other SCI-driven changes, it leads to an

increase in the bladder frequency and amplitude in SCI animals (Cruz and Cruz, 2011). In order to safeguard kidneys and regain quality of life, several pharmacological treatments and surgical approaches have been developed. In our study, the intrathecal administration of BTX-A counteracted the increased bladder amplitude associated with SCI without causing urinary retention. Maximal detrusor pressure was reduced resulting in reduced amplitude of bladder contractions. In fact, previous work from Coelho et al. demonstrated NDO improvement after intrathecal administration of botulinum toxin A (Coelho et al., 2014) in SCI animals, by targeting sensory afferents (Coelho et al., 2016b). Also, it suggested that limitation of the toxin activity to sensory afferents is enough to control bladder amplitude in SCI animals.

Neurochemical changes after intrathecal BTX-A

To infer about the underlying mechanisms of the effects of intrathecal BTX-A, the levels of SNAP25, TRPV1 and GFAP in the lumbosacral spinal cord were resolved by western blotting. These markers were chosen because SNAP25 is the established target of BTX-A (Coelho et al., 2012), TRPV1 is a marker of nociceptive fibers known to decrease following BTX-A (Park and Chung, 2018) and GFAP is a marker for astrocytes, considered by some as potentially affected by BTX-A (Vacca et al., 2012). Surprisingly, we found no significative changes in TRPV1, GFAP and SNAP25 expression in the lumbosacral spinal cord, despite a non-significant trend for a decrease of TRPV1 and SNAP25. This likely reflects the limited number of samples from SCI animals which received BTX-A injection used for western blotting assay. Therefore, the results obtained could eventually reach significance if the number of samples increased.

As mentioned, TRPV1 expression was expected to decrease following BTX-A administration (Park and Chung, 2018). In fact, Matak and his co-workers reported that botulinum toxin A injection might prevent SNARE-mediated translocation of TRPV1 receptors to the axonal membrane, being TRPV1 expression reduced due to the delivery of the toxin (Matak and Lackovic, 2014). In our study, we found a slight decrease of TRPV1 expression. TRPV1 is strongly

activated by noxious signals (Tominaga et al., 1998). Thus, improved pain levels could reflect changes in the expression of this polymodal noxious stimuli receptor.

SNAP25 expression was also assessed to assure toxins' action and was expected to decrease after BTX-A injection. As spinal cord injury leads to abnormal firing at lower thresholds of sensory neurons, BTX-A would be easier internalized, cleaving SNAP25 resulting in a decreased SNAP25 expression. SNAP25 relative expression was not altered between all experimental groups. As the cleavage of SNAP25 is the ultimate product of BTX-A action (Matak and Lackovic, 2014) and given the results obtained, measurement of cleaved SNAP25 would be the most accurate marker to assure the toxins' action.

GFAP expression is considered by some authors influenced by BTX-A action (Vacca et al., 2012). In the present study, GFAP expression did not decrease in SCI animals which received the BTX-A injection. In fact, there is a tendency for increased GFAP expression in SCI animals at the lumbar spinal cord, remaining elevated even after intrathecal treatment., Increased GFAP expression at the lumbosacral level, a distant location from the injury site, is unexpected and the reasons for this remain elusive. Although some authors support the idea that astrocytes may be influenced by BTX-A action ((Marinelli et al., 2012)), others report that BTX-A injection only appears to have a slight, if any, effect on astrocytes (Piotrowska et al., 2017). This is supported by a lack of astrocytic expression of SNAP25 (Parpura et al., 1995; Hepp et al., 1999; Wang et al., 2014) but does not exclude an indirect effect of the toxin.

VI. Conclusions & Future perspectives

Conclusions & Future perspectives

The present study aimed to establish and characterize an incomplete spinal cord injury model as a helpful tool to further understand the mechanisms underlying neuropathic pain and NDO arising after SCI. Our main goal was to intrathecally inject Botulinum toxin A (BTX-A) and analyse its action on mechanical allodynia, thermal sensitivity and bladder function. We found improvement of pain levels and bladder function. At a cellular level, TRPV1, GFAP and SNAP-25 relative expression in the lumbosacral spinal cord was determined but none of them was significantly different between experimental groups.

Albeit interesting, the present work had varied limitations and needs further improvement:

- A reduced number of SCI animals receiving intrathecal BTX-A was used in the present study. In addition, the protocol of establishing an incomplete injury is not entirely fully replicable between animals, due to inherent animal variability and technical difficulties. Thus, in order to increase the power of the study, additional animals should be included to reduce variability between animals.
- Due to the lack of time, samples from the bladder and dorsal root ganglia were not analysed. We expect to conclude tissue analysis in the near future which could help explain behavioural effects without spinal neurochemical changes.
- As the Burrowing behaviour did not seem the most appropriate test to assess spontaneous pain, the place escape/avoidance paradigm test (PEAP) could be a proper test for the task. This test is based on the active choice of the animal between its natural preference (hide from light) for pain relief. Baastrup et. al. had shown that SCI animals, under analgesics treatment, remained more time in the dark environment when mechanically stimulated when compared to SCI animals which received saline solution (vehicle) (Baastrup et al., 2011). Although promising, PEAP can only be performed once, not allowing studies of disease progression.

- To assess thermal sensitivity, the Hargreaves' test could also be performed. This test allows testing of individual paws, contrary to the Hot Plate/Cold Plate test. This test also gives in detail which limb has reduced thermal withdrawal.

- Locomotor function was assessed by performing the Catwalk gait analysis. Since the software available only allowed the quantification of paw print intensity, the data was only used to show representative images of animals' locomotion. Further analysis would require the use of the Basso, Brenahan and Beattie scale (Basso et al., 1995).

- SCI is known for the disruption of several ascending and descending pathways that are important in the CNS. Like in the spinal cord, several changes may occur in supraspinal centers, either in the thalamus, periaqueductal grey area, hypothalamus or cerebral cortex. Neuronal loss is present in these supraspinal centers as well as activation of microglia. Cysteine-cysteine chemokine ligand 21 (CCL21) is known to be upregulated 4 weeks after SCI in the thalamus. SCI triggers upregulation of this neuroimmune modulator, inducing microglial activation. As microglia is believed to contribute to altered nociceptive processing in the thalamus, enzyme immunoassays should be performed to assess tissue levels of CCL21 or other proinflammatory cytokines like IL-6 or TNF-α either in animals that received vehicle or BTX-A injection and verify its effect in supraspinal centers (Zhao et al., 2007; Wu et al., 2014).

- As observed in previous studies from our lab (Coelho et al., 2014), intrathecal BTX-A reduced the expression of spinal calcitonin gene-related peptide (CGRP). CGRP is an important mediator of noxious stimuli with an important role in central sensitization. (Karami et al., 2013; Coelho et al., 2014). Another important neuropeptide, co-expressed with CGRP is substance P, also involved in pain perception (Maiaru et al., 2018). We suggest that CGRP and substance P levels should also be quantified in the spinal cord, dorsal root ganglia and bladder after intrathecal BTX-A. Likewise, the receptors for both neuropeptides, (Receptor activity modifying protein 1- RAMP1 for CGRP and NK1 for substance P) should be quantified as well (Hay and Walker, 2017; Maiaru et al., 2018).

- As prevention is better than cure or management of a chronic condition, we forward that BTX-A could be injected at early stages of disease progression and assess if it prevents the development of pain and NDO.

Currently, the available formulations of botulinum toxin A are not specific and target sensory, parasympathetic and sympathetic fibres (Coelho et al., 2012). Recently, a study forwarded that the use of modified botulinum toxins could be used to reduce pain (Maiaru et al., 2018). Future studies aiming to present new treatment options for SCI-induced pain and NDO should investigate the use of these modified botulinum toxins, possibly adjusted to specifically silence TRPV1-expressing neurons, widely considered pivotal players in the emergence of pain and NDO (Cameron et al., 2006; Shimizu et al., 2018).

VII. References

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