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Dorsal root ganglion electrostimulation in a rat model of neuropathic pain: Protocol implementation and improvement

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The only thing that overcomes hard luck is hard work

Harry Golden

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Abstract

Neuropathic pain (NP) is a chronic syndrome of disordered sensory perception and a disabling condition that can arise from various diseases, which manifest as a matrix of symptoms and signs. Spinal cord stimulation (SCS) is recommended as the first option for neuropathic pain, in the USA. Nevertheless, this approach has some limitations that reduce its efficacy or even lead the patients to give up from treatment. This prompted the need to try new targets for electrostimulation, namely the dorsal root ganglia (DRG). DRG are potential targets since the pathological changes that characterize chronic pain states occur within the sensory neurons in these structures. Multiple published clinical studies demonstrated that DRG electrical stimulation (DRG-S) is effective in reducing pain states in humans. However, the mechanisms underlying DRG-S analgesia are still largely unknown. Acknowledging these clinical outcomes, we aimed at better understanding the DRG-S mechanisms by implementing a successful protocol of DRG-S that could be effective in reducing the nociceptive behavior in a rat model of chronic neuropathic pain.

For that purpose, we developed a surgical approach for implanting bipolar platinum/iridium electrodes nearby the L4 DRG of adult Wistar rats, and used the neurostimulation parameters replicated from the clinical practice (20Hz, 150µs pulse width, and current at 80% of motor threshold). The neuropathic pain model was induced by the spared nerve injury (SNI) surgery. On different time-points of SNI development (7, 14, and/or 21 days), the nociceptive behavior was assessed by the von-Frey, Pin-Prick, and Acetone tests, and the motor coordination was also evaluated by the Rotarod test. Spontaneous pain (and the affective dimension of pain) was analyzed by the Conditioned Place Preference test. In addition, we analyzed the expression of the neuronal injury markers Activating Transcription Factor 3 (ATF3) and Neuropeptide Y (NPY) by immunohistochemistry in the ipsilateral DRG.

Although the number of animals in each group is still low, data showed that electrode insertion *per se* had no evident behavioral effects on mechanical or cold allodynia nor on mechanical hyperalgesia and causes no histological evidence of DRG neuronal

damage. DRG-S appears to decrease the neuropathy-induced hypersensitivity, particularly for mechanical allodynia, as the paw withdrawal threshold for von Frey filaments most times increased after DRG-S. In a rat receiving a repeated DRG-S protocol until a later time-point of SNI (21 days), data suggest a recovery of the sensitivity when this was lost due to the neuropathic condition. On the other hand, motor coordination was impaired after DRG-S. The Conditioned Place Preference test showed that DRG-S had not a rewarding effect. Finally, the percentage of ATF3 immunoreactive cells was not altered by DRG-S, in comparison to the labeling found in SNI rats without electrostimulation. In contrast, the NPY levels showed a decrease after repeated DRG-S protocol in an intermediate SNI time-point.

Data suggest that the electrodes implantation surgery and the electrodes maintenance do not affect the nociceptive behavior of a naïve animal, as well as do not impair the motor coordination throughout the experimental period. The results also show that acute and repeated DRG-S of the L4 DRG appears to decrease the neuropathy-induced hypersensitivity. In the molecular evaluation, data suggest that repeated DRG-S of the L4 DRG decreases the expression of NPY in sensory neurons, possibly implying this molecule in the mechanisms of DRG-S induced analgesia. This study still needs improvement in some parts of the protocol to increase the success rate of electrodes implantation and neurostimulation. This would enable future studies in animal models aiming to understand the intrinsic mechanisms associated with the analgesic effects of DRG-S.

Keywords: Neuropathic pain (NP); Dorsal Root Ganglia (DRG); Dorsal Root Ganglion Stimulation (DRG-S); Electrode Implantation Surgery; Analgesic Stimulation; Conditioned Place Preference; Activating Transcription Factor 3 (ATF3); Neuropeptide Y (NPY).

Resumo

A dor neuropática (*NP*, de Neuropathic Pain) é uma doença crónica caraterizada por uma perceção sensorial anormal, sendo uma condição incapacitante que pode ter origem em várias doenças, que se manifestam como uma matriz de sintomas e sinais. A estimulação elétrica da medula espinhal (SCS, de *Spinal Cord Stimulation*) é recomendada como primeira opção para a dor neuropática, nos EUA. No entanto, esta abordagem tem algumas limitações que reduzem a sua eficácia ou levam mesmo os doentes a desistir do tratamento. Isto provocou a necessidade de experimentar novos alvos para a electroestimulação, nomeadamente os gânglios raquidianos (DRG, de *Dorsal Root Ganglia*). Os DRG são potenciais alvos, uma vez que as alterações patológicas que caracterizam os estados de dor crónica ocorrem nos neurónios sensoriais destas estruturas. Vários estudos clínicos publicados demonstraram que a estimulação elétrica dos DRG (DRG-S, de *Dorsal Root Ganglion Stimulation*) é eficaz na redução dos estados de dor em humanos. No entanto, os mecanismos subjacentes à analgesia do DRG-S são ainda largamente desconhecidos. Reconhecendo estes resultados clínicos, procurámos compreender melhor os mecanismos da DRG-S, implementando um protocolo bem-sucedido de DRG-S que poderia ser eficaz na redução do comportamento nociceptivo num modelo animal de dor neuropática crónica.

Para esse efeito, desenvolvemos uma abordagem cirúrgica para a implantação de elétrodos bipolares de platina/irídio nas proximidades do DRG L4 de ratos Wistar adultos, e utilizámos os parâmetros de neuroestimulação replicados da prática clínica (20Hz, 150µs de largura de pulso, e corrente a 80% do limiar motor). O modelo de dor neuropática foi induzido pela cirurgia de *Spared Nerve Injury* (SNI). O comportamento nociceptivo foi avaliado em diferentes momentos do desenvolvimento do SNI (7, 14, e/ou 21 dias), pelos testes *von-Frey*, *Pin-Prick*, e Acetona, e a coordenação motora foi também avaliada pelo teste Rotarod. A dor espontânea (e a dimensão afetiva da dor) foi analisada pelo teste *Conditioned Place Preference* (CPP). Além disso, analisámos a expressão do Fator de Ativação da Transcrição 3 (ATF3, de *Activating Transcription*

Factor 3) e do Neuropeptídeo Y (NPY, de *Neuropeptide Y*), dois marcadores de lesão neuronal, por imunohistoquímica nos DRG ipsilaterais.

Embora o número de animais em cada grupo seja ainda baixo, os dados demonstraram que a inserção dos elétrodos por si só não teve efeitos comportamentais evidentes na alodínia mecânica ou ao frio nem na hiperalgesia mecânica e não causa qualquer evidência histológica de danos neuronais nos DRG. A DRG-S parece diminuir a hipersensibilidade induzida pela neuropatia, particularmente para a alodínia mecânica, uma vez que o limiar de retirada da pata dos filamentos de *von Frey* aumentou na maioria das vezes após o DRG-S. Num animal com mais tempo de evolução de SNI (21 dias), que recebeu um protocolo de neuroestimulação (DRG-S), os dados sugerem uma recuperação da sensibilidade quando esta estava perdida pela condição de dor neuropática. Por outro lado, a coordenação motora foi afetada após a DRG-S. O teste de CPP mostrou que a DRG-S não teve um efeito de recompensa. Finalmente, a percentagem de células imunorreativas ao ATF3 não foi alterada pela DRG-S, em comparação com a percentagem de células marcadas em animais SNI sem electroestimulação. Em contraste, os níveis de NPY mostraram uma diminuição após neuroestimulação (DRG-S) repetida num animal com 14 dias de evolução de SNI.

Os resultados sugerem que a cirurgia de implantação dos elétrodos e a manutenção destes não afetam o comportamento nociceptivo num animal naïve, assim como não prejudicam a coordenação motora durante todo o período experimental. Os resultados mostram também que as neuroestimulações (DRG-S) agudas ou repetidas, do DRG L4, parecem diminuir a hipersensibilidade induzida pela neuropatia. Na avaliação molecular, os dados sugerem que a neuroestimulação (DRG-S) repetida do DRG L4 diminui a expressão do NPY nos neurónios sensoriais, implicando possivelmente esta molécula nos mecanismos da analgesia induzida pela DRG-S. Este estudo precisa ainda de ser melhorado em algumas partes do protocolo para aumentar a taxa de sucesso da implantação de elétrodos e da neuroestimulação. Isto permitiria estudos futuros em modelos animais com o objetivo de compreender os mecanismos intrínsecos associados aos efeitos analgésicos dos DRG-S.

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List of abbreviations

ATF3 – Activating Transcription Factor 3

Aα – A-Alpha

Aβ – A-Beta

Aδ – A-Delta

Aγ – A-Gamma

BOLD – Blood-Oxygen-Level-Dependent

Ca – Calcium

CCI – Chronic Constriction Injury

CGRP – Calcitonin Gene-Related Peptide

CNS – Central Nervous System

CRPS – Complex Regional Pain Syndrome

CSF – Cerebrospinal Fluid

DRG – Dorsal Root Ganglia

DRG-S – Dorsal Root Ganglion Stimulation

fMRI – Functional Magnetic Resonance Imaging

GFAP – Glial Fibrillary Acidic Protein

GS – Glutamine Synthetase

HTMRs – High-Threshold Mechanoreceptors

I – Current Intensity

IASP – International Association for the Study of Pain

IR – Immunoreactive

K – Potassium

LTMRs – Low-Threshold Mechanoreceptors

mA – Milliamperes

Na – Sodium

NGS – Normal Goat Serum

NMDA – N-methyl-D-aspartate

NP – Neuropathic Pain

NPY – Neuropeptide Y

PAF – Peripheral Afferent Fiber

PBS – Phosphate buffered saline

PBS-T – Phosphate buffered saline with Triton X-100

PFA – Paraformaldehyde

PHN – Postherpetic Neuralgia

PNS – Peripheral Nervous System

PTFE – Polytetrafluoroethylene

R – Resistance

SC – Spinal Cord

SCS – Spinal Cord Stimulation

SGCs – Satellite Glial Cells

SNI – Spared Nerve Injury

TCAs – Tricyclic Antidepressants

TENS – Transcutaneous Electrical Nerve Stimulation

TTX – Tetrodoxin

V – Voltage

Y1R – Neuropeptide Y1 Receptor

Y2R – Neuropeptide Y1 Receptor

Introduction

Pain

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage"(IASP, 2020). Pain is a subjective experience that comprises two complementary components: the sensory perception, a localized sensation in one determined part of the body with discrimination concerning its time, space, intensity, and physical qualification, and the unpleasant emotional experience that is responsible for the behavioral response to pain (Wall and Melzack 1989). Pain can be reported in the absence of tissue injury, but in the great majority of cases, it is initiated by the activation of the nociceptors, specialized peripheral sensory neurons that work as detectors of noxious stimuli capable of producing tissue damage. Pain can also have origin in a lesion of the peripheral nervous system (PNS) or central nervous system (CNS) associated with somatic and autonomic reflexes (Ossipov 2012; Loeser and Melzack 1999; Dubin and Patapoutian 2010). In this case, we are in the presence of neuropathic pain. In the biological context, the perception of pain is useful for signaling the presence of an injury, thus acting as a warning system, and is evoked only at pressures and temperatures extreme enough to produce tissue lesion or injury, and by toxic molecules. However, pain often outlives its protective utility and becomes chronic, persisting after acute injury and causing alterations in the pain pathways. Pain classification comprises three different levels: symptoms, mechanisms, and syndromes, in which the latter is defined taking into account what is known about its etiology, location, frequency, intensity, genetics, clinical symptoms, history, and response to treatment. Beyond these criteria, pain can be also classified according to its duration into acute or chronic pain (Pergolizzi, Raffa, and Taylor 2014; Ossipov 2012; Dubin and Patapoutian 2010).

Acute pain, usually of short duration, is the "normal" pain since it has a physiological role, alerting the organism, and therefore being necessary for survival (Ossipov 2012). It

is normally elicited by a substantial lesion of the non-neuronal tissue and consequent activation of the nociceptive transducers, an event that can be viewed as a biochemical and behavioral cascade that is initiated by tissue injury (Loeser and Melzack 1999; Clifford J Woolf 2010). It is this pain that teaches us how to recognize and avoid objects and situations that have the potential to produce lesion (Ossipov 2012). On the other hand, when the pain has a duration of more than 3 to 6 months and loses the warning function becoming pathological itself, it is called chronic pain.

Chronic Pain

Chronic pain is commonly initiated by a lesion or disease, but persists beyond the time of treatment since pain responses are not suppressed because the body is unable to restore the physiologic functions (Loeser and Melzack 1999; Pergolizzi, Raffa, and Taylor 2014). This type of pain is considered maladaptive and a public health problem that is highly prevalent in developed countries like Portugal, where it is estimated that around 36.7% of the adult population suffers from this disease, which does not have any biological value (Ossipov 2012; Cristóvão and Reis-Pina 2019). It is well-established that acute pain can originate chronic pain, through a process named chronification, and that the cascade that initiates chronification may be interrupted by the treatment for acute pain. The biological mechanisms behind chronification include neuroplasticity, pain modulation, central sensitization, and a neuromatrix of pain with several areas of the CNS interplaying (Pergolizzi, Raffa, and Taylor 2014).

Chronic pain can also be referred to as persistent pain, and can be subdivided according to its cause and its local of origin into nociceptive or neuropathic pain (Kerstman et al. 2013). Nociceptive pain is related to the detection of the noxious stimulus by the nociceptors, like for example something too hot, cold, or sharp, which is essential to maintain the integrity of the body, and arises after an excessive stimulation of the nociceptors in the skin, viscera and others organs, which may cause damage to non-neuronal tissue (Clifford J Woolf 2010; Castro-Lopes 2004). Nociceptive pain then results

from the transduction of the noxious stimuli in action potentials and the transmission of these action potentials through a normally functioning somatosensory nervous system. The action potentials are generated by a sodium (Na) current depolarization that is followed by a potassium (K) repolarization and elevation of intracellular calcium (Ca). In situations of chronic nociceptive or inflammatory pain, the nociceptors have a notable plasticity that amplifies the non-neuronal signals, as a consequence of the delivery of agents with sensitizer effects that increase the sensitivity of the nociceptors, thus reducing the nociceptors activation threshold and increasing the neuronal excitability. These alterations in the nociceptor's activity are manifested as allodynia and hyperalgesia (Merskey and Bogduk 1994; Ossipov 2012; Krames 2014b; Esposito et al. 2019). Allodynia is defined as "pain due to a stimulus that does not normally provoke pain" and in the clinical context is used to refer to mechanical brush-evoked allodynia mediated by A-beta (Aβ) fibers. Hyperalgesia is defined as "increased pain from a stimulus that normally provokes pain" (Kerstman et al. 2013; C. J. Woolf et al. 1998; Castro-Lopes 2004; Wall and Melzack 1989; Merskey and Bogduk 1994). In contrast to nociceptive pain, neuropathic pain arises from a lesion or disease of the nervous system, either peripheral or central, and when it happens in the CNS it is also called central pain.

Neuropathic Pain (NP)

Neuropathic pain (NP) is a chronic syndrome of disordered sensory perception and a disabling condition that can arise from various diseases, which manifest as a matrix of symptoms and signs, including metabolic diseases (diabetes), infections (HIV), vascular diseases (stroke), traumas (spinal cord injury), immune diseases (multiple sclerosis) and cancer (Bouhassira et al. 2008; Campbell and Meyer 2006; Jensen et al. 2011; Castro-Lopes 2004; Guha and Shamji 2016; Kerstman et al. 2013). After lesion, pain is felt either without any stimulus or, when in the presence of a stimuli, the sensation of pain is felt at a higher intensity than it would normally, due to the activation of both injured and uninjured afferent nociceptors that innervate the affected member, which triggers the

development of spontaneous and ectopic activity (Campbell and Meyer 2006; Guha and Shamji 2016).

The peripheral NP disease differs from the other types of peripheral pain by the presence of deafferentiation – permanent loss of primary afferent fibers. When this happens, the most common positive sensory phenomena are spontaneous pain, allodynia, and hyperalgesia. In fact, NP has two main components, stimulus-evoked pain and stimulus-independent pain, being that the latter can be steady or intermittent. Spontaneous pain (or stimulus-independent pain) is commonly described as shooting, lancinating, or burning pain and depends on the activity of the sympathetic nervous system. On the other hand, stimulus-evoked pain is common for peripheral nerve injury and is characterized by hyperalgesia and allodynia (Clifford J. Woolf and Mannion 1999; Dworkin et al. 2003; Kerstman et al. 2013). Beyond the neuronal injury, in the presence of a peripheral NP condition, there is also non-neuronal tissue injury, like in the skin. This lesion and the nociceptors that remain intact may give rise to ongoing pain and two different types of hyperalgesia – primary and secondary. In primary hyperalgesia the area that is affected is the area where the lesion occurs, leading to sensitization of the primary afferent nociceptors, and is manifested as spontaneous pain and an increased response to heat. The secondary hyperalgesia occurs in the uninjured non-neuronal tissues that surround the affected area, and is a consequence of the sensitization of the CNS, being characterized by mechanical hyperalgesia (Campbell and Meyer 2006).

Pain Perception

The perception of pain results from the activation of nociceptors that detect and transmit noxious stimuli from the periphery to the CNS. Three major functional domains constitute the peripheral mechanisms for the perception of pain: transduction, transmission, and modulation. The first functional domain is transduction, which happens when noxious stimuli are received by the nociceptors as information in the form of electrical signals that are sent to the dorsal horn of the spinal cord via the central

branch of the nociceptive neurons. This process that transforms the nociceptive stimuli into action potentials depends on the properties of specific ion channels and receptors that are present in the peripheral terminations of the nociceptors. When the signals arrive to the dorsal horn, they are carried to supraspinal regions in the CNS through various ascending pathways, like the spinothalamic tract, an event called transmission. In the higher regions, the nociceptive information is processed and integrated with cognitive and limbic information, generating the sensation of pain. However, the inputs that are generated in the spinal cord are controlled by inhibitory and facilitatory spinal interneurons and also by descending (inhibitory and facilitatory) projections that will ultimately inhibit or facilitate the transmission of nociceptive inputs through the ascending tracts, an event called modulation. The process of pain perception is illustrated in figure 1 (Bingham et al. 2009; Miller 2009).

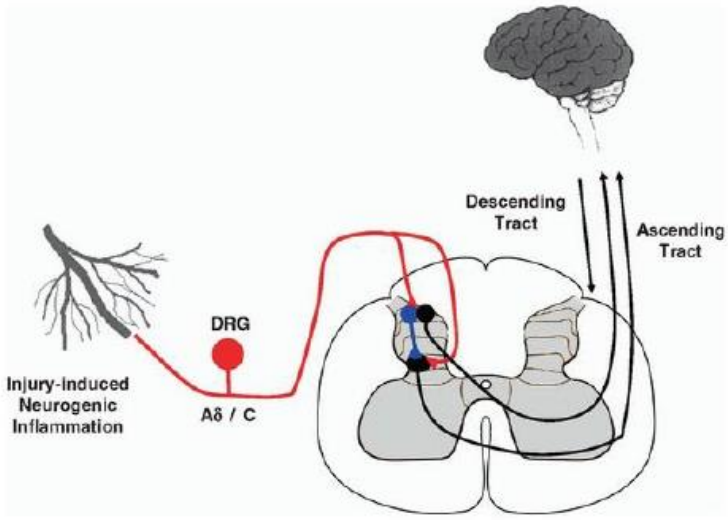

Figure 1: Pain Perception. Activation of peripheral pain receptors (nociceptors) by noxious stimuli generates signals that travel through peripheral nerves to the cell bodies of primary afferent neurons located at sensory ganglia, such as dorsal root ganglia (DRG). From there, the action potentials are transmitted to the dorsal horn of spinal cord via the primary afferent neurons' central axonal branch. From the dorsal horn, the inputs are carried to higher regions in the CNS through various ascending tracts, where they are processed and integrated, generating the pain sensation. Descending inhibitory and facilitatory supraspinal projections to

the spinal cord modulate the quality of the signal that is processed at the spinal cord and transmitted to supraspinal regions (Miller 2009).

Dorsal Root Ganglia (DRG)

Nociceptors are primary afferent neurons that have their cell bodies (perikarya or somas) located in sensory ganglia named dorsal root ganglia (DRG), or trigeminal ganglia, in the case of innervation from the face and head. The DRG are located in the proximity of neural foramina at each adjacent vertebrae level and are constituted by highly heterogeneous structures that contain many types of cells, in which around 15% are the primary afferent neurons (Castro-Lopes 2004; Esposito et al. 2019; Dubin and Patapoutian 2010; Rush and Waxman 2009). These are pseudounipolar neurons, as illustrated in figure 2, with an axon that bifurcates into two different processes, one proximal or central branch that ends in the dorsal horn of the spinal cord, and one distal or peripheral branch, which ends in the diverse peripheral organs, like the skin, joints, muscle and viscera. The axons that end in the periphery constitute the primary afferent sensory nerves and the local where the axon bifurcates is called the T-junction, which normally may impede the propagation of some action potentials. In reality, this structure can play three different roles: a) can act as an impediment to electric impulses that go from the periphery to the dorsal root entry zone; b) can participate in the electric pulse propagation; and c) can act as low pass filter to the information that comes from the periphery to the CNS (Krames 2014b; Esposito et al. 2019). The DRG are considered the gateway for processing peripheral sensory signals, including touch or proprioception, and also inflammatory or somatic pains arising from any part of the body except the face and head, and as such, they are also considered as the structures that allow the communication between the PNS and the CNS (along with the trigeminal ganglia).

Figure 2: Pseudounipolar neuron (adapted from https://qbi.uq.edu.au/brain/brain-anatomy/types-neurons).

Neurons in the DRGs are encircled by an envelope of glial cells, namely the satellite glial cells (SGCs) around the somas and the Schwann cells that surround either unmyelinated or myelinated fibers (forming in this case the myelin sheath), as illustrated in figure 3. The communication between the neurons within each DRG is not direct, being the SGCs that ensure this intra-ganglionic crosstalk. In fact, these non-neuronal cells mediate the interaction between glia-glia and neuron-glia through the activation of chemokine receptors, the release of cytokines and gliotransmitters, and the response to neuronal signaling. For this, the SGCs express the receptors of numerous neuroactive agents and respond to the received signals from other cells that influence the DRG neurons. They also act as a barrier since they are critical in situations of response to chemical stimuli. Taking this into account, it is comprehensible that these non-neuronal cells participate in the transducing processes within the DRG (Sapunar et al. 2012). These glial cells can be easily identifiable since they are characterized by the presence of certain specific proteins. Therefore, it is possible to detect SGCs by performing immunohistochemistry with antibodies against the glial fibrillary acidic protein (GFAP), a cytoskeleton protein, or glutamine synthetase (GS), which catalyzes the conversion of glutamate to glutamine. GS is produced in visible amounts in normal conditions and is used as a selective marker

of the cells, while, on the other hand, GFAP is only detectable after nerve damage or inflammation, since in normal conditions the protein is only expressed at low basal levels (Hanani 2005). On the other hand, Schwann cells can be detected by performing immunohistochemistry against S100, an homodimeric protein that is often found in cells derived from the neural crest, or against SOX10, a transcription factor that directs neural stem cells towards the glial lineage. Both proteins are selective markers and are detectable in normal conditions (Liu et al. 2015).

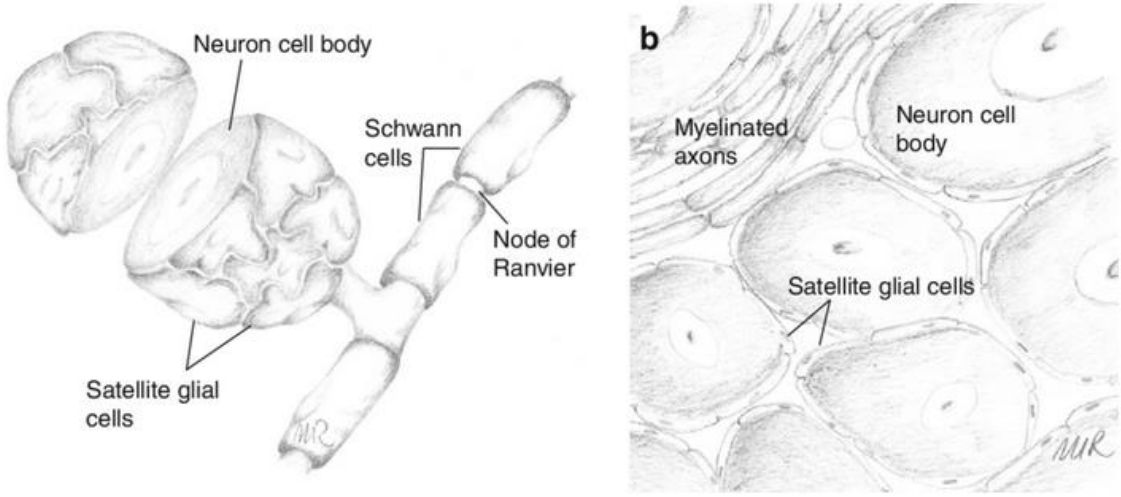

Figure 3: Dorsal Root Ganglion surrounded by Satellite Glial Cells (SGCs) and Schwann cells. Pseudounipolar neuron surrounded by SGCs, forming a neuron-SGCs unit. The axon of the pseudounipolar neuron bifurcates and projects one peripheral branch, which extends to the peripheral target, and one central branch, which extends to the spinal cord (a). Primary sensory neurons surrounded by SGCs (b) (adapted from (Richner et al. 2014))**.**

Even though primary afferent neurons are protected by SGCs, they are susceptible structures to chemokines, leukocytes infiltrations, and also to macrophages, which lead to a neuroinflammatory environment that triggers mechanisms of neuronal hyperexcitability. These responses of the immune system occur because the DRG, although being considered as belonging to the PNS, are not protected by neither blood-brain nor blood-nerve barriers (Dubin and Patapoutian 2010; Guha and Shamji 2016;

Rush and Waxman 2009; Krames 2014b; Liem et al. 2016; Esposito et al. 2019). Therefore, the DRG are important in the initial phase and in the maintenance of the chronic pain states. It was showed that after peripheral afferent fiber (PAF) injury, the SGCs within DRG become activated and proliferate, which leads to a cascade release of pro-inflammatory cytokines, an abnormal flux of Ca^{++} and K^{+} ions, alterations in the immune system and the expression of early and late genes, and even of growth factors. All of these alterations have as a consequence the generation of neuronal hyperexcitability within the DRG neurons and the development of neuropathic pain states (which originate from injury to the nervous tissue). Indeed, SGCs have an important role in pathologic states like pain, and/or inflammation. One of the biggest determinants of neuronal excitability is the extracellular concentration of K^{+}, which is regulated by SGCs within the sensory ganglia. Some studies have shown that this impairment of glial potassium homeostasis contributes to pain. Beyond the alterations in the potassium, an increase in the expression of neurotrophic factors was also showed after axotomy, along with the increase in the activation and proliferation of SGCs. This increase in the SGCs that surround the DRG neurons triggers a persistent mechanical allodynia, which shows that ganglia-derived neurotrophins are a source of nociceptive stimuli for neuropathic pain (Sapunar et al. 2012; Esposito et al. 2019). In addition, also the Schwann cells contribute to this sensitizing environment. When a lesion in a peripheral nerve happens, the Schwann cells are the first responders. In normal conditions, these non-neuronal cells maintain a homeostatic interaction with the associated axons. However, after lesion, the homeostatic state suffers a break, which results in the activation of the Schwann cells and subsequent release of pro-inflammatory cytokines, trophic factors, and chemoattractants that recruit neutrophils, macrophages, and other inflammatory cells. All together, these factors contribute to sensitization, and to the hypersensitivity of nociceptive neurons, and the consequent spontaneous generation of ectopic discharges, which underlie the sensation of neuropathic pain (Krames 2014b; Kuffler 2020).

Taking into account the above, we can conclude that DRG have an important role in the modulation of peripheral and central sensory mechanisms that include the generation

and development of nociceptive and neuropathic pain (Krames 2014b). In the past two decades, a lot of work that was developed showed that alterations in the excitability of the DRG neurons are representative of the associated alterations in the related fibers and that this is important for many of the pathological conditions (Rush and Waxman 2009)

Nociceptors

Nociceptors are defined by IASP as a high-threshold sensory receptor of the peripheral somatosensory nervous system and are known to be anatomically, electrophysiologically, and neurochemically heterogeneous. According to the diameter, degree of myelination of their axons, and conduction velocity, the cutaneous sensory fibers can be divided into four groups: A-Alpha (Aα), A-Beta (Aβ), A-Delta (Aδ), and C-fibers. The conduction velocity is directly proportional to the diameter of the axons, and the fact of these being, or not, myelinated. Most of the nociceptors are constituted by fibers of the C type that are small in diameter and unmyelinated, and for that reason have a slow conduction velocity (0.4-1.4 m/s). On the other hand, the A-fibers are those that mediate the initial fast-onset sensation since they are myelinated and have a higher rate of conduction velocity (5-30 m/s). Within the group of A-fibers, the Aδ fibers are thinner than Aβ fibers and have a lower conduction velocity (Dubin and Patapoutian 2010; Rush and Waxman 2009). In normal physiological conditions, only the Aδ and the C-fibers transmit nociceptive information when a noxious stimulus is applied to the skin, in such a way that the Aδ fibers are responsible for the conduction of the initial, well-localized, immediate or first acute pain sensations that are followed by the more diffuse, poorly localized, slow painful sensations that normally remain after the stimulus (second pain), and which are caused by the activation of C-fibers, as illustrated in figure 4 (Castro-Lopes 2004; Esposito et al. 2019). However, in the presence of peripheral nerve lesion or even tissue inflammation, the Aβ neurons undergo plastic changes that make them also sensitive to the high threshold stimuli, facilitating the transduction and encoding of nociceptive stimuli by these primary afferents.

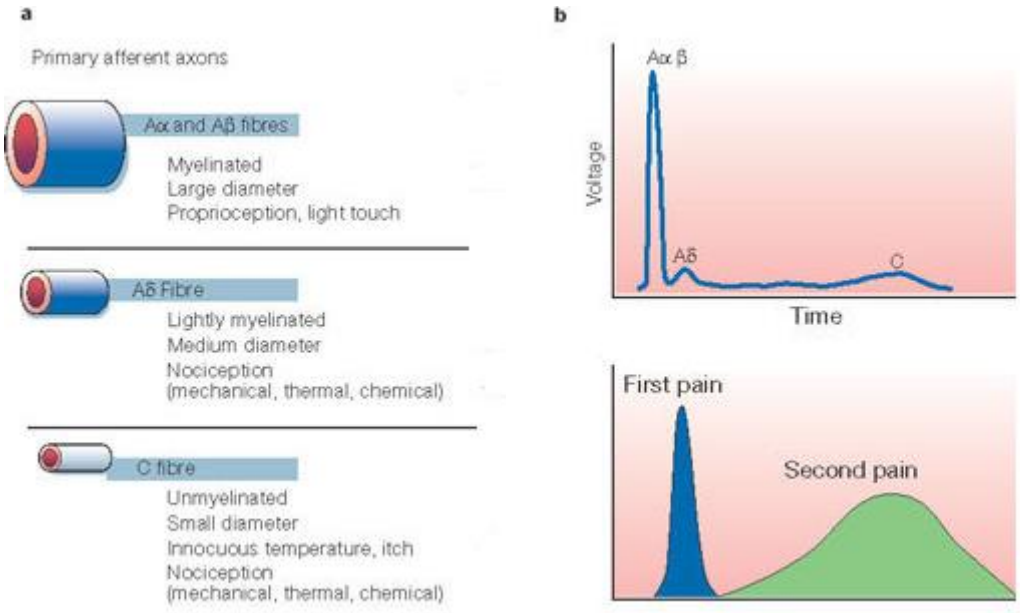

Figure 4: Sensitive fibers. Differences between sensitive fibers regarding to diameter, myelination degree and the type of the information they convey (a), and their relation with first and second pain (b) (adapted from (Basbaum 2001)).

Besides to the conduction velocity, nociceptors are also classified taking into account the sensitivity and the response threshold to the stimulus. Thus, they can be classified into mechanical, thermal, or chemical. The nociceptors that respond to any type of stimulus are called polymodal nociceptors and are, in the majority, C-fibers nociceptors. In addition to these, there are also silent nociceptors, usually identified as stimulus insensitive nociceptors, which only respond after they have been sensitized by inflammatory mediators (Dubin and Patapoutian 2010). When the nociceptors are activated by mechanical forces they are labeled as mechanoreceptors, which can be divided into low-threshold mechanoreceptors (LTMRs) and high-threshold mechanoreceptors (HTRMs). The LTMRs encode a continuous range of stimulus intensities below the noxious thresholds, mediating the innocuous touch. On the other hand, HTMRs are nociceptive neurons that respond to intense mechanical stimuli that impose the risk of tissue injury, mediating mechanical pain, being therefore indicated as the nociceptors that mediate the acute pain produced by the pin-prick stimulus. In addition, they respond to chemical stimuli, having high heat thresholds. In the case of

tissue injury, they have their thresholds lowered to both mechanical and chemical stimuli. These mechanosensitive neuronal fibers transmit pain and exhibit little or no adaptation, in such a way that the stimulus duration can persist for several minutes or even hours (Olson et al. 2016; Delmas, Korogod, and Coste 2018; IASP 2014).

The last categorization of nociceptors, specifically of the C-fibers, is related with their neurochemical properties. Those C nociceptors that can release neuropeptides, like substance P (SP) or calcitonin gene-related peptide (CGRP), are classified as peptidergic, while the non-peptidergic nociceptors do not release peptides (IASP 2014).

After peripheral lesion, the C-fibers exhibit aberrant and ectopic firing and the A-fibers contribute to the maintenance of chronic pain and clinical allodynia (Guha and Shamji 2016). The A-fibers are, in their majority, heat and or mechanosensitive, although A-fibers sensitive to noxious cold have also been described. Some studies demonstrate that the cooling of the skin to 4°C leads to the activation of A- and C-fibers sensitive to innocuous cooling and cold-sensitive nociceptors, while the perception of the pin-prick stimulus was related with the capsaicin-insensitive A-fibers activity (Dubin and Patapoutian 2010).

Mechanisms behind NP

Spontaneous pain, allodynia, and hyperalgesia all arise as consequences of the sensitization mechanisms that include ectopic generation of impulses, *de novo* expression of neurotransmitters, and their receptor and ions channels, as illustrated in figure 5 (Dworkin et al. 2003). Indeed, the most important changes responsible for the disease are related with the altered gene/protein expression in the primary sensory neurons (Sapunar et al. 2012; Esposito et al. 2019). Chronic neuropathic pain is characterized by a decreased threshold to the firing of action potentials of nociceptors and by central amplification of a normal peripheral sensory activation that can result in allodynia and hyperalgesia (Guha and Shamji 2016; Krames 2014b). Different studies showed that primary hyperalgesia is related with peripheral mechanisms, collectively

termed as peripheral sensitization, that lead to the sensitization of nociceptors and activation of silent nociceptors. On the other hand, secondary hyperalgesia depends on sensitization mechanisms that act in the dorsal horn of the spinal cord, known as central sensitization. Stimulus-evoked hyperalgesias are commonly subdivided based on the modality, which may be mechanical, thermal, or chemical. The mechanical can be divided into brush-evoked (dynamic), pressure-evoked (static) and punctate (Clifford J. Woolf and Mannion 1999; Castro-Lopes 2004). Stimulus-evoked pain acts by a mechanism that is highly related with the cutaneous sensitive fibers of the nociceptors. The A-fibers are implicated in mechanical hyperalgesia, as a consequence of central sensitization, which also originates cold allodynia, and punctate mechanical hyperalgesia. Brush-evoked hyperalgesia is a direct consequence of the increased central response to the inputs of Aβ fibers, while punctate mechanical hyperalgesia arises from central sensitization to the inputs of Aδ fibers (Clifford J. Woolf and Mannion 1999; Campbell and Meyer 2006). In those conditions of sensitization, the Aβ fibers are allowed to transduce pain signals since the neurons express receptors for and release glutamate, SP, and CGRP (Esposito et al. 2019). In addition to these mechanisms, the development of peripheral NP involves the peripheral immune system and different non-neuronal cell types, like SGCs and Schwann cells, at the periphery, as well as other glial cells of the CNS, as astrocytes and microglia, that make the whole process very complex. After a situation of tissue lesion, glial cells responses alter the neuronal function in the PNS and CNS, namely by becoming activated and releasing cytokines and pro-inflammatory molecules that lead to inflammation and neuronal hyperexcitability, and therefore to pain. Furthermore, there is also the production of several pain mediators that contribute to peripheral and central sensitization, which are crucial events in the development and maintenance of peripheral NP. The central sensitization is clinically associated with dynamic mechanical allodynia evoked by light brush stimulus and allodynia and hyperalgesia evoked by cold objects. On the other hand, peripheral sensitization includes ectopic and spontaneous discharges, ectopic hyperexcitability via the induction of changes in the expression of neurotransmitters, neuromodulators, growth factors, receptors, and neuroactive molecules, as well as alterations in the ion

channels expression. As consequence, the firing threshold of Aδ and C nociceptors is lowered to the non-noxious range, which turns these fibers sensitive to moderately warm temperature and capable of producing a sensation of burning pain. It is also associated with weak mechanical stimuli (touch), like static mechanical allodynia, which can result from peripheral sensitization of C-fibers, and also pin-prick mechanical hyperalgesia. In addition to these, peripheral sensitization also enhances nociception, which results in spontaneous or paroxysmal pain and heat hyperalgesia (Backonja 2003; Kerstman et al. 2013; Gierthmuhlen and Baron 2016).

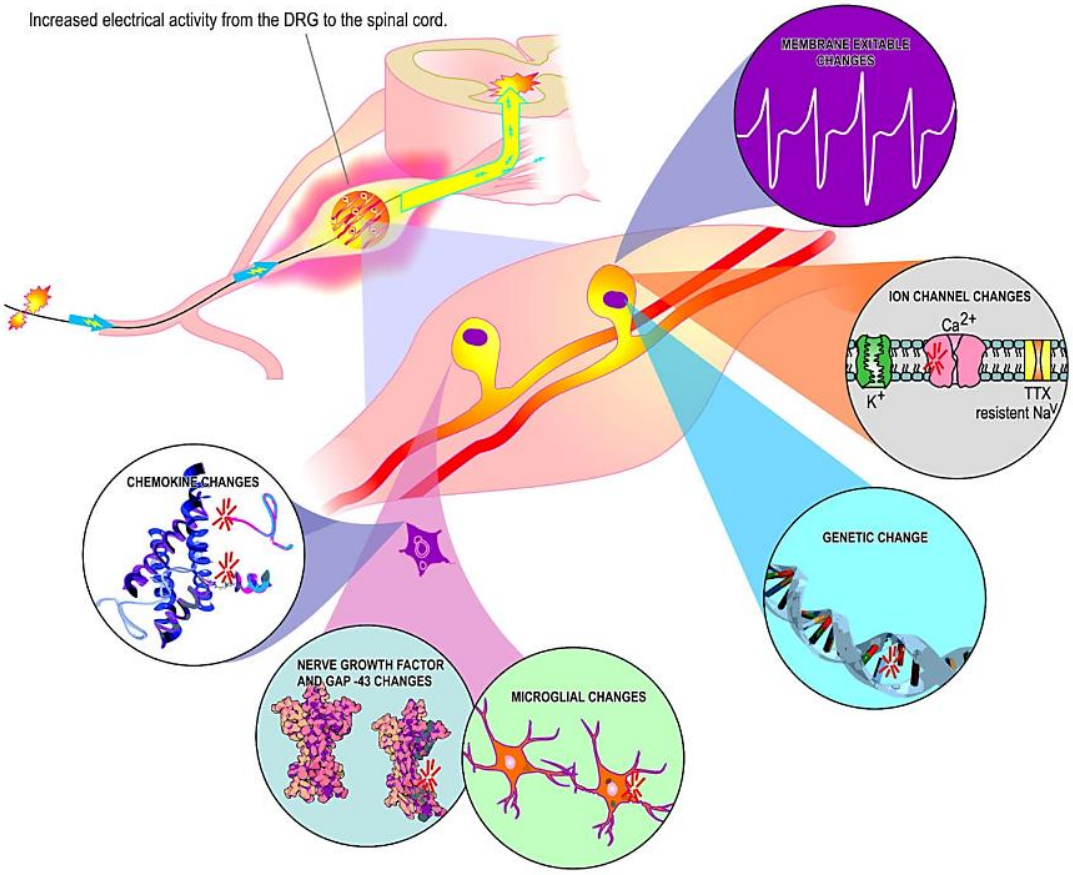

Figure 5: Dorsal root ganglion responses after injury. Release of various factors and changes in glial cells, gene expression, nerve growth factors, chemokines, and ion channels (adapted from Krames 2014a).

Activating transcription factor 3 (ATF3)

The DRG are responsible for the synthesis of signaling molecules that are involved in a cascade of reactions that lead to a phenomena as survival or regeneration (Seijffers, Mills, and Woolf 2007). In fact, after lesion, the regeneration of peripheral nerves involves the assembly of cytoskeletal proteins, the formation of new membrane, and expression of multiple adhesion molecules, and receptors in the growth cones. This process is initiated by retrograde signals in the lesion site provoked by the alteration of multiple transcription genes in the neuronal cell body. This is accompanied by PNS sensitization, which is implicated in pain mechanisms and involves various mediators that trigger particular signal-transduction pathways. The majority of these pathways imply also the direct activation of transcription factors (Patodia and Raivich 2012). Among the various transcription factors that are up-regulated and also activated in the DRG after peripheral injury, is the activating transcription factor 3 (ATF3), which acts as an adaptative-response gene thanks to its ability for responding differentially according to the cellular context (Cachemaille et al. 2012) .

ATF3 is a member of the activating transcription factor/cAMP-responsive element-binding protein (ATF/CREB) family of transcription factors. In the nervous tissue, ATF3 is either not expressed or expressed at very low levels in physiological conditions and intact neurons. Indeed, ATF3 mRNA is only detected in DRG neurons after axotomy, without constitutive expression in naïve sensory neurons, after electrical stimulation or intense natural stimuli (Obata et al. 2003; Tsuzuki et al. 2001). Thus, some studies demonstrated that ATF3 is induced, in the PNS, in DRG neurons that were axotomized after transection of the spinal nerve or sciatic nerve. ATF3 up-regulation occurs in the DRG, not only in neurons but also in SGCs and Schwann cells (Isacsson, Kanje, and Dahlin 2005), of animals induced with different models of neuronal injury, such as peripheral nerve compression (Isacsson, Kanje, and Dahlin 2005), chronic constriction injury (CCI) (Obata et al. 2003), and spared nerve injury (SNI) (Tsujino et al. 2000a). In addition, the induction of ATF3 expression in DRG sensory neurons was also reported in animal models of joint inflammation (Nascimento et al. 2011; Nascimento, Castro-Lopes, and

Moreira Neto 2014) and/or degeneration (Ferreira-Gomes et al. 2012; Adães et al. 2015). So, ATF3 expression can be quite useful since the axotomized and the intact neurons coexist in the same DRG, and it is essential, in animal models of peripheral nerve injury, to identify the axotomized neurons. In agreement, ATF3 was postulated and assumed as a unique marker of neuronal injury (Tsuzuki et al. 2001; Obata et al. 2003; Tsujino et al. 2000a). However, some studies show that not all the populations of DRG neurons are immunoreactive (IR) to ATF3 and that the increase of ATF3-IR cells appears to be dependent on the pathological painful condition. Thus, while in the monoarthritis chronic inflammatory model ATF3 immunoreactivity was mainly found in the peptidergic medium- and small-size sensory neurons (Nascimento et al. 2011), in the CCI animals ATF3-IR cells were detected, in the majority, in peptidergic medium- and large-size and not in small-size neurons (Obata et al. 2003). Indeed, the study conducted by Isacsson, in 2005, demonstrated that after compression of the sciatic nerve of the rat, ATF3 was translocated to the nucleus of sensory neurons and of the surrounding SGCs, and also of Schwann cells (Isacsson, Kanje, and Dahlin 2005). This indicates that Schwann cells are activated through a series of reactions and signal transduction cascades that include ATF3. Moreover, some studies have shown that although ATF3 is highly induced in the DRG neurons after peripheral lesion, it is down-regulated during re-innervation of the peripheral target, being the only transcription factor known with these characteristics (Seijffers, Mills, and Woolf 2007; Tsuzuki et al. 2001). These studies suggested that ATF3 promotes neurite outgrowth and enhances peripheral nerve regeneration (Seijffers, Mills, and Woolf 2007). In addition, ATF3 is induced in all of the injured DRG neurons, at early time points after model induction. In fact, in an animal model of nerve transection, ATF3 was induced in both sensory and motor neurons as early as 12 hours after (Isacsson, Kanje, and Dahlin 2005). Moreover, it also persists until 2 weeks in nerve injury models and, in some cases, although the expression gradually decreases with time, it is still visible at 10 weeks after model induction. This temporal pattern of ATF3 induction implies this transcription factor as a unique neuronal marker of axonal injury that is useful in degeneration and regeneration related studies.

Neuropeptide Y (NPY)

Neuropeptide Y (NPY), also known as pancreatic polypeptide-related neuropeptide Y or neuropeptide tyrosine, is a 36 amino acid peptide rich in tyrosine, which belongs to the pancreatic polypeptide (PP) family. NPY was isolated from the porcine brain and is broadly distributed over the CNS and PNS, acting as a regulator molecule (Frisén et al. 1992; Solway et al. 2011; Magnussen, Hung, and Ribeiro-da-Silva 2015; Mantyh et al. 1994; Malet et al. 2017a; P. R. Brumovsky et al. 2002). To initiate the cellular signaling, NPY activates G-protein coupled Y receptors with seven-transmembrane domains which are implicated in diverse homeostatic roles, including pain modulation. Among the five cloned NPY receptors (Y1, Y2, Y3, Y4, and Y5), the Y1 (Y1R) and Y2 (Y2R) receptors, located at key pain signaling centers across the nervous system, are the most implicated in pain mechanisms in mammals, particularly in the dorsal horn of the spinal cord (P. R. Brumovsky et al. 2002; Magnussen, Hung, and Ribeiro-da-Silva 2015; Malet et al. 2017a; Intondi et al. 2008). Both NPY and their receptors are highly conserved in vertebrates, including humans (P. Brumovsky et al. 2005).

In naïve rats, the expression of NPY is nonexistent or occurs only in a few DRG neurons. On the other hand, NPY expression is upregulated after axotomy or peripheral nerve injury, particularly in large neurons and, as consequence, there is an increase in the immunoreactive fibers, mostly in the deeper laminae of the dorsal horn. However, the immunoreactivity for NPY is not exclusively found in the ipsilateral DRG neurons, since Sterne and colleagues showed that less than 1% of the contralateral DRG neurons also show immunoreactivity against NPY (Sterne et al. 1998; P. R. Brumovsky et al. 2002; Malet et al. 2017a). The expression of the Y1 and Y2 receptors is different from that of NPY since they are regularly expressed in the DRG neurons where they exhibit a cell-specific distribution pattern, with the Y1R being found in the small size DRG neurons, and the Y2R in the medium- and larger-size DRG neurons (Malet et al. 2017a).

Several studies showed that after nerve lesion, the DRG large neurons exhibit a profound synthesis of *de novo* NPY, consistent across the different models of neuropathic pain (Magnussen, Hung, and Ribeiro-da-Silva 2015). Additionally, in models of neuropathic

pain, the NPY expression appears to be correlated with the nerve lesion extension (Magnussen, Hung, and Ribeiro-da-Silva 2015). In Solway and colleagues' studies, it has been demonstrated that the increase of NPY lasts until 24 weeks after lesion, which indicates that the peptide was temporarily available to grant a long-term inhibition of the pronociceptive transmission (Solway et al. 2011). Other studies showed the NPY upregulation happens on a time-scale, which appears progressively in the first 5 days after lesion and reaches the peak after 15 days (Sterne et al. 1998). The mechanism that leads to *de novo* expression is not already known but it has been proposed that the origin might be the trauma and not the pain *per se*. The trauma could be the triggering factor since painful inflammation did not increase the immunoreactivity of NPY in the DRG neurons (Magnussen, Hung, and Ribeiro-da-Silva 2015). Some *in vivo* studies demonstrated that the NPY can potentially inhibit the release of diverse neurotransmitters from sensory neurons. These studies, together with other *in vitro* studies, suggested that NPY may have a role in the regulation of the transmission of nociceptive inputs from DRG neurons, particularly after peripheral nerve lesion (Mantyh et al. 1994).

The excitatory effect of NPY was reported in DRG cells withdrawn from animals induced with nerve injury. In this study, Ossipov and colleagues reported that the nerve injury-induced *per se* an upregulation of NPY expression in afferent fibers, particularly in medium- and large-size DRG neurons, as previously referred. In the same study, the time scale of NPY expression was consistent with previous reports, since the upregulation of NPY was noted three days after the nerve injury, and was also consistent with the development of tactile hypersensitivity (Ossipov et al. 2002). Nelson and colleagues, in 2019, reported that the intrathecal administration of both NPY and an agonist of Y1R reduced the mechanical and the cold hypersensitivity in the SNI model (Nelson et al. 2019). Also, both a conditional knock-down of NPY and an intrathecal administration of an antagonist of Y1R elicited a robust and reliable increase in the cold and mechanical hypersensitivity (Nelson et al. 2019; Solway et al. 2011; Intondi et al. 2008). These two different approaches demonstrated that the neuropathic hyperalgesia is tonically inhibited by the NPY that is endogenously released within the dorsal horn (Nelson et al,

2019). Furthermore, the intrathecal administration of NPY also reduced the gene expression evoked by a stimulus in the neuron population that expressed Y1R, suggesting that Y1R has a great capacity to endogenously alleviate pain (Nelson et al. 2019; Magnussen, Hung, and Ribeiro-da-Silva 2015). The particular effect of the Y1R agonists in the inhibition of pain is quite robust in SNI models (Nelson et al. 2019; Intondi et al. 2008). However, the NPY can also play a pro-nociceptive role, as above mentioned. Some studies showed that the intrathecal application of NPY exacerbated the hyperalgesic behavior after nerve injury, through activation of the Y1R in myelinated nerve fibers. Another study showed that the subcutaneous application of NPY in the animal hind paw, induced with CCI, exacerbated the hyperalgesic response to nociceptive stimulation. These contradictory results could be explained by the participation of different subtypes of NPY receptors and also by the type and local of application/administration (Intondi et al. 2008; Solway et al. 2011). Nevertheless, the hypothesis that NPY relieves the experimental neuropathic pain stays plausible since Sapunar and colleagues showed that the overexpression of NPY reduces pain-induced behavior and the DRG neurons hyperexcitability after lesion (Sapunar et al. 2005).

As previously referred, also Y2R plays a role in the modulation of pain. Some studies showed that the activation of Y2R by NPY in DRG neurons leads to an inhibitory effect of Ca^{++} currents (Sapunar et al. 2005). Besides that, a direct correlation between neuronal excitability and the activation of different receptors showed that the Y2R activation is associated with increased firing, particularly in small- and medium-size DRG neurons (P. Brumovsky et al. 2005). Another study has shown that the intrathecal administration of a Y2R agonist reduced not only the behavioral response to the noxious stimulus but also the hyperalgesia associated with inflammation. On the other hand, and in accordance, the administration of a Y2R antagonist reversed the anti-allodynic effect of NPY in the SNI model. Additional data, showing that the axotomy increased the inhibition of Ca^{++} currents mediated by Y2R in DRG neurons and that NPY attenuated the excitatory post-synaptic currents via Y2R, suggested that NPY acts in the Y2R pre-synaptic receptor to inhibit the release of pro-nociceptive neurotransmitters (Intondi et al. 2008). In contrast to the Y1R, the Y2R is mostly present in small- and medium-size

DRG neurons, and after axotomy, their expression is increased, especially in small-size neurons (P. Brumovsky et al. 2005).

Animal models of NP

Although researchers know that the NP condition develops after axotomy in somatic nerves, the underlying and related mechanisms are still not fully understood. Thus, experimental animal models, using species such as rats or even mice, have been developed (Bourquin et al. 2006; Payne, Belleville, and Keast 2015). Animal models allow the researchers to correlate different characteristics of clinical pain syndromes, like neuropathic pain, with quantification of pain behaviors, which permits the understanding of some of the mechanisms involved in pain processing (acute or chronic). However, pain in animals can only be assessed by the evaluation of behavioral cues, like simple withdrawal reflexes and complex voluntary and intentional behaviors. Behavioral tests may use electrical, mechanical, or even thermal stimuli, but electrical stimulation is not as well accepted as the others since the animals have to be restrained and this could lead to high levels of anxiety and stress, which can trigger modulatory effects on pain sensitivity (Baliki et al. 2005).

The Spared Nerve Injury or SNI model was established by Décosterd and Woolf, in 2000. This neuropathic pain model is induced through the partial denervation, in which two of the three-terminal distal branches of the sciatic nerve, the tibial and common peroneal nerves, are cut distally from the sciatic trifurcation and the third branch, the sural nerve, is spared. This model was developed as a direct method to investigate changes in both injured primary sensory neurons and neighboring intact sensory neurons. This model was also developed taking into account a previously existing model, the CCI model developed by Wall in 1979, that consists of doing a complete transection of a major peripheral nerve (sciatic nerve with an accompanying lesion to the saphenous nerve) to produce an entire denervation of the distal hind limb (Decosterd and Woolf 2000). In addition to these two models, also the Spinal Nerve Ligation (SNL), described by Kim and

Chung in 1992, is one of the most used. All these neuropathic pain animal models, in relation to the respective authors, are illustrated in figure 6.

The SNI model has a stable prolonged duration, lasting until 12 months, and has been described as a model that induces prolonged changes in mechanical sensitivity and thermal responsiveness in animal species like rats and mice. These behavioral dysfunctions are caused by modifications in neuronal cells firing activity at the supraspinal and spinal levels (neuron-glia cells communication) (Boccella et al. 2018). This model allows the researchers to test distinct sensory modalities in the hind paw of the rat since the sural innervation is intact. Moreover, the model also allows the study at the biochemical and cellular levels in injured and non-injured territories, in a separated way, and at peripheral or central levels (Pertin, Gosselin, and Decosterd 2012). The surgery is relatively easy to perform, reliable and it is associated with a reproducible, long-lasting, and sound hypersensitivity, manifested as hyperalgesia and allodynia. The model is also effective since it does not involve excessive inflammation, as it occurs in the CCI model (Pertin, Gosselin, and Decosterd 2012; Shields, Eckert III, and Basbaum 2003). Studies showed that sparing the sural branch in rats is the most robust and used model (Shields, Eckert III, and Basbaum 2003).

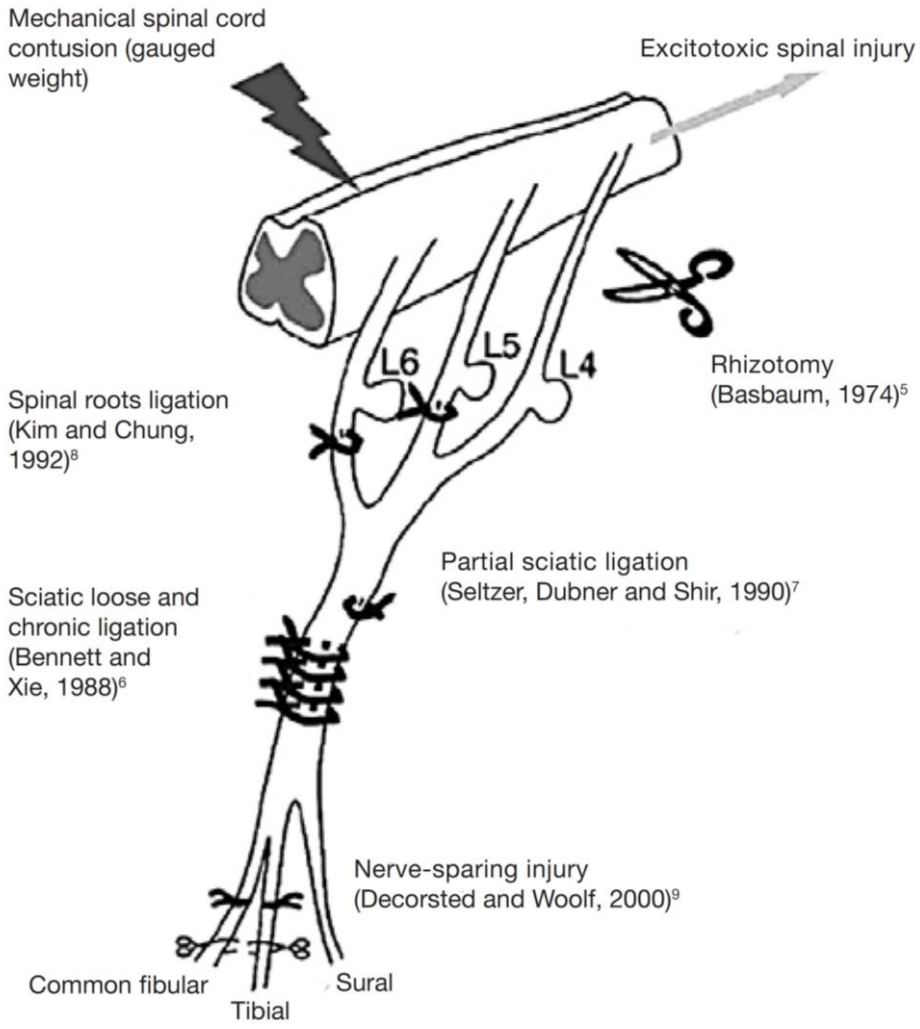

Figure 6: The various animal models that mimic the neuropathic condition (adapted from (Garcia-Larrea and Magnin 2008)).

Current management of NP

The first problem that physicians face in pain management is the fact that there are no therapeutic approaches that can prevent the occurrence of neuropathic pain conditions (Clifford J. Woolf and Mannion 1999). Although many neuropathic pain treatment possibilities exist, only some of them were investigated using mechanism-based research. Most of the treatments focus on the proven efficacy in the neuropathic pain states, neglecting the evidence for therapeutic strategies targeting distinct mechanisms (Gierthmuhlen and Baron 2016).

In the current guidelines for the pharmacological treatment of neuropathic pain emerge three main categories of drugs: first-line, second-line, and third-line medications. The first-line medications includes antidepressants with both norepinephrine and serotonin reuptake inhibitors (Dworkin et al. 2003). The tricyclic antidepressants (TCAs) are drugs that block the reuptake of norepinephrine (noradrenaline) and serotonin, block the hyperalgesia induced by N-methyl-D-aspartate (NMDA) agonists, and also have sodium channel blocking properties. They are the first medication category that showed to be effective in neuropathic pain when compared with placebo (Kerstman et al. 2013; Dworkin et al. 2003). Another subcategory of first-line medications is the calcium channel alpha2-sigma ligands, which include both the antiepileptic drugs gabapentin and pregabalin, and which bind to presynaptic voltage-gated calcium channels in the dorsal horn, leading to a decrease of excitatory neurotransmitters release such as glutamate and SP (Kerstman et al. 2013; Dworkin et al. 2003). As seen the first-line medications act at central levels of the nervous system, and for that they usually produce central adverse-effects that could limit their use (Gierthmuhlen and Baron 2016).

The second-line medications are opioids and tramadol, and are used when the first-line ones do not have a satisfactory response. Opioids have been shown to produce as much analgesia as TCAs. It was also demonstrated that opioids decrease pain, disability, and allodynia, and that they also improve the performance in daily tasks and sleep (Kerstman et al. 2013; Dworkin et al. 2003; Gierthmuhlen and Baron 2016). On the other hand, tramadol is a unique analgesic agent that showed low-affinity binding for the mu-opioid receptor and inhibits the reuptake of norepinephrine and serotonin. So, taking this into account, tramadol is considered as a weak opioid agonist that mimics some properties of TCAs. Studies that compared it with placebo showed that tramadol decreases pain and allodynia, and also improves the quality of life. Both for opioids and tramadol the adverse effects include nausea, constipation, and somnolence (Kerstman et al. 2013; Dworkin et al. 2003).

The third-line medications includes some antidepressants and anti-epileptic medications (Kerstman et al. 2013). Besides these classical approaches, Woolf and

Manion proposed, in 1999, an alternative treatment target, the sodium channels insensitive to tetrodotoxin (TTX). These channels have the particularity that they are expressed in specific sensory neurons that avoid the adverse effects of other membrane stabilizers such as TCAs, sodium-blockers channels, and anticonvulsants (Clifford J. Woolf and Mannion 1999).

The treatment of neuropathic pain is very complex and for that reason, the guidelines recognize that the medication combination has a greater analgesic effect than monotherapy. However, the use of more than one drug is associated with an increase of the side-effects (Kerstman et al. 2013). Moreover, although all the drugs have their efficacy proven, they are not satisfactory neither for the patients nor the physicians, since they only offer a modest reduction of the perception of pain (Jensen et al. 2011; Kerstman et al. 2013). The drugs usually used for the treatment of NP, like TCAs and other first-line medications, have their efficacy limited and unwanted drug-related adverse-effects (Clifford J. Woolf and Mannion 1999). So, taking these into account, new approaches were developed to restore the function and reduce disability with the minor adverse-effects possible and with a lesser resistance or insensitivity than seen with the pharmacological approaches.

Non-pharmacological treatment for NP

Diverse techniques are necessary to help the patients that suffer from various chronic neuropathic pain syndromes. Various stimulation techniques have been found promising in the relief of pain in various types of chronic neuropathic syndromes. Some of these techniques are transcutaneous electrical nerve stimulation (TENS), peripheral nerve stimulation, nerve root stimulation, and also spinal cord and deep brain stimulation (Gierthmuhlen and Baron 2016).

The spinal cord stimulation (SCS) may be indicated for the treatment of certain neuropathic pain diagnoses. This technique showed positive results in patients that have tried other therapies that have failed, principally in low back pain syndrome and

complex regional pain syndrome (CRPS). The results also showed a cost-effectiveness after 2.5 years of follow-up and that the patients treated with SCS returned to work more often than the patients treated with conventional medical therapies (Kerstman et al. 2013).

Spinal Cord Stimulation (SCS)

Spinal cord stimulation (SCS), also known as epidural electrical stimulation of the dorsal columns of the spinal cord or conventional spinal cord stimulation of the dorsal columns, was firstly described by Shealy et al., in 1967, motivated by the gate control theory (Slavin 2014; Tilley et al. 2016; Koetsier et al. 2019). Meanwhile, this approach has gained support as a therapeutic option to treat chronic pain, since it is an optimal treatment for patients experiencing chronic neuropathic pain. SCS works by blocking or modulating electrical signals that encode painful stimuli while reducing its potentiation, and the leads are traditionally placed in the posterior epidural place, as illustrated in figure 7 (Pan et al. 2016; Tilley et al. 2016; Caylor et al. 2019). Although SCS is mostly used alone, some studies show that it can be used as a complementary treatment option for pharmacological therapy (Koetsier et al. 2019). In the USA, the guidelines from the American Society of Regional Anesthesia and Pain and the American Society of Anesthesiologists recommended SCS as the first option for neuropathic pain, since clinical studies showed that SCS reduces pain in patients with failed back surgery syndrome, CRPS type I and II, and post-herpetic neuralgia (PHN). Moreover, studies with traditional SCS, in animals, showed improvement in pain-like behaviors (Gong, Johanek, and Sluka 2016).

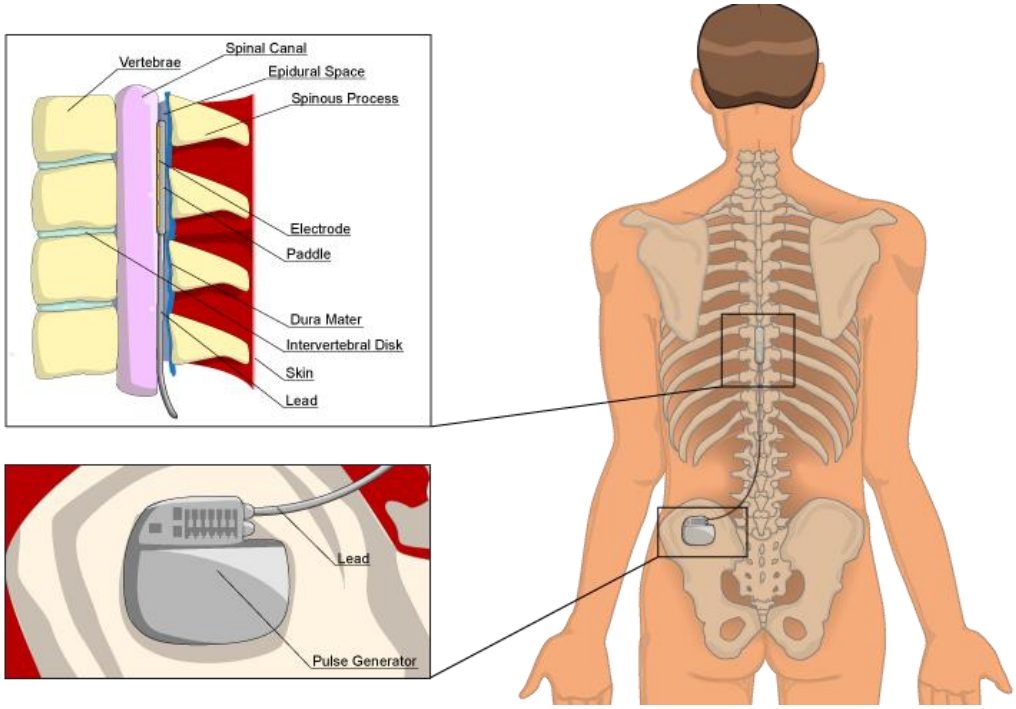

Figure 7: Epidural placement of spinal cord electrodes for SCS (adapted from https://www.ecpc1.com/patients_spinalcordstimulator.php)**.**

The electrical basis of stimulation is the Ohm's Law: Voltage (V) = Current Intensity (I) x Resistance (R), where we can control de V and I, but not the R. The resistance is affected by intrinsic patient's factors like the composition of the cerebrospinal fluid (CSF), other fluids, dural thickness, among others. The stimulation pulse is the energy applied to the target point and is characterized by the amplitude (strength) and pulse width (duration). There are three parameters, illustrated in figure 8, that can be controlled: frequency, pulse width, and amplitude. Pulse frequency is defined as the number of stimulation pulses delivered *per* second, where higher frequencies increase the number of action potentials delivered, and therefore changes in frequency result in changes in sensation. The pulse width is the parameter that defines the duration of the stimulation pulse, typically measured in microseconds (µs). A greater pulse width will result in depolarization of fibers with higher thresholds since stimulation stays on longer. Lastly, amplitude is the strength of the stimulus measured in volts or milliamps (mA). The higher

the amplitude, the greater the size of the electrical field, which results in the depolarization of nerves over a large area (Pain 2014, IASP).

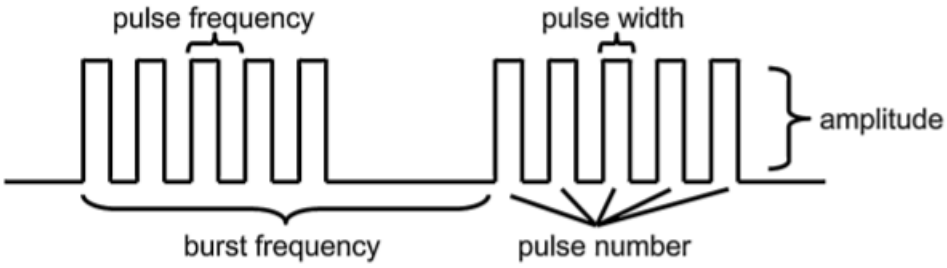

Figure 8: Stimulation parameters that define the shape of the stimulation: pulse number, pulse frequency, pulse width, burst frequency, and amplitude (adapted from (Crosby et al. 2015))**.**

The spinal cord stimulation technique has multiple modalities: traditional, burst, and high-frequency. The traditional SCS uses a tonic paradigm to deliver continuous pulses of various frequencies, pulse widths, and intensities to the spinal cord (SC). The adverse-effect that disturbs the patients the most is paresthesia (abnormal sensation, whether spontaneous or evoked) in the stimulated dermatomes. Consequently, as an alternative to this adverse-effect two different techniques have emerged: the burst SCS and the high-frequency SCS. The burst SCS technique uses small bursts of pulses of stimulation, instead of continuous pulses like in traditional SCS, which allows avoiding the sensation of paresthesia. Recently, it was showed that burst SCS reduces neuropathic pain better than traditional SCS, in the majority of patients, with the benefit of not generating paresthesia (Crosby et al. 2015). The burst SCS waveforms replicate endogenous burst-firing Na^{+} spikes that ride on a Ca^{++}-dependent plateau. The pulses *per* burst and pulse width parameters constitute the charge density, which has been shown to work as a predictor of burst SCS efficacy. Snider and colleagues showed, in 1998, that burst SCS has a ceiling effect since less effects were seen at greater than 6-7 spikes *per* burst (Caylor et al. 2019). In both SCS techniques – traditional and burst – the parameters can be modulated, along with electrode configuration and placement on the SC, to maximize

pain relief taking into account the needs of the individual patient (Crosby et al. 2015). The high-frequency SCS is based on delivering kilohertz-frequency impulses via percutaneously placed epidural leads. The paresthesia-free is achieved by employing a charge delivery strategy with high frequency and low amplitudes pulses to maximize the total charge delivery without having the paresthesia effect (Caylor et al. 2019). In general, SCS techniques show the ability to facilitate changes at the molecular level, which may be behind the physiological mechanisms that result in the relief of pain sensation and improvement in the quality of life (Tilley et al. 2017). The three different modalities deliver energy to the posterior spinal cord tracts instead of delivering to the sensory neurons (Caylor et al. 2019).

SCS techniques were shown to be effective for treating an entire limb pain, and diffuse neuropathies, like failed back surgery and CRPS. Nevertheless, it can make the treatment of specific targets, such as feet or even pelvic pain, difficult. Another major difficulty, being even considered as an adverse-effect, is the sensation of paresthesia, which can lead patients to give up from trials (Pan et al. 2016; Tilley et al. 2017; Caylor et al. 2019). These limitations are related to postural effects, given that the leads can move with changes in postural position. Additionally, other factors can contribute to the eventual loss of effectiveness with time, such as the inherent plasticity of the central nervous system synaptic mechanisms and the compensatory spinal plasticity/habituation (Esposito et al. 2019; Pan et al. 2016). The majority of diseases or lesions that affect PNS are local, rather than systemic, and for that reason, the targets of the therapies should also be regional (Sapunar et al. 2012).

Taking these factors into account, the need to try new targets for electrostimulation has emerged, namely the dorsal root ganglia.

Dorsal Root Ganglion Stimulation (DRG-S)

In clinical settings, DRG stimulation (DRG-S), also known as ganglionic field stimulation, is an approach of electrical stimulation (neuromodulation) that can be used in

combination with SCS or alone. It has already been used for several years, being firstly described in 1995 and 1998, by Wright and Colliton, and also by Alo and colleagues (Aló et al. 1999; Wright and Collition 1995). However, the majority of reports in the literature only started to appear within the past 5 years. Nowadays, DRG are a primordial choice as clinical targets for neuromodulation since they have an important role in the modulation of peripheral and central sensory processing. As earlier mentioned, they contain the cell bodies of several pseudounipolar afferent neurons that detect peripheral stimuli, transduce them into action potentials and transmit these to the spinal cord dorsal horn. These cell bodies are surrounded by SGCs, forming together a structural and functional unit that is known to participate in the mechanisms leading to neuropathic and inflammatory pain, together with the immune system. Thus, DRG are a potential target since the pathological changes that characterize chronic pain states occur within the sensory neurons in these structures (Caylor et al. 2019). In addition, DRG have the advantage of being anatomically and physically accessible (Krames 2014a; Liem et al. 2016). Indeed, the electrode implantation is similar to the procedure for SCS, since it involves accessing the epidural space, guiding the electrode into the neuroforamen, and positioning it in an adjacent position to the DRG, as illustrated in figure 9. Neuromodulation of DRG is an emerging neurotechnology also because the electrodes are more steerable and flexible. Accordingly, there are, currently, multiple published clinical studies demonstrating that DRG electrical stimulation was effective in reducing pain states in humans (Krames 2014a; Caylor et al. 2019). The results indicate a high success rate in terms of postural effects, pain relief, and paresthesia intensity when compared with SCS, improving the quality of patients' life (Caylor et al. 2019; Krames 2014a; Liem et al. 2016). Gemes et al., in 2013, has shown that the field stimulation can lead to the block of the passage of impulse trains through the T-junctions of the sensory neurons, where the central and peripheral processes join the stem process (Gemes et al. 2013). Additionally, it has been proposed that DRG stimulation may reduce the net nociceptive input to the spinal cord, restoring the DRG filtering function that may have been altered following peripheral nerve damage (Krames 2014a; Koopmeiners et al. 2013b).

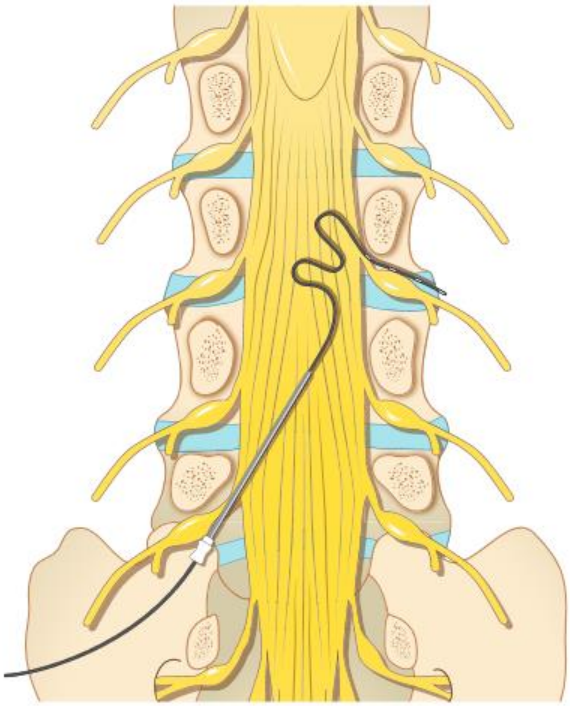

Figure 9: Leads implantation for dorsal root electrostimulation (adapted from (Esposito et al. 2019))**.**

Hypothetical mechanisms of action and pain relief

In 2014, Krames has proposed a hypothetical mechanism for the action of neuromodulation of the DRG. This mechanism, which results in chronic pain relief and amelioration of secondary symptoms, is based on a few main topics. The first one is related with the vasodilation in the periphery, and it was proposed that it happens as a result of downstream effects. The second suggests that neuromodulation leads to the stabilization of peripheral nociceptors sensitization, also resulting from a downstream effect, and to the release of neuromodulators in the spinal dorsal horn, an upstream effect. The decrease in hyperexcitability of DRG neurons happens as a consequence of the down-regulation of abnormal TTX-sensitive Na^+ channels, upregulation of K^+ channels, and also the restitution of the normal Ca^{++} current flow. The third topic is related to the deactivation of sensitized dorsal-horn wide-dynamic-range neurons, through the potential modulation of supraspinal brain regions involved in the

development and maintenance of chronic pain, namely the activation of supraspinal centers and/or the turning off of other brain centers that have been triggered by injury of peripheral afferent fibers or inflammation. The last point is related with the trigger of potential genetic effects at DRG and SC, and with the stabilization that occurs in the activation of microglia, responsible for the release of cytokines, such as chemokines and interleukins, and nerve growth factors, among others, which were abnormally released in response to PAF injury or inflammation (Krames 2014a).

Another mechanism proposed by Koopmeiners and colleagues, in 2013, is based on an *in vitro* study of a combined neuromodulation using DRG-S – SCS. The mechanism of action is based on the fact that the combined neuromodulation altered the Ca^{++} influx, which slowed the nerve conduction velocity, reduced action potentials propagation, and the neuronal excitability when compared with a baseline before stimulation. So, the main idea in this mechanism is that the DRG stimulation provides analgesia by blocking action potentials, reducing the somatic excitability, as well as the pathologic ectopic activity in the neuronal cell body. The results also showed that DRG-S limited the action potentials propagation through the amplification of the T-junction activity since this structure is the natural process where the action potentials are filtered. This is particularly important in neuropathic pain, since in this condition there is loss of the filtering ability at the T-junction in the nociceptive sensory units (Koopmeiners et al. 2013b).

In 2016, Pawela and colleagues decided to investigate the effect of the DRG-S, not only at the peripheral level but also in the brain regions that respond to the analgesic effect after neuromodulation at the DRG level, by using functional magnetic resonance imaging (fMRI). The results showed that the DRG-S attenuates the Blood-Oxygen-Level-Dependent (BOLD) signal response to noxious electrical hind paw stimulation, in brain regions that are directly related with acute pain, and which are considered as part of the pain matrix. Besides these ascending tract regions, also areas in the pain control descending tracts were implicated in this decreased BOLD signal response. Pawela speculated that this negative BOLD response could be the result of the activation of both opioid and dopamine receptors, which causes vasoconstriction in the presence of

increased neuronal plasticity. Additionally, it also corroborated the previous hypotheses that implicate the T-junction in the main mechanism. In fact, the results of the neospinothalamic activity were consistent with the mechanism hypothesized by Koopmeiners (Koopmeiners et al. 2013a). The use-dependent process at the T-junction is based on the actions of the Ca^{++}/calmodulin-dependent protein kinase II (CaMKII), which is widely involved in neuronal plasticity. Once activated, this signaling molecule remains persistently activated, which is in accordance with the continuing reduction of the nociceptive response in brain regions after the neuromodulation of DRG (Pawela, Kramer, and Hogan 2017).

In addition to these hypothetical mechanisms, other mechanisms have been proposed by distinct authors, but none contradicts the hypothetical mechanisms mentioned above, because rather, on the contrary, they even overlap and complement them. Some of the hypothetical mechanisms were summarized by Kramer and colleagues, in 2018, and in the majority of them, the basic idea is that the two main mechanistic structures are the Ca^{++} channels and the T-junction. Apart from the key idea of the involvement of the T-junction and Ca^{++} channels, the authors also refer to the theories based on the implication of the modulation of neural activity in supraspinal regions and of the modulation of sympathetic pathways, which are corroborated by different authors, as illustrated in figure 10 (Kramer et al. 2018).

In spite of all these views, in truth, the mechanisms underlying DRG-S analgesia are still largely unknown.

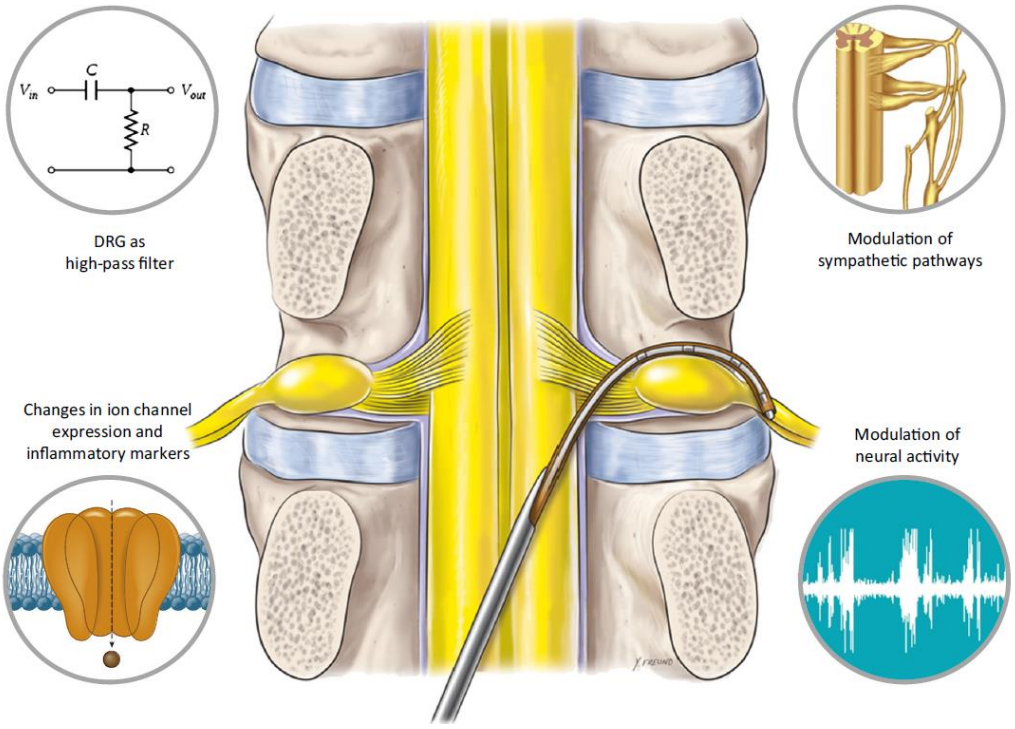

Figure 10: Involvement of the T-junction, calcium channels, modulation of sympathetic pathways, as well as modulation of neural activity as hypothetical mechanisms underlying the efficacy of DRG-S (adapted from (Kramer et al. 2018)).

DRG-S versus SCS: advantages and adverse-effects

The DRG-S is a clinically effective intervention for analgesic purposes and its efficacy is greater after initiating treatment when compared with the SCS. This could be due to the greater mechanical stability of DRG-S and to the fact that it uses lower stimulation frequencies (20 Hz) in comparison to SCS (40-60 Hz) (Pan et al. 2016). As referred before, SCS presents some difficulties in reaching specific areas such as the foot and the groin. This is related to the fact that the relevant dorsal column fibers are difficult to reach in the epidural access and may require stimulation at higher amplitudes than the usual, which can generate painful nerve root activation. DRG-S is a neuromodulation technique that overlaps this problem, since in DRG-S the recruitment is at the somata, which allows avoiding these issues. In accordance, DRG-S showed to be more effective in treating diseases such as low back pain, phantom limb pain, causalgia, diabetic peripheral

neuropathy, and perineal pain, among others. When evaluated in terms of efficacy in releasing the pain states, DRG-S has shown to be statistically superior to the SCS after three months of treatment, and also when compared in terms of pain relief and superiority throughout 12 months of treatment. This sustained efficacy is supported by the greater specificity of stimulation for painful areas, less variation in stimulation intensity with postural variation, and also by the ability to deliver paresthesia-free analgesia. The paresthesia effect reported by the SCS patients is highly related with changes in posture that alter the distance between the electrodes and the dorsal columns because of their motion within the CSF. This alteration in the distance can give rise to two different outcomes: a) patients can decrease the stimulation amplitude in an attempt to avoid the painful stimulation; or b) the distance between the electrodes and the dorsal columns will result in less dorsal column recruitment and insufficient stimulation. Both outcomes will result in decreased therapeutic/pain relief. In the DRG-S the postural effects are minimized and much less vulnerable perturbations are reported. This happens because the DRG-S electrodes are placed, as said before, in juxtaposition with the DRG and the foramen, with a little space filled with CSF between them, which results in fewer perturbations in their placement due to physical movement. This improvement in the location of the placement of the electrodes does not just solve the paresthesia problem but it also decreases the probability of the patients requiring the reposition or replacement of the electrode, minimizing the health care costs, the risks, and, more important, the patient dissatisfaction. Another advantage of DRG-S regarding to the SCS is the lower rate of energy consumption. In the SCS the layer of CSF impedes the stimulation to reach its dorsal column targets. Because of this, the energy needed to overlap this layer in SCS has to be high. In the DRG the layer of CSF is very thin, and thus less current intensity is needed for the stimulation to reach its target. This results in a lower amplitude required for reaching the stimulation. In comparison, DRG-S requires up to 93% less power than SCS. One positive outcome is the life-time of the batteries and the possibility of increasing the invisibility of the treatments since the patients have fewer interactions with the neuromodulation

system, which would otherwise be perceived as costly and result in overall poorer outcomes (Esposito et al. 2019).

Despite of these advantages, the major obstacle in DRG-S is the placement of the DRG electrodes within the neural foramen. The electrode implantation involves accessing the interlaminar contralateral epidural access and the guiding of the electrode into the neuroforamen toward the inferior aspect of the contralateral pedicle. The flexible electrode is guided and positioned adjacent to the DRG (Esposito et al. 2019). In the clinical studies, it is widely accepted that the technical approach is difficult, with a higher learning curve than the placement of the traditional SCS electrodes. Apart from this, also the placement of the electrode is associated with a risk of nerve or DRG injury; however, recent studies report that the safety in DRG-S is equivalent to that of SCS (T. Deer et al. 2020). This could be explained by the fact that DRGs are considered resilient structures that are surrounded by a high density of capillaries, which, at least, contribute to its robustness (Caylor et al. 2019).

An approach to dorsal root ganglion neuromodulation

The DRG is a structure that emerges bilaterally in each sensory dorsal root, near to the convergence point of the peripheral nerve with the motor efferent fibers. So, both anatomically and functionally, it is localized in the junction of the two nervous systems, central and peripheral, as illustrated in figure 11. Taking this into account, the DRG is the perfect target with multimodal opportunities for the management of pain (Liem et al. 2016). Before the launch of neuromodulation, the DRG was already the primordial choice as a therapeutic target for the delivery of anti-inflammatory steroids, for surgery ablation (ganglionectomy), and both radio-frequency ablation and radio-frequency surgery (Krames 2014a). Later, some studies have shown that the electric field stimulation of the dissociated DRG cell bodies reduced the frequency and the amplitude of the ectopic discharges, which characterize neuropathic pain. Thus, it was

hypothesized that the neuromodulation applied to the DRG could be an effective approach to the management of pain (Liem et al. 2016).

In humans, the DRG can be accessed both from the outside of the neural foramen inside to the epidural space, and in the opposite, from the epidural space to the outside of the neural foramen (Krames 2014a). The approach for implantation of the electrodes for SCS is done along the epidural space. For the DRG-S, the approach suffers some modifications, which allow the electrodes to be placed near to the dorsal nerve roots. Recently, an SCS system was re-designed and incorporates extremely flexible and small diameter electrodes. This improvement allows the electrodes to be introduced within the DRG cell bodies in the vertebral foramen, through the percutaneous retrograde placement with fluoroscopic guidance (Liem et al. 2016). The access is facilitated using a standard loss-of-resistance technique. The electrode position is determined through an intraoperative programming of the device, which confirms the paresthesia overlap with the painful regions (Liem et al. 2013).

On the other hand, in animal models, like the rat, the DRG are much smaller and the surgery to access them is methodologically challenging. Two different techniques may be used to access the DRG cells bodies of the rats. The first consists of removing the soft tissue and doing the approach through the intervertebral foramen. The second one, consists in, apart from the excision of soft tissue, also removing the laminar bone, exposing the DRG caudal pole. The first approach is more challenging and successful results take longer to be achieved, but it is less traumatic. Small differences in the surgical approach may result in significant behavioral differences because it was shown that rats submitted to extensive laminectomy show hyperalgesia, while rats with minimal laminectomy did not show any increase in pain-related behaviors (Sapunar et al. 2012).

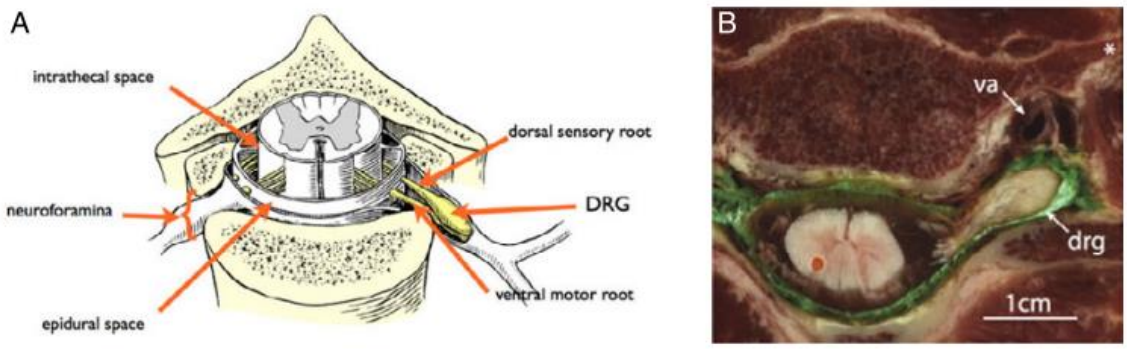

Figure 11: Position of dorsal root ganglion outside of the intervertebral neural foramen showed in a cartoon (A) and in a real picture (B) (Krames 2014b)**.**

In conclusion, animal pain models of neuropathic pain may provide complementary results that can be used to understand some of the neurobiological mechanisms implicated in the positive effect of DRG-S on pain relief. Additionally, research on experimental animals is an important part of translational research and previous studies showed that DRGs are easily accessible in rats (Puljak et al. 2009b; 2009a). However, there are some limitations associated with the size of DRG in rats, much smaller than in humans, and with the electrodes/leads implantation protocol.

Objectives

Acknowledging the clinical results, where an effective relief of neuropathic pain was observed after dorsal root ganglion stimulation protocols, we aimed at better understanding the DRG-S mechanisms by implementing a successful protocol of DRG electrical neurostimulation that could be effective in reducing the nociceptive behavior in a rat model of chronic neuropathic pain. For that purpose, the following objectives were pursued:

- **To build and implant suitable electrodes devices for *in vivo* neurostimulation –** To achieve this goal we performed literature research and the acquisition of the wires for the electrodes construction as well as the external neurostimulator. We also performed several pilot implantation surgeries in order to improve the surgical approach.
- **To evaluate the effect of electrodes implantation surgery and their maintenance on the nociceptive behavior –** To achieve this, the electrodes implantation surgery was performed in a naïve animal, and 7, 14 and 21 days after surgery the nociceptive behavior was evaluated by using the von-Frey, Pin-Prick, and Acetone tests. Also, the motor coordination was evaluated by the Rotarod test.
- **To evaluate the effect of DRG-S on the nociceptive behavior of SNI animals –** We performed the electrodes implantation surgery and 7 days after we induced SNI in the rats. We evaluated the nociceptive behavior and motor coordination at 7, 14 and 21 days after SNI induction, on animals with a neurostimulation protocol.
- **To evaluate the effect of DRG-S on spontaneous pain of SNI animals –** We assessed the spontaneous pain by performing the Conditioned Place Preference (CPP) test on the days between the two-time points of behavioral evaluation.
- **To evaluate the effect of DRG-S on the expression of molecules known to be implicated in neuropathic pain mechanisms at the DRG –** To achieve this, the

expression of ATF3 and NPY proteins were evaluated by immunohistochemistry in the DRG of control, SNI, and SNI with DRG-S animals.

Materials & Methods

Animal Handling

Experiments were carried out in adult male Wistar rats weighing between 270gr and 320gr at the beginning of the experiments. A total of 44 rats were used: 23 were from Charles River (France) while the remaining 21 were bred in Biotério Geral do Centro de Investigação Médica da Faculdade de Medicina da Universidade do Porto. From the 44 animals, a total of 13 animals did not follow the nociceptive behavior, motor coordination, and/or neurostimulation protocols since they reached the humane endpoints and were euthanized. Other 18 animals were not included here as they did not follow the protocols until the end or data regarding nociceptive behavior did not validate the correct development of a neuropathic pain condition. Thus, only a total of 13 animals followed the protocols and were included in the scheme of figure 12.

All the animals were housed two or three *per* cage at Biotério Geral do Centro de Investigação Médica da Faculdade de Medicina da Universidade do Porto, with water and food *ad libitum*, and under controlled conditions of temperature and humidity, 22±2ºC and 55±5%, respectively. The lightning was also controlled (12-hour light/12-hour dark cycle). The animals from Charles River required a period of adaptation to the animal facility conditions of 5-7 days. Afterward, all the animals went through a period of 5 days of habituation to the elevated grid, and manipulation by the researcher. During the whole experimental period, the health of the animal was monitored, with particular attention to weight loss, loss of mobility, poor healing, opening of stitches, and self-mutilation (autotomy). All the experimental procedures were performed according to the ethical norms for the study of experimental pain in conscious animals (Zimmermann 1983), as well as the regulation of local authorities on the use of animals for scientific purposes. The experiments were approved by the Ethical Committee for animal experimentation and welfare (ORBEA - *Órgão Responsável pelo Bem-Estar dos Animais*) from the Faculdade de Medicina da Universidade do Porto.

Experimental Groups and Experimental Design

The experimental groups are schematized in figure 12. One animal was subjected to the electrodes implantation surgery, as a *Sham* control of this surgical approach, but did not receive any DRG neurostimulation protocol (**Sham-DRG-S**). SNI was induced in different sets of animals, as a model of peripheral neuropathic pain. In one group of SNI rats the disease was allowed to develop until 14 days after induction and the animals were only behaviorally tested to monitor the development of neuropathic pain hypersensitivity (**SNI 14d**). Two groups of SNI animals, each maintaining the neuropathic condition for 7 or 14 days after induction, received DRG neurostimulation protocols (**SNI+DRG-S 7d** and **SNI+DRG-S 14d** groups). One SNI animal was allowed to develop the neuropathic condition until 21 days after induction and received DRG neurostimulation protocols (**SNI+DRG-S 14d-21d**). Mechanical allodynia and hyperalgesia, cold thermal allodynia and motor coordination were assessed in all animals through adequate standard behavioral tests. In addition, spontaneous pain behaviors were also evaluated in SNI animals that were allowed to maintain the neuropathic condition for longer than 7 days.

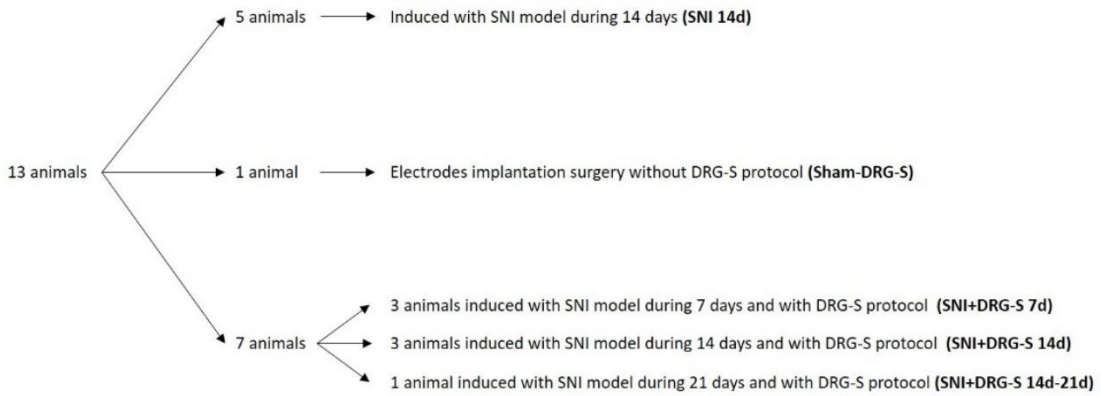

Figure 12: Distribution of the animals by the different experimental groups.

The experimental design of behavioral testing in each set of animals is schematized in figures 13-17, and is briefly described as follows:

a) **Sham-DRG-S animal:** on day -1 before the surgery for electrodes implantation (baseline), and on days 7, 14, and 21 after surgery, the animal was submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the nociceptive behavior and motor coordination. After nociceptive assessment, the animal was sacrificed by perfusion (figure 13);

b) **SNI 14d group:** on day -1 before SNI induction (baseline), and on days 7 and 14 after neuropathic pain model induction, the animals were submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the nociceptive behavior and motor coordination. Between days 9 and 12, the animals went through the conditioning days of Conditioned Place Preference (CPP), without any conditioning. On day 14, after nociceptive assessment, the animals were sacrificed by perfusion (figure 14);

c) **SNI+DRG-S 7d group:** On days -8, and on day 7, the animals were submitted to von-Frey, Pin-Prick, and Acetone tests to evaluate the nociceptive behavior and motor coordination. On day 7, the animals were also tested for the motor threshold induced by neurostimulation and received a session of 30 minutes of neurostimulation. The animals were sacrificed by perfusion, after the behavioral assessment (figure 15).

d) **SNI+DRG-S 14d group:** On days -8, and every 7 days up to 14 days, the animals were submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the nociceptive behavior and motor coordination. On days 7 and 14, the animals were also tested for the motor threshold induced by neurostimulation and received a session of 30 minutes of neurostimulation. Between the day 9 and 12, the animals were conditioned on the CPP, receiving a daily neurostimulation session in these days. On day 14, the SNI+DRG-S 14d animals were sacrificed by perfusion (figure 16).

e) **SNI+DRG-S 14d-21d animal:** On days -8, and on 14 and 21 days, the animal was submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the

nociceptive behavior and motor coordination. On days 14 and 21, the animal was also tested for the motor threshold induced by neurostimulation and received a session of 30 minutes of neurostimulation. Between the day 16 and 19, the animal was conditioned on the CPP, receiving a daily neurostimulation session in these days. On day 21, the animal was sacrificed by perfusion (figure 17).

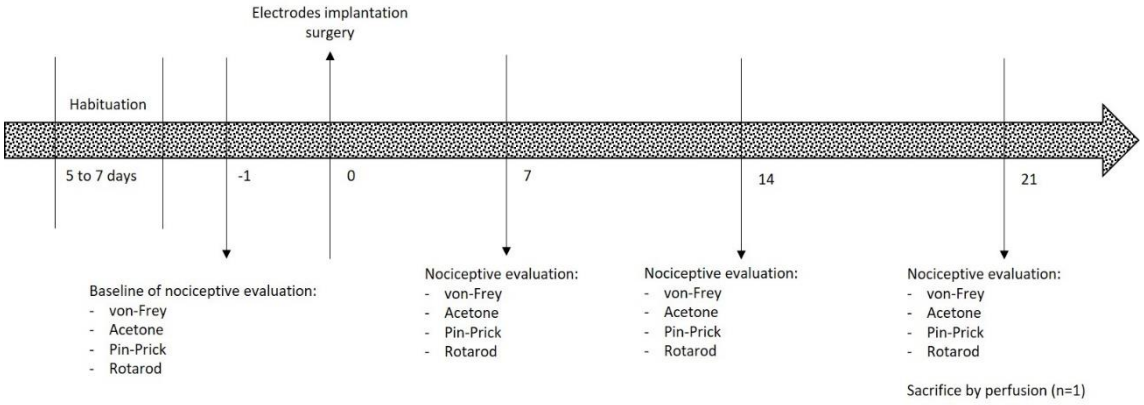

Figure 13: Timeline for behavioral analyses of Sham-DRG-S animal (n=1) (animal with electrodes without neurostimulation protocol). On days -1, and every 7 days up to 21 days, the animal was submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the nociceptive behavior and motor coordination. On day 21 the animal was sacrificed by perfusion.

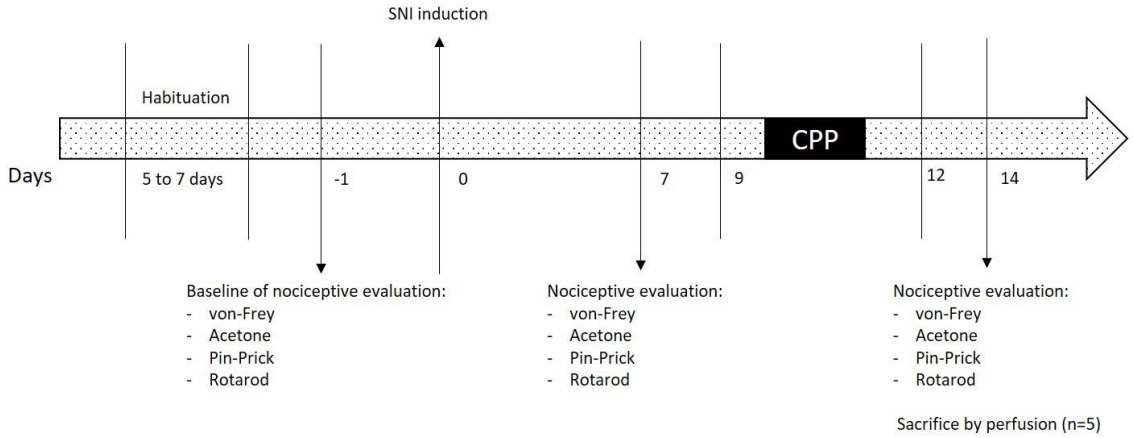

Figure 14: Timeline for behavioral analyses of the SNI 14d group (n=5). On days -1, and every 7 days up to 14 days, the animals were submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the nociceptive behavior and motor coordination. Between days 9 and 12, the animals were placed on the CPP apparatus but without any conditioning. On day 14 the SNI animals were sacrificed by perfusion.

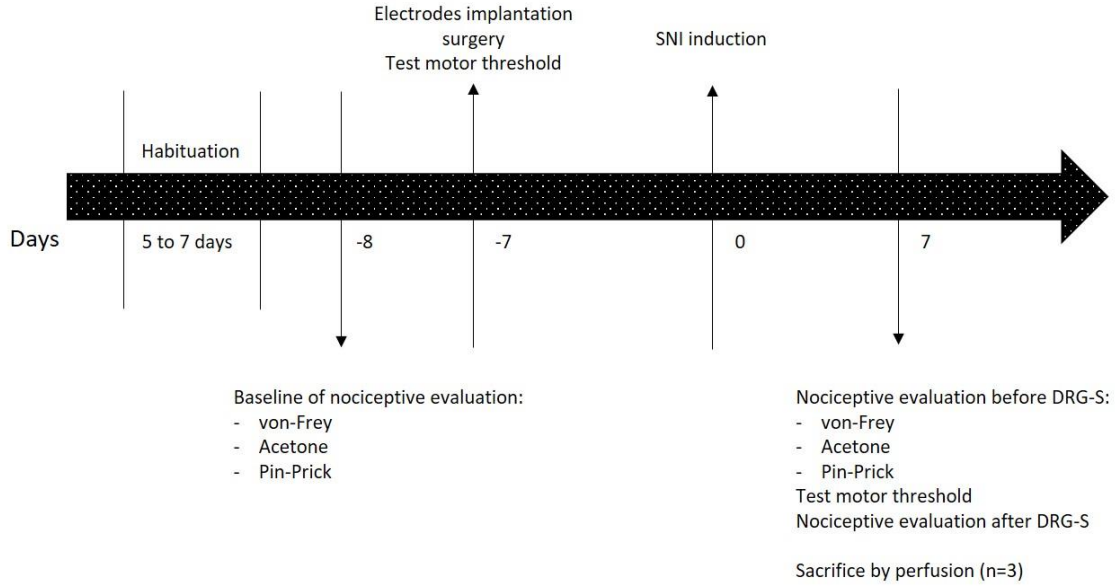

Figure 15: Timeline for behavioral analyses of SNI+DRG-S 7d group (n=3) (SNI animals with neurostimulation protocol). On days -8 and day 7, the animals were submitted to von-Frey, Pin-Prick, and Acetone tests to evaluate the nociceptive behavior and motor coordination. On day 7

the animals were also tested for the motor threshold induced by neurostimulation and received a session of 30 minutes of neurostimulation. After behavior evaluation, the SNI+DRGS 7d animals were sacrificed by perfusion.

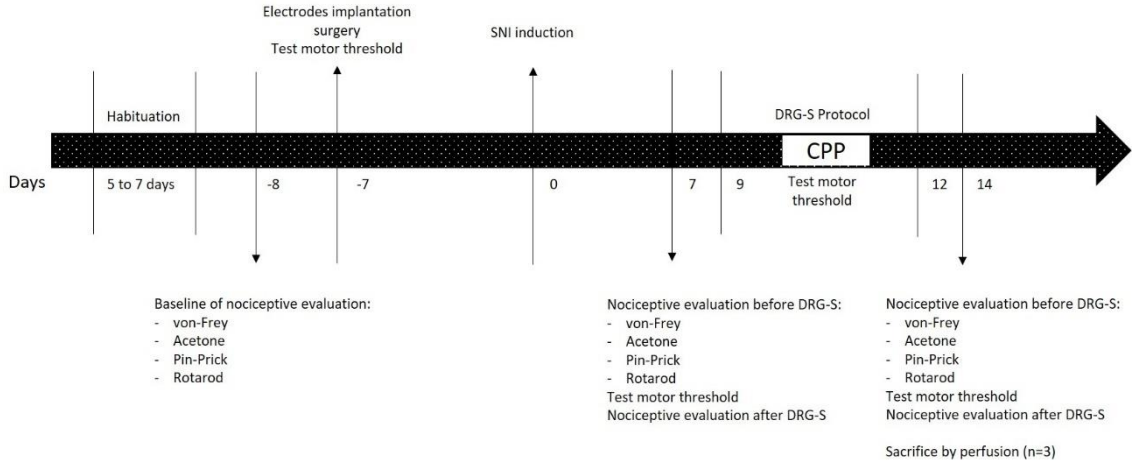

Figure 16: Timeline for behavioral analyses of SNI+DRG-S 14d group (n=3) (SNI animals with neurostimulation protocol). On days -8 and every 7 days up to 14 days, the animals were submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the nociceptive behavior and motor coordination. On days 7 and 14, the animals were also tested for the motor threshold induced by neurostimulation and received a session of 30 minutes of neurostimulation. Between the day 9 and 12, the animals were conditioned on the CPP, receiving a daily neurostimulation session in these days. On day 14 the animals were sacrificed by perfusion.

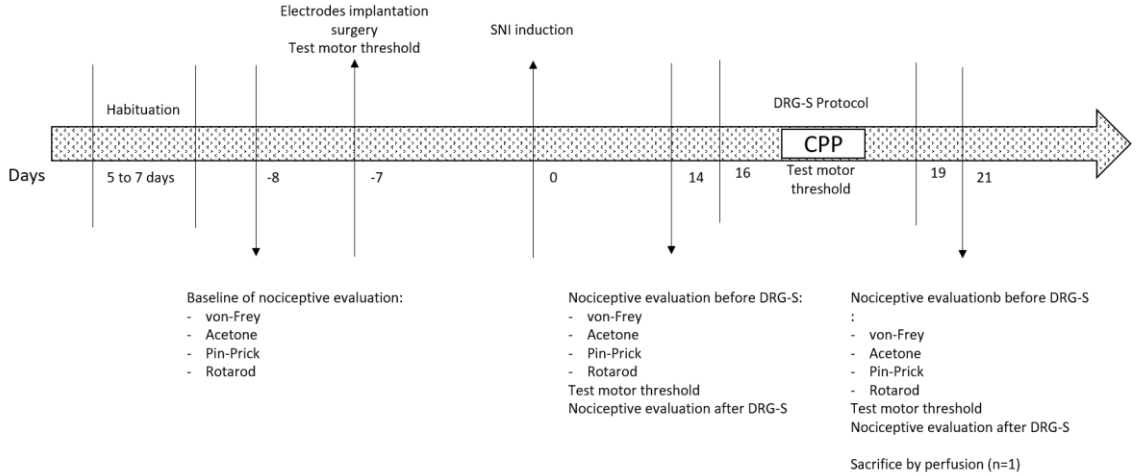

Figure 17: Timeline for behavioral analyses of SNI+DRG-S 14d-21d animal (n=1) (SNI animal with neurostimulation protocol). On days -8 and days 14 and 21, the animal was submitted to von-Frey, Pin-Prick, Acetone, and Rotarod tests to evaluate the nociceptive behavior and motor coordination. On days 14 and 21 the animal was also tested for the motor threshold induced by neurostimulation and received a session of 30 minutes of neurostimulation. Between the day 16 and 19, the animal was conditioned on the CPP, receiving a daily neurostimulation session in these days. On day 21 the animal was sacrificed by perfusion.

Induction of neuropathic pain - spared nerve injury (SNI) model

To induce neuropathic pain, the SNI model, illustrated in figure 18, was chosen (Decosterd and Woolf 2000). The animals were deeply anesthetized with isoflurane (5% for induction and 2-2.5% for maintenance), and then the right tight of the animal was trichotomized and the skin was sterilized with 70% ethanol and iodopovidone (Betadine®, Meda Farma, Lisboa-Portugal). A vertical incision of 3-5 cm was made along the skin, and a blunt-ended scissor was used firstly to dissect the underlying connective tissue layers and then to remove the muscle fibers without tearing them. The muscle fibers were separated in layers, in such a way that when the second layer was reached an extra care was needed because the nerve was just under it. After carefully peeling off the fibers, the sciatic nerve and their ramifications could be observed. With the help of a small curve clamp, the peroneal and tibial branches were reached and isolated, and

each one was tightly ligated in two different regions of the nerve, separated by 2-4 mm, with 5-0 non-absorbable silk thread. Afterwards, the space between the ligated regions was cut, to not allow the regeneration of the nerve fibers. At the end of the axotomy, the muscle was sutured in layers, and this was followed by suture of the skin, both with surgical sutures (4-0 Sutura cirúrgica de seda, Silkam®, B. Braun, Portugal). Subsequently, the local incision was sterilized with iodopovidone (Betadine®, Meda Farma, Lisboa-Portugal) and the animals were placed in the cages to recover from the anesthesia and the procedure.

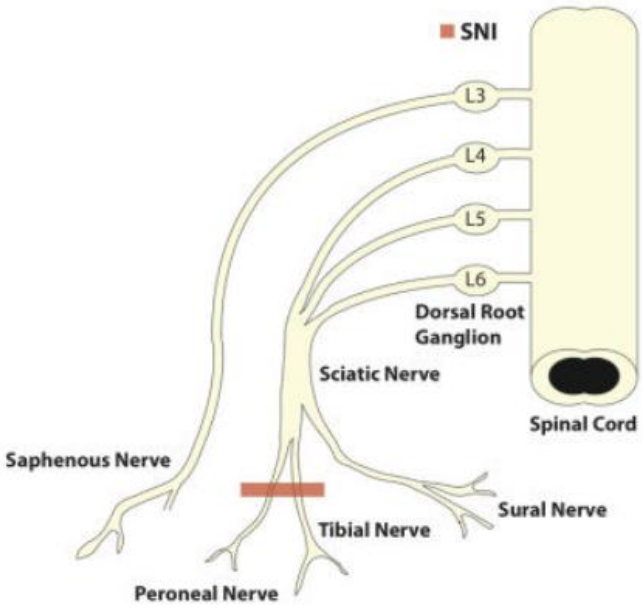

Figure 18: The Spared Nerve Injury (SNI) procedure. This procedure consists of sparing the adjacent sural and saphenous nerves and doing the transection and displacement of the tibial and common peroneal nerves. The SNI model leads to a complete denervation of the tibial area but leaves intact the saphenous and sural sides (adapted from Duraku et al. 2012).

Implantation of bipolar platinum/iridium electrodes in DRG

Seven days before the induction of the SNI model, the animals were submitted to a surgical procedure for insertion of bipolar platinum/iridium electrodes next to the L4 DRG. Before the surgery, 0.1 mL of tramadol (Tramal®, Grünenthal, Amadora-Portugal)

was orally administered to alleviate post-surgical pain. After, an intraperitoneal injection of medetomidine hydrochloride (0.5mg/Kg, Domitor®, Esteve Farma, Carnaxide, Portugal) and ketamine hydrochloride (75mg/Kg, Imalgene®, Merial, Lyon, France) was administered to induce deep anesthesia. For the surgery to implant the electrodes, here termed ganglionic surgery, a stereotaxic frame was used as support for the animal. Before the animals were placed in the stereotaxic frame, the fur of their back was trichotomized to allow better visibility of the area of interest and reduce the surgical site of infection. Subsequently, the skin was sterilized with 70% ethanol and iodopovidone (Betadine®, Meda Farma, Lisboa-Portugal), and the animal was then placed in the stereotaxic frame. A scalpel was used to make a small incision in the skin just to the right of the dorsal midline starting from the superior iliac crest and parallel to the spine. As soon as the muscle was exposed, the superficial muscular fascia was incised and the paraspinal muscles were separated by a combination of several incisions, always lateral to the bone, until the right transverse processes of the lumbar vertebrae L4 was reached. A surgical retractor was used to maintain the apophysis exposed, and this was done with the help of a binocular surgical magnifying glass for better visualization and definition of the apophysis. This allowed the fracture of the L4 transverse processes with a rongeur to access the L4 ganglion that was beneath. The L4 comprises the cell bodies of the sensory neurons that innervate the hind paw. Muscle and bone removal were always minimized only to the enough to perform the implantation of the electrodes next to the ganglia.

After the L4 ganglion was exposed, two electrodes that were made from high conductivity platinum-iridium wires (CFW2047288, 0.254 mm, California Fine Wire Company®, California, USA), with the insulation coating removed at the point of contact with L4 DRG, were used. The insertion of electrodes, which were sterilized using 70% ethanol, was performed aseptically. The first wire was inserted under the L4 ganglion with the aid of a small curve clamp, then the second wire was placed over the L4 ganglion and finally, the two wires were stitched together with non-absorbable surgical suture (7-0 prolene surgical suture, Ethicon) and glued with Hystoacryl® to the bone. The Histoacryl® was also used to fix the places where the wires were joined with 7-0 prolene.

The tips of the wires that were maintained inside the animals were isolated with a drop of Histoacryl®. After the wires were positioned, the tips of the wires, which were then externalized, were ligated to the connector of the external neurostimulator (N'Vision® 8840, Medtronic®, Porto-Portugal; kindly freely provided by Medtronic Portugal). To assure that the wires were well implanted and working, the neurostimulation was tested. For this, the neurostimulation protocol, as described below, was initiated and the amplitude (mA) was increased until the right hind paw presented an involuntary movement. After the stimulation protocol was tested, a small incision was made in the skin, about 1 cm above and perpendicular to the skull base, where the tips of the electrodes were externalized with the help of a cannula, as illustrated in figure 19. The tips were protected with a small plastic tube that was stitched, by using surgical sutures (4-0 Sutura cirúrgica de seda, Silkam®, B. Braun, Portugal), and glued with Histoacryl® to the skin, and then a small drop of normal glue was used to seal the opening of the tube. At the end of this procedure, the wound was closed in layers (muscle and skin) using surgical sutures (4-0 Sutura cirúrgica de seda, Silkam®, B. Braun, Portugal). The intervened area was sterilized with iodopovidone (Betadine®, Meda Farma, Lisboa-Portugal). All the animals were rehydrated with a subcutaneous injection of 2 mL of 0.9% saline (Braun, Queluz de Baixo-Portugal). The sedative effect of the anesthesia was reverted by an intramuscular injection of atipamezole hydrochloride (1mg/kg; Antisedan®, Esteve Farma, Carnaxide-Portugal).

During the five post-surgery days, the animals received administration of antibiotics (Enrofloxacin, 25mg/mL, Baytril®, Bayer, Portugal) to prevent an inflammatory reaction from the surgery and from the wires that remained inside the animal, in contact with the muscle and nervous tissue.

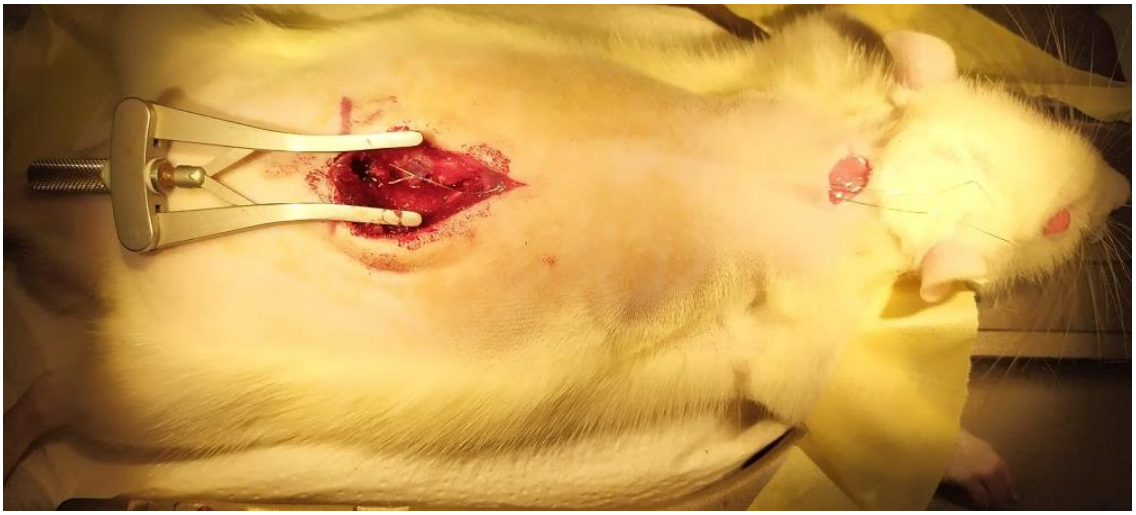

Figure 19: Picture taken after electrode implantation and before the wound being closed in layers.

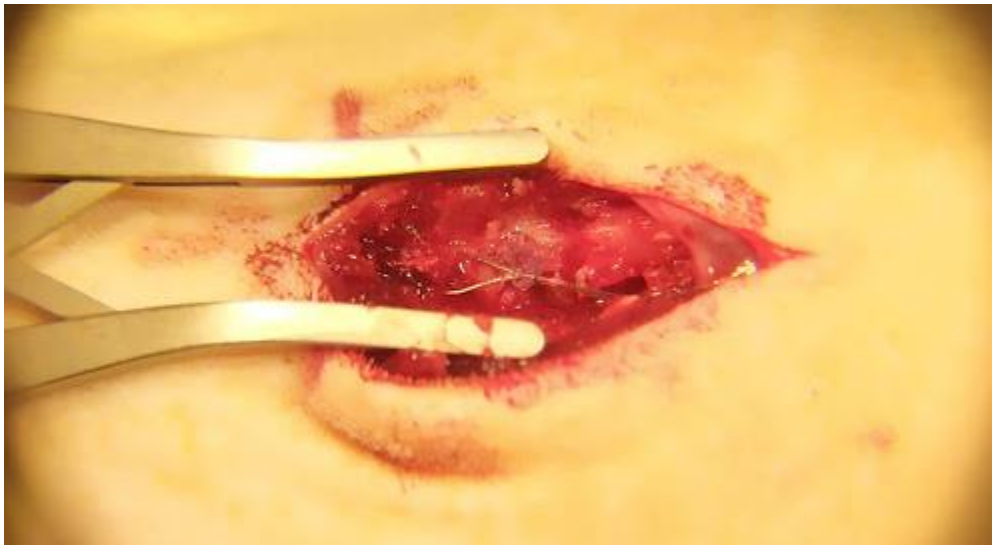

Figure 20: Amplified picture to show in more detail the electrodes placed above and under the L4 DRG.

Dorsal Root Ganglion Stimulation (DRG-S)

DRG-S was performed in SNI rats with different durations of the neuropathic condition, as explained above. The current intensity for DRG-S was chosen as follows: the amperage, in mA, was determined in anesthetized rats, as being that for which any further increase resulted in perceptible movement of the hind limbs during stimulation at 2 Hz and with a pulse width of 150 μs. For the DRG-S protocol in awake and unrestrained rats, the pulses were delivered at a current set at 80% of this motor threshold and at a 20 Hz pulse rate, which are parameters similar to those currently used to produce analgesia in clinical trials (Eldabe et al. 2015).

Nociceptive, motor coordination and spontaneous pain–like behavioral testing

All animals were subjected to four tests to assess the nociceptive behavior as well as motor coordination, which were always performed in the following order: 1st- von-Frey test (VF); 2nd-Pin-Prick test (PP); 3rd - Acetone test (AC) and 4th- Rotarod test (RR). Before the behavioral tests, the animals were accustomed to the elevated metal grid for a minimum period of at least three days. The timeline of behavioral assessment is illustrated above in figures 13-17, in the *Experimental Groups and Experimental Design* section.

von-Frey test: assessment of mechanical allodynia

To assess mechanical allodynia, the animals were placed in an elevated metal grid with small holes that allowed the application of the von-Frey monofilaments to the lateral plantar surface of the hind paws, as illustrated in figure 21. The filaments are calibrated for ascendant forces ranging from 0.008g to 60g (0.008g; 0.02g; 0.04g; 0.07g; 0.16g; 0.4g; 0.6g; 1.0g; 1.4g; 2.0g; 4.0g; 6.0g; 8.0g; 10.0g; 15.0g; 26.0g; and 60.0g) and were tested by applying them in a position lateral to the skin and in such a way that they were

pressed slowly until they bent. The test was performed by applying each filament 5 times at intervals of 1-4 seconds. The first filament in the series that evoked at least 1 response (from the 5 applications) was considered as the threshold. The criterion for considering it as a positive response was the existence of a reflexive withdrawal. The withdrawal could, sometimes, be accompanied by other responses like licking or grooming of the tested hind paw (Tal, Michael and Bennett 1994).

Pin-prick test: assessment of mechanical hyperalgesia

This test was performed using a blunt 25G needle, to indent but not to penetrate the skin of the plantar hind paw. For that purpose, a rapid and precise movement was applied to the lateral plantar region of the hind paw of the animals, as illustrated in figure 21. A positive response was achieved when the animal reacted with pain-like behavioral signs, like licking, raising, or shaking the tested hind paw, being the time of response counted with the help of a timer. The time started counting when the stimulus was applied and ended when the animal did not present any sign of pain response, namely an elevated hind paw (withdrawal time) (Raja, Campbell, and Meyer 1984).

Acetone test: assessment of thermal allodynia

Like in the previous two behavioral tests, the acetone test was applied at the same lateral-plantar region of the hind paws of the animals (figure 21). This test was performed by applying drops of 40µL of acetone with the help of a micro-pipette. Extra care was taken so that the tip of the micro-pipette did not touch the paw, since this could be taken as a mechanical stimulus that could interfere with the assessment of the thermal allodynia. The time the animals spent with their hind paws elevated, or licking and/or shacking them was registered. The timer was stopped only when the hind paw was placed in the metal grid without any kind of reaction elicited by the acetone drop (Choi et al. 1994).

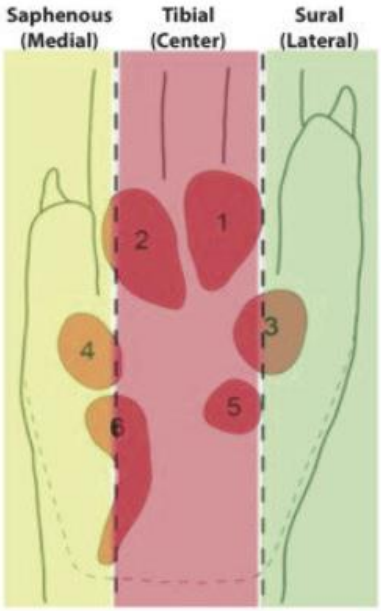

Figure 21: Area in rat plantar hind paw for the behavioral assessment. The area that received the mechanical and thermal stimuli was the lateral region (green), innervated by the spared sural branch of the sciatic nerve (adapted from (Duraku et al. 2012)).

Rotarod test: assessment of motor coordination

The rotarod test evaluates functional parameters, like the motor coordination of the rats. In this test, each animal was placed on a horizontal rod that rotated around its long axis, and each trial was finished when the animal fell off the rotarod. The apparatus was configured to accelerate from 4 rpm to 40 rpm in 180s and the time that animals stayed in the rotarod without falling was recorded. Before the test, each animal was trained for 5 minutes, on the day before the baseline assessment, at a constant speed of 10 rpm, as previously described (Piel et al. 2014).

Conditional Place Preference (CPP): assessment of spontaneous pain–like behaviors

Spontaneous pain–like behaviors were assessed by evaluating the preference of the animals for a chamber where they received the neurostimulation protocol or a neighbor chamber where they did not receive any stimulation. This CPP test was performed in the days between the assessment of nociceptive behavior (days 10 to 13 (SNI 14d and SNI+DRG-S 14d groups) or days 16 to 19 (SNI+DRG-S 14d-21d)). We used a "home-made" CPP apparatus with 3 chambers in which 2 removable doors separate the central chamber from the side chambers. The two side chambers differ between them by the orientation of the stripes in the walls and by the type of grid that covers the bottom of the chamber. The procedure consisted of three different phases. The first was the pre-conditioning day, when the animals were allowed to explore the 3 chambers for 15 minutes, and the time spent in each side chamber was recorded (phase 1). The second phase comprised the four following days, when the place condition was conducted. On these days, the animals received two 30-minutes sessions separated by 6h in which ganglion neurostimulation or no stimulation were administered. The chamber paired with ganglion neurostimulation was consistent on all 4 days for a given animal but was randomly assigned for different animals (phase 2). The acquisition of conditioned place preference was tested on the day after the last conditioning session. At these final session, each animal was allowed to freely explore the 3 chambers for 15 minutes, and the time spent on each side was recorded (phase 3) (Pan et al. 2016).

In phase 1, all the animals that showed a predetermined level of preference for one chamber (>67% of total time) at this pre-conditioning stage were excluded from the test. The preference score was calculated based on the preference recorded in phase 3, as the total time spent in the chamber in which the animal received the neurostimulation protocol, minus the total time spent in the other side chamber, as previously described (Pan et al. 2016).

Tissue preparation

Tissue processing for immunohistochemistry

For immunohistochemistry, the Sham-DRG-S, SNI, and SNI+DRG-S (7d, 14d, and 14d-21d) animals were intraperitoneally anesthetized with sodium pentobarbital (100 mg/kg, Eutasil ®, Ceva, Algés, Portugal) diluted in 0.9% saline and then perfused through the ascending aorta. Briefly, an incision in the abdominal and thoracic cavities forming a V shape was performed to expose the heart. After this, 0.1mL of heparin was injected into the left ventricle, to prevent the blood from clotting. A cannula was then inserted in the ascending aorta through an incision in the same ventricle to promote the circulation of 300mL of oxygenated Tyrode's solution and allow the cleaning of the whole blood remaining in the body. When this solution was over, 900mL of a fixative solution containing 4% paraformaldehyde (PFA) diluted in phosphate buffer saline 0.1M (PBS 0.1M) circulated on the same cannula. In the end, the animals were dissected and the L3, L4, L5, and L6 spinal cord segments were collected along with their roots as well as the respective ipsilateral and contralateral DRGs. This material was post-fixed for 4 hours in the same fixative solution and kept in 30% sucrose with 0.1% sodium azide until they were processed.

The DRGs were then serially sliced in 12µm transverse sections, using a cryostat (Leica Instruments GmbH, CM3050, Germany) at -20ºC, and collected into poly-L-Lysine coated slides. Each DRG was collected in 8 sequential series, with an average of 10 sections *per* slide. The slides were dried on a heating plate for 1 hour at 37ºC and thereafter were stored at -20ºC until use in immunohistochemistry reactions.

Immunohistochemistry against ATF3 or NPY

The slides containing the L4 and L5 DRG slices of Sham-DRG-S (21d), SNI (14d), and SNI+DRG-S (7d, 14d, and 14d-21d) animals, sacrificed 7, 14, or 21 days after model induction or implantation electrode surgery (Sham-DRG-S), were thawed and incubated

firstly in 50% ethanol, during 30 minutes, and then washed in PBS 0.1M, followed by washes in PBS containing 0.3% of Triton X-100 (PBS-T). Sections were afterwards incubated with the blocking solution (10% Normal Goat Serum, NGS, diluted in PBS-T), to avert unspecific ligations. After, the slides with the sections of DRG of Sham-DRG-S, SNI, and SNI+DRG-S (7d, 14d, and 14d-21d) animals were incubated with the primary antibody against ATF3 (polyclonal rabbit anti-ATF3; 1:200; C-19; SC-188; Santa-Cruz Biotechnology, USA) or NPY (polyclonal rabbit anti-NPY; 1:8000; N9528; Sigma-Aldrich), diluted in PBS-T containing 2% NGS, during two overnights at 4ºC. Two days after the incubation with the primary antibody, the same sections were washed in 2% NGS diluted in PBS-T and were then incubated, in the dark, with the secondary antibody (Alexa 594 goat anti-rabbit, A11012, for ATF3 and NPY, from Invitrogen, Thermo Fisher Scientific) diluted 1:1000 in PBS-T containing 2% NGS, respectively, at room temperature, for 1h. From the moment of incubation with the secondary antibody, the slides were always protected from light and were then washed in PBS-T followed by PBS. After, slides were coverslipped with a phosphate glycerol solution (3 parts of glycerol and 1 part of PBS 0.1M) and they were stored at -20°C until visualization.

Microscopic analyses were performed by using a fluorescent microscope (AXIO MRm, Zeiss, Germany) coupled to a digital camera (Axiocam MRm) and an image software (Axiovision 4.8 software, Carl Zeiss MicroImaging) to capture the images. The acquisition conditions, such as amplification of the objective, light intensity, contrast, and hue, were maintained constant. The total number of cells was counted as well as the number of ATF3 labeled cells (ATF3 positive) or NPY labeled cells (NPY positive). This was performed with the support of the FIJI ® computer program (Version 1.53 c). Data are presented as the percentage of cells expressing ATF3 or NPY in both the L4 and L5 DRG.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism Software Version 8.0.2 for Windows. Results were present as the mean ± SEM. For the Conditioned Place Preference Test a multiple unpaired t-test was performed to investigate differences at the time spent on the DRG-S chamber between the pre-test and test days. In all statistical analysis the level of significance was considered as $p<0.05$.

Results

The surgical approach for electrodes implantation was successfully implemented

The process began with the choice of the more appropriate electrodes for implanting, which needed also to be suitable for the DRG electrical stimulation in rats. For this purpose, extensive research on published articles both on human and animal studies was performed, taking into account different properties of the electrodes, namely the conduction properties and the coating of the wire. After deciding the type of wire needed, we contacted different selling companies, as most did not provide electrodes suitable for implanting in rats. The wire chosen was composed of a mixture of Platinum and Iridium (90% and 10%, respectively), which has excellent conduction properties, with PTFE (Polytetrafluoroethylene) coating, since it is biologically compatible and easy to peel off, and was purchased from California Fine Wire Company.

The second step consisted in establishing the most appropriate and safe surgical approach for the electrodes implantation (Figure 22). This was the most challenging process since different obstacles and kinds of problems occurred during and after the surgeries. These were solved step by step, from one surgery to another, so that the whole process took more than three months until we had a surgery without any intraoperative and/or postsurgical complications. Only after this was achieved, we started performing the complete surgeries, which included the implantation of the electrode wires, and started testing the stimulation protocol, based on literature research on clinical cases.

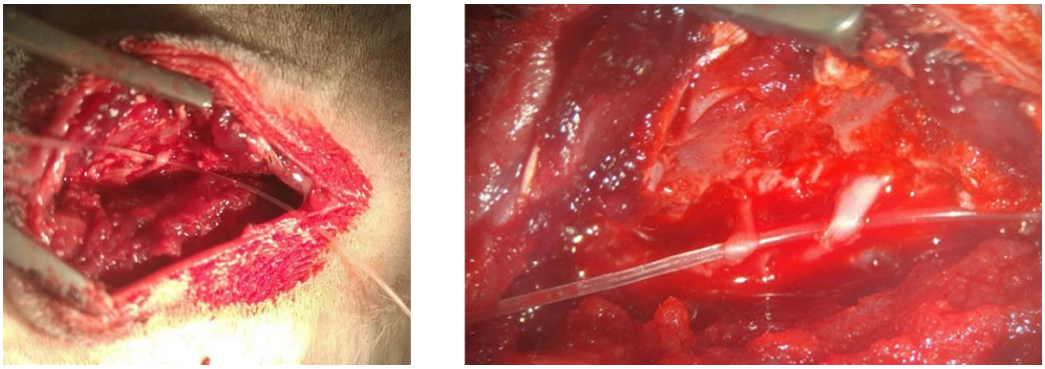

Figure 22: Picture illustrative of a surgery in which a catheter was implanted underneath the L4 and L5 DRG. This was performed as a pilot surgical approach for electrodes implantation.

To implement the stimulation protocol, a third challenge was overpassed, which consisted in the choice of the external neurostimulator. This is a fundamental instrument for delivering the electrical stimuli to the DRG. For this, we contacted Medtronics® Portugal, which kindly gave us an external neurostimulator that allows the definition by the user of the different stimulation parameters, such as the pulse frequency, the intensity, and the pulse width, among others.

The placement of the electrodes underneath the DRG and their maintenance appear to not affect the animal's nociceptive behavior and motor coordination

To evaluate if the electrodes implantation and their maintenance *per se* were harmful and could affect the nociceptive behavior, the sensory function was evaluated 7 days after the surgery for electrodes implantation, and every 7 days until day 21, in one rat subjected to the electrodes implantation surgery, but not receiving any DRG neurostimulation protocol (Sham-DRG-S). For that purpose, the von-Frey, Pin-Prick and the Acetone tests, which evaluate the mechanical allodynia, mechanical hyperalgesia and cold allodynia, respectively, were performed at different time points. Moreover, the motor coordination was also assessed by the Rotarod test.

There were no differences in the threshold of allodynic responses to von-Frey (Figure 23A) and Acetone (Figure 23C) tests throughout the 21 days in comparison to the pre-surgical baseline. However, the hyperalgesic responses to noxious mechanical stimuli (Pin-Prick test, Figure 23B) show that the animal developed some hypersensitivity on day 7, with a withdrawal duration of 10s, which reversed to baseline values (0s) on day 14. These baseline values were then maintained on day 21. Together, these findings appear to indicate that the implantation and permanence of the electrodes, during 21 days and without the neurostimulation protocol, are well tolerated and produce minimal hypersensitivity responses.

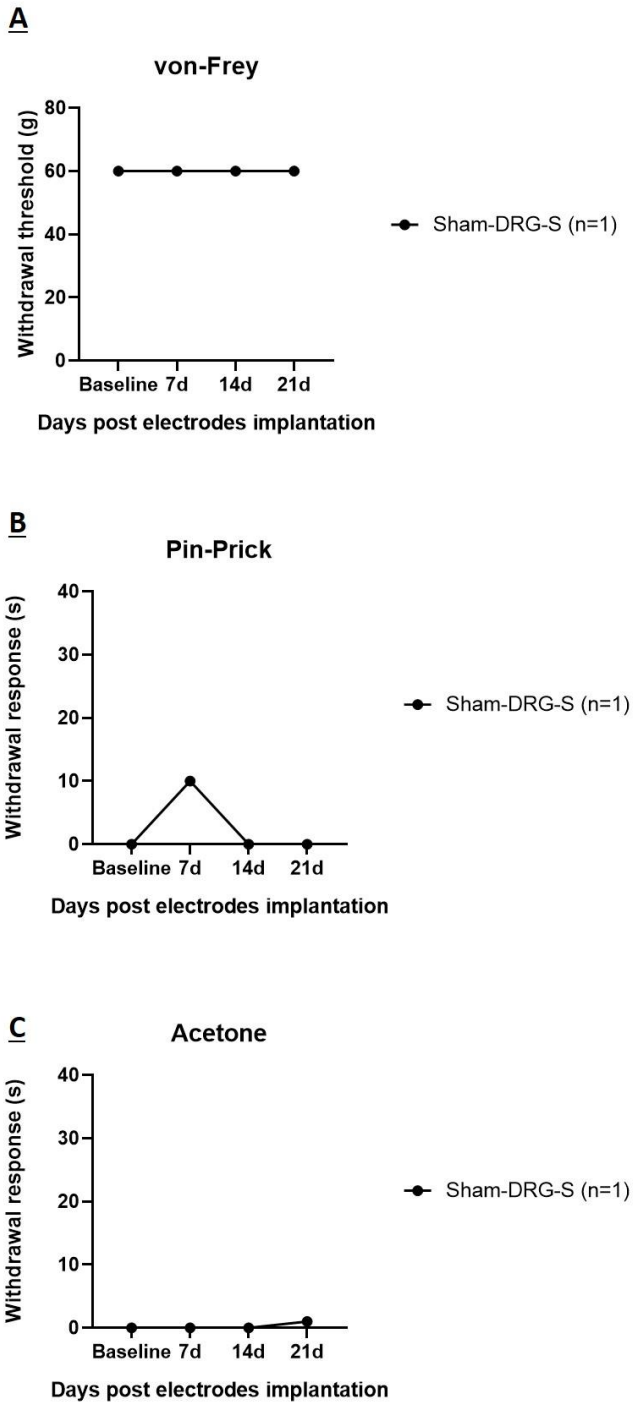

Figure 23: Nociceptive behavioral testing. Testing of allodynic responses to mechanical stimuli (von-Frey, **A**), and cold stimuli (Acetone, **C**) showed no differences throughout the 21 days after the electrodes implantation surgery. On the other hand, the hyperalgesia responses to noxious mechanical stimuli (Pin-Prick, **B**), show an increase in the ipsilateral threshold on day 7, when compared with the baseline and to the 14 and 21 days. This value can be representative of the

habituation of the DRG to the electrode's maintenance. Due to the low number of animals a statistical analysis was not performed.

The animal showed an increase in the time of permanence in the rotarod apparatus since the values increased from 98s, at baseline, to 110s, at 7 days after the electrode implantation surgery. On days 14 and 21, the duration of time the animal stayed in the Rotarod without falling was of 138s and 135s, respectively (Figure 24). This result appears to indicate that the implantation and maintenance of the electrodes next to the DRG do not impair the motor coordination throughout the experimental period.

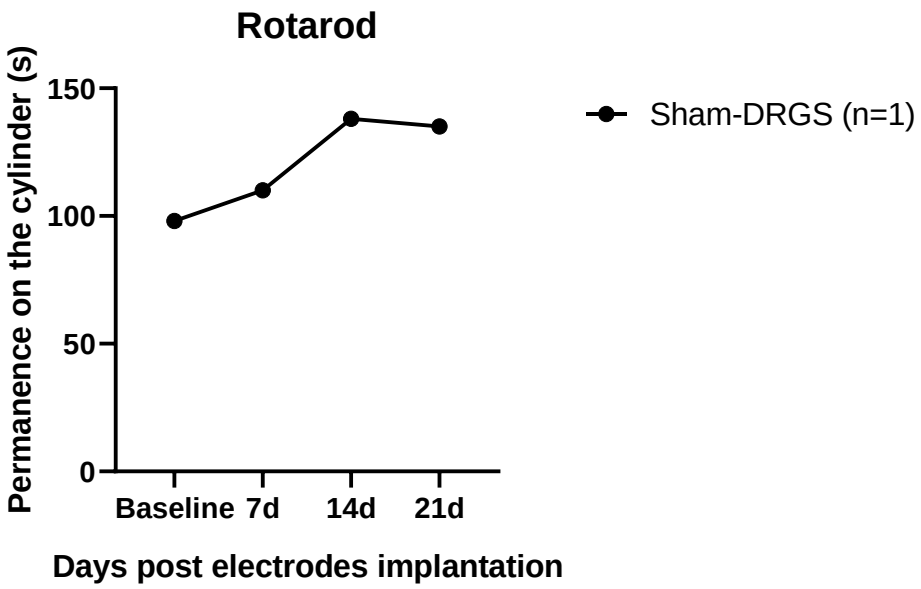

Figure 24: Time of permanence in the Rotarod, in seconds (s), of an animal subjected to the electrode implementation surgery but not receiving DRG electrical stimulation. Due to the low number of animals a statistical analysis was not performed.

Dorsal root ganglion stimulation (DRG-S) appears to attenuate the hypersensitivity to mechanical stimuli

The effect of the electrical stimulation of the DRG (DRG-S), and consequent neuronal activation, on the nociceptive behavior was evaluated in awake, unrestrained rats, at different time points of development and maintenance of the neuropathic pain condition induced by the SNI. For that purpose, the von-Frey, Pin-Prick, and Acetone tests were performed. Moreover, the motor coordination was also assessed through the Rotarod test.

Acute DRG-S of the L4 DRG appears to decrease the neuropathy-induced hypersensitivity

Mechanical allodynia: von-Frey test

The SNI was performed 7 days after the surgery for electrodes implantation, and 7 days after that, the nociceptive behavior was assessed (SNI+DRG-S 7d). The hypersensitivity was evidenced by the reduced threshold of the withdrawal response to mechanical stimulation (von-Frey), which showed a mean of 60 ± 0g at baseline and decreased to 22 ± 19g at 7d (Figure 25).

The withdrawal thresholds to von-Frey filaments were then evaluated at 0, 15, 30, 45, and 60 minutes after implementing the neurostimulation protocol only once (a single event neurostimulation or acute neurostimulation) at 7d of SNI. The withdrawal thresholds increased to 40 ± 19g at the 15 min time-point (7d(15m)), as compared to pre-stimulation values (22 ± 19g at 7d). At 30 min after neurostimulation (7d(30m)), there was a decrease to values similar to those of 7d, followed again by an increase to values of 40 ± 20g at 45 min (7d(45m)) after the end of the neurostimulation. The last evaluation at 60 min (7d(60m)) showed a withdrawal threshold mean similar to that observed at 7d. The results appear to show that the residual analgesia resulting from

electrical DRG stimulation attenuates the hypersensitivity to von-Frey filaments stimuli in neuropathic pain conditions.

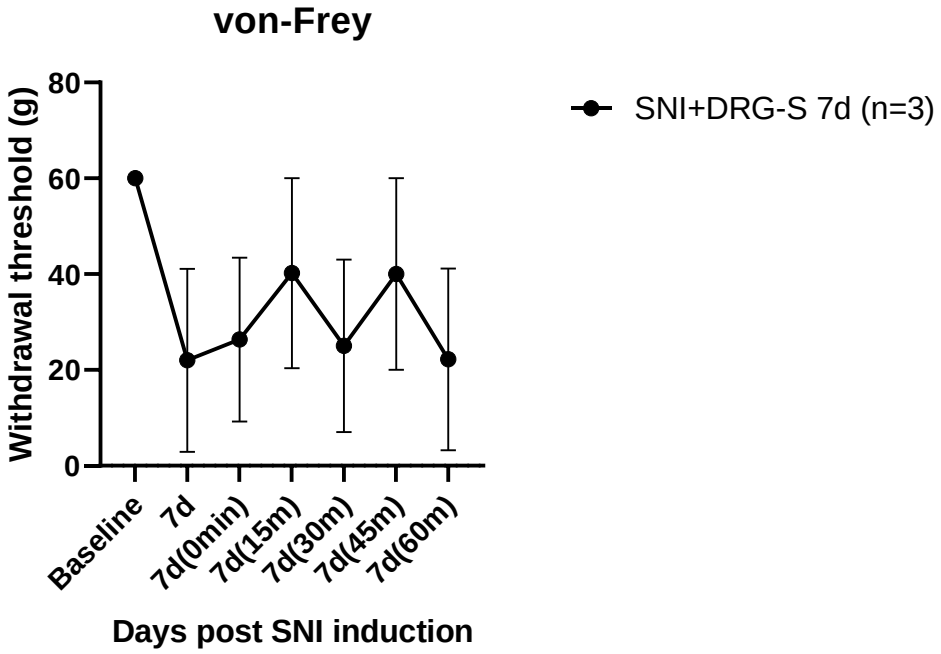

Figure 25: Effect of an acute dorsal root ganglion neurostimulation (DRG-S) on the withdrawal responses, in grams, to mechanical allodynic stimuli (von-Frey filaments) on rats with SNI. Nerve injured animals receiving DRG-S appear to show an attenuation of SNI-induced mechanical allodynia at specific time-points after neurostimulation. Due to the low number of animals a statistical analysis was not performed.

Mechanical hyperalgesia: Pin-Prick test

At 7 days after SNI induction, the animals showed a hyperalgesic response to noxious mechanical stimuli (Pin-Prick, Figure 26), as indicated by the increase in the mean of withdrawal duration, in seconds, from 0 ± 0s (Baseline, pre-SNI induction) to 8 ± 5s (7d). However, right after the neurostimulation protocol, the response withdrawal decreased to a mean value of 1 ± 0s (7d(0min)) and these low withdrawal duration values were

maintained until the last evaluation (7d(60m)). This appears to indicate that the DRG-S attenuates the hypersensitivity to Pin-Prick stimuli in neuropathic pain conditions.

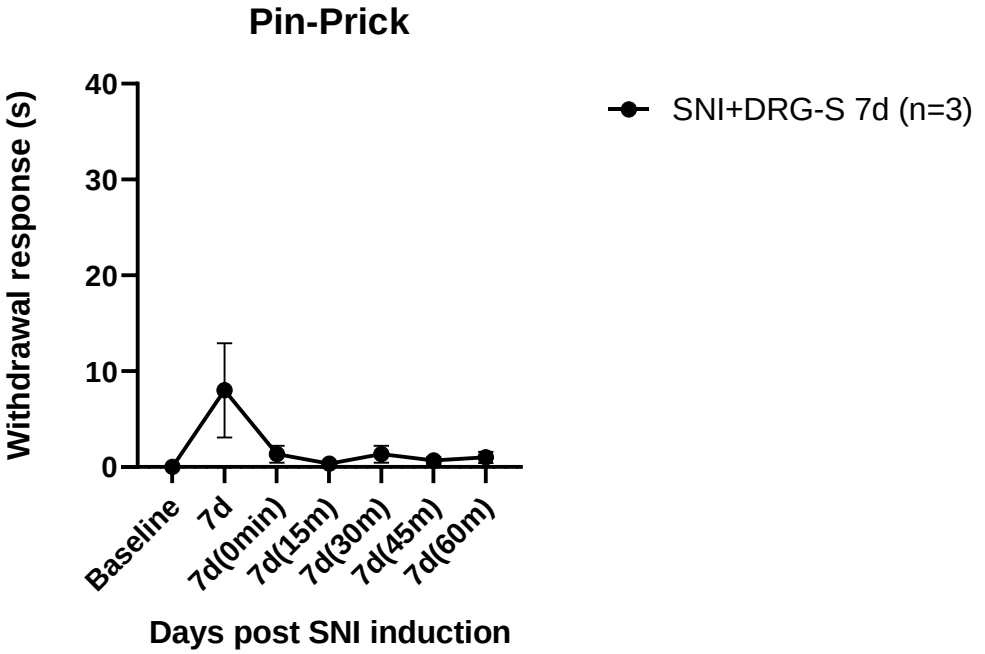

Figure 26: Effect of an acute dorsal root ganglion neurostimulation (DRG-S) on the withdrawal response, in seconds, to noxious mechanical stimulus (Pin-Prick) on rats with SNI. Nerve injured animals receiving DRG-S appear to show an attenuation of the SNI-induced mechanical hyperalgesia. Due to the low number of animals a statistical analysis was not performed.

Cold allodynia: Acetone test

As in the previous nociceptive test, 7 days after SNI induction the animals showed also a hypersensitivity to cold stimuli that was indicated by the elevated values of withdrawal duration at 7d (14 ± 3s), when compared to 0 ± 0s at baseline, as assessed by the Acetone test (Figure 27). Also, for this nociceptive test, the DRG-S protocol appears to have attenuated the hypersensitivity associated with the SNI, since the duration of the withdrawal responses decreased to mean values of 5 ± 3s, just after the end of the neurostimulation (7d(0min), and these reduced values were maintained in mean

withdrawal responses of 3 ± 1s to 6 ± 4s until the end of the behavioral assessment (7d(60m)). These results appear to indicate that the DRG-S attenuates the withdrawal responses to cold stimuli in neuropathic pain conditions.

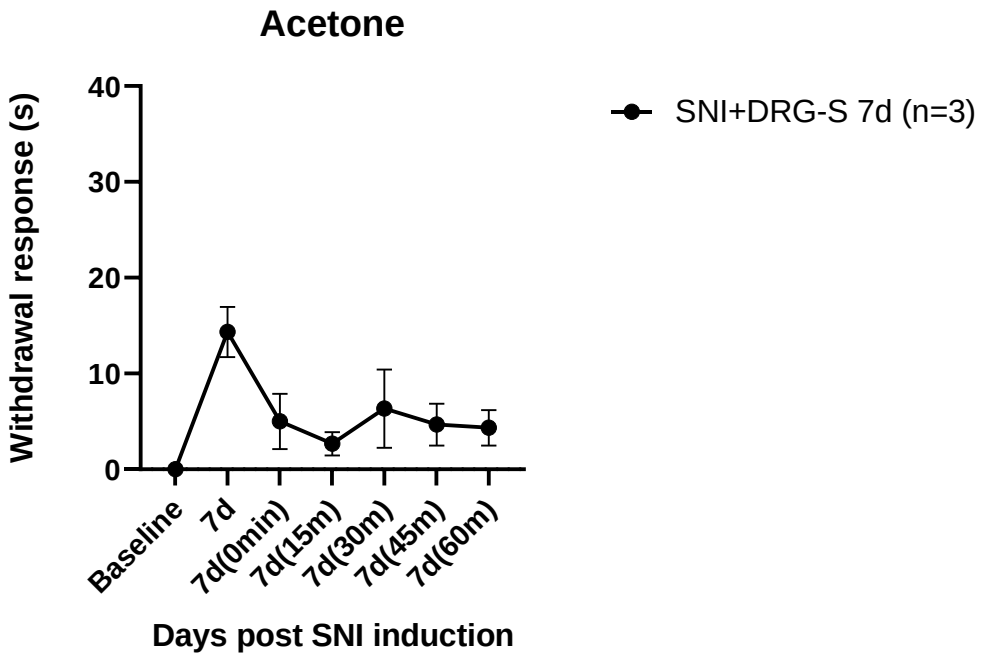

Figure 27: Effect of an acute dorsal root ganglion neurostimulation (DRG-S) on the withdrawal response, in seconds, to cold allodynic stimuli (Acetone test) on rats with SNI. Nerve injured animals receiving DRG-S appear to show an attenuation of SNI-induced cold allodynia. Due to the low number of animals a statistical analysis was not performed.

Repeated DRG-S of the L4 DRG appears to decrease the neuropathy-induced hypersensitivity

After observing that DRG-S of the L4 DRG at a single time-point (7d, acute neurostimulation) had the potential to reduce the hypersensitivity in SNI rats, we decided to study the effect of repeated neurostimulations. Thus, a group of SNI animals received a 30 minutes session of neurostimulation on days 7 and 14 of SNI, which was followed by behavioral testing at 0, 15, 30, 45 and 60 min after the electrical stimulus,

and also received a daily neurostimulation session between the days 9 and 12, during the CPP test (SNI+DRG-S 14d group).

Mechanical allodynia – von-Frey test

The withdrawal thresholds to the application of the von-Frey filaments are shown in Figure 28. None of the animals belonging to either group showed mechanical hypersensitivity at the baseline, as indicated by withdrawal thresholds of 60 ± 0g in the SNI 14d group, which was not subjected to the electrodes implantation surgery, and of 49 ± 11g in the SNI+DRG-S 14d group. As expected, seven days after SNI induction, both the SNI 14d and the SNI+DRG-S 14d groups exhibited withdrawal thresholds of 1 ± 1g (for the SNI 14d) and 2 ± 2g (for the SNI+DRG-S 14d), which indicates a higher mechanical sensitivity characteristic of peripheral neuropathic pain conditions. This mechanical hypersensitivity was maintained in the SNI 14d group, which presented a withdrawal threshold mean of 0.06 ± 0g on day 14. In the SNI+DRG-S 14d group, neurostimulation on day 7 appeared to increase the mechanical thresholds immediately after discontinuing the DRG-S (25 ± 18g at 7d(0min)), as well as at 15 minutes after (31 ± 16g at 7d(15m)). Nevertheless, 30 minutes after ending the electrical stimulation, the mechanical thresholds decreased to 10 ± 3g, and maintained low (5 ± 3g) until the last evaluation at 60 minutes.

On day 14, the withdrawal responses of the SNI+DRG-S 14d rats, were similar to those of day 7 (means of 2 ± 2g for 7d and 4 ± 2g for 14d), which indicates that the period of neurostimulation that occurred during the conditioning for the CPP test (between days 9 and 12 of SNI) did not have a sustained effect. After the neurostimulation on day 14, the animals presented a mean of 18 ± 8g just after discontinuing the DRG-S (at 14d(0min)), suggesting an analgesic effect, which was sustained until 15 minutes after, as indicated by the mechanical thresholds of 17 ± 9g (at 14d(15m)). However, 30 minutes after discontinuing the DRG-S, the analgesic effect appears to have been

reversed, as the threshold mean decreased to 8 ± 4g and was maintained in low values of 4 ± 3g to 5 ± 2g until 60 minutes after the end of neurostimulation.

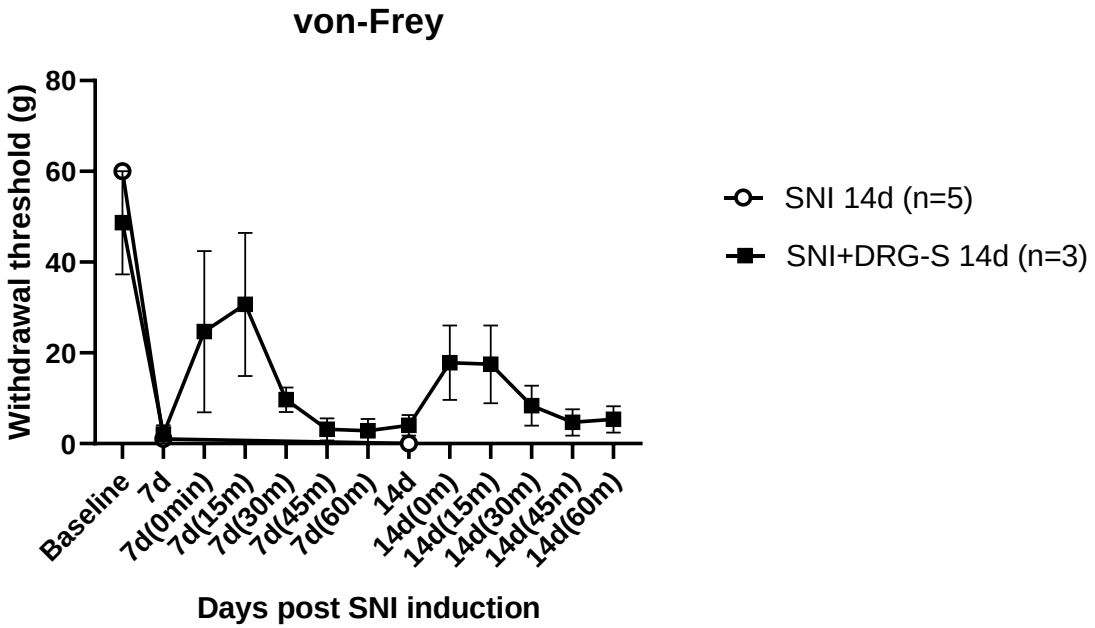

Figure 28: Effect of repeated DRG-S on the withdrawal responses, in grams, to mechanical allodynic stimuli (von-Frey filaments) on rats with SNI. Nerve injured animals receiving DRG-S showed an attenuation of SNI-induced mechanical allodynia just after discontinuing DRG-S, both when this occurred at 7d and 14d of SNI. This analgesic effect was reversed 30 minutes after discontinuing DRG-S. Due to the low number of animals a statistical analysis was not performed.

Mechanical hyperalgesia – Pin-Prick test

The effect of DRG-S on the withdrawal responses to noxious mechanical stimuli were then evaluated and are shown in Figure 29. On the baseline testing sessions, the SNI 14d and SNI+DRGS 14d animals showed no mechanical hypersensitivity, as indicated by withdrawal durations of 0 ± 0s for both groups. At 7 days after SNI induction, the groups showed a slight increase of the withdrawal responses to means of 4 ± 1s, for the SNI 14d, and 2 ± 1s for the SNI+DRG-S 14d groups, indicating the development of some hyperalgesia to noxious mechanical stimuli. On day 14, the SNI 14d group exhibited an

increased mean value of withdrawal responses, of 13 ± 4s, which characterizes the neuropathic pain state. The administration of a 30 minutes session of neurostimulation in the SNI+DRG-S 14d animals on day 7, provoked a decrease in the withdrawal response to mean values of 0 ± 0s just after discontinuing DRG-S (at 7d(0min)) that was maintained until, at least, 15 minutes after the end of DRG-S. At 30 minutes after neurostimulation and until the end of the behavioral assessment the values of withdrawal responses gradually increased to 7 ± 6s, 6 ± 3s and 4 ± 1s (for 30min, 45min and 60min after neurostimulation, respectively).

Seven days after, at 14 days of SNI, the pre-DRG-S withdrawal responses showed a mean value of 6 ± 3g (at 14d), which indicates that the DRG-S administered between days 9 and 12 of SNI, during the CPP conditioning, did not evoke a sustained analgesic effect on mechanical hyperalgesia-induced behavior (although one could expect that this value would be much higher, close to that found in the SNI 14d group at 14 days of SNI). DRG-S on day 14, caused a decrease of withdrawal responses to 1 ± 0s immediately after discontinuing DRG-S (at 14d(0min)), suggesting a short-term analgesic effect, which was less evident at 15 minutes after stimulation (4 ± 1s at 14d(15m)), but observed again at 30 minutes after the end of the neurostimulation session (1 ± 0s, at 14d(30m)). At the 45- and 60-minutes time-points after DRG-S, the values were again slightly increased to 2 ± 1s and 3 ± 0s, respectively.

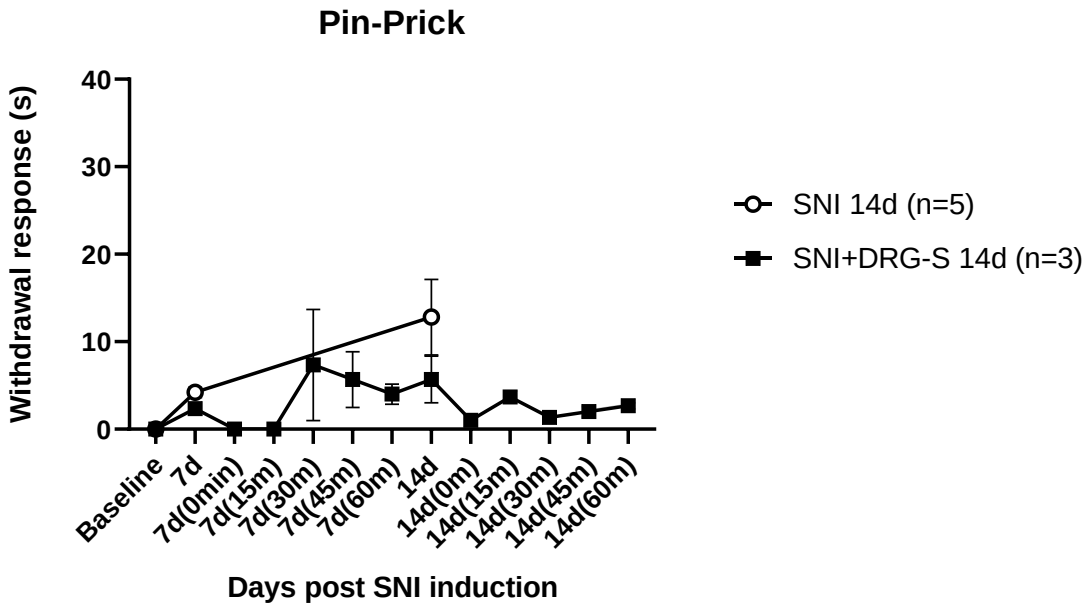

Figure 29: Effects of repeated dorsal root ganglion neurostimulation (DRG-S) on the withdrawal response, in seconds, to noxious mechanical stimulus (Pin-Prick) on rats with SNI. Nerve injured animals receiving DRG-S appear to show a short-term attenuation of SNI-induced mechanical hyperalgesia just after discontinuing DRG-S, both when this occurred at 7d and 14d of SNI. This analgesic effect was reversed 30 minutes after discontinuing DRG-S, particularly on day 7. Due to the low number of animals a statistical analysis was not performed.

Cold allodynia – Acetone test

The Acetone test was used to access the withdrawal response of the animals to cold stimuli (Figure 30). At baseline, all the animals showed mean values for the withdrawal responses of 0 ± 0s, which indicates no hypersensitivity to cold stimuli, as expected. On day 7 after SNI induction, the SNI 14d group showed an increase to 9 ± 2s, and then a further increase on day 14, to mean values of 12 ± 4s, which characterize the neuropathic pain states. The SNI+DRG-S 14d group exhibited a mean withdrawal duration of 3 ± 2s on day 7, indicating the existence of some cold allodynia. Immediately after the end of the 30 minutes DRG-S session, the SNI+DRG-S 14d animals showed a reduction of the withdrawal duration values to 1 ± 0s (at 7d(0min)), suggesting a short-

term analgesic effect, which was reversed 15 minutes after discontinuing DRG-S (7d(15m)), when a mean value of 3 ± 3s was found. On day 14, the withdrawal response mean was even further increased to 9± 1s, as compared to day 7 (3s), which indicates that the period of neurostimulation protocol between days 9 and 12 of SNI, during conditioning for the CPP test, did not have a sustained analgesic effect on cold allodynia-induced behavior. However, subsequent neuronal activation induced by the DRG-S session at 14d of SNI resulted in withdrawal responses of 2 ± 1s just after discontinuing DRG-S (at 14d(0min)), suggesting an analgesic effect for cold allodynia that was maintained until 60 minutes after discontinuing DRG-S but that was gradually becoming weaker (mean values of 4 ± 1s, at 15min, 3 ± 1s at 30min, 4 ± 3s at 45min, and 5 ± 2s at 60min).

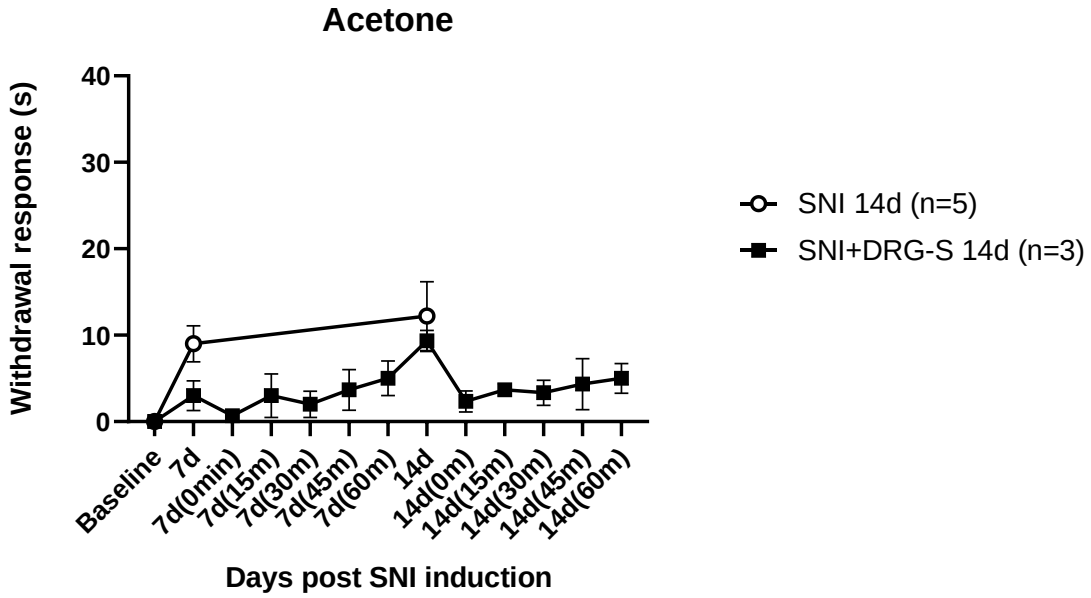

Figure 30: Effects of repeated dorsal root ganglion neurostimulation (DRG-S) on the withdrawal response, in seconds, to cold allodynic stimuli (Acetone test) on rats with SNI. Nerve injured animals receiving DRG-S appear to show an attenuation of SNI-induced cold allodynia just after discontinuing DRG-S, both when this occurred at 7d and 14d of SNI. This analgesic effect appears to have been somewhat maintained until the 60 minutes after discontinuing DRG-S. Due to the low number of animals a statistical analysis was not performed.

Motor coordination – Rotarod test

The motor performance in the Rotarod test is shown in Figure 31. All the animals, both from the SNI 14d and SNI+DRG-S 14d groups, showed worsened motor coordination after SNI induction, as inferred from the decreases in the time of permanence in the rotarod apparatus, from 92 ± 7s (at baseline) to 38 ± 13s (at 7d of SNI, for the SNI 14d group) and 120 ± 26s (at baseline) to 53 ± 18s (at 7d of SNI, for the SNI+DRGS 14d group). The SNI 14d animals maintained these low durations of permanence on the rotarod on day 14, with a mean of 31 ± 6s. The DRG-S at 14 days of SNI did not change the motor coordination of the SNI+DRG-S 14d animals.

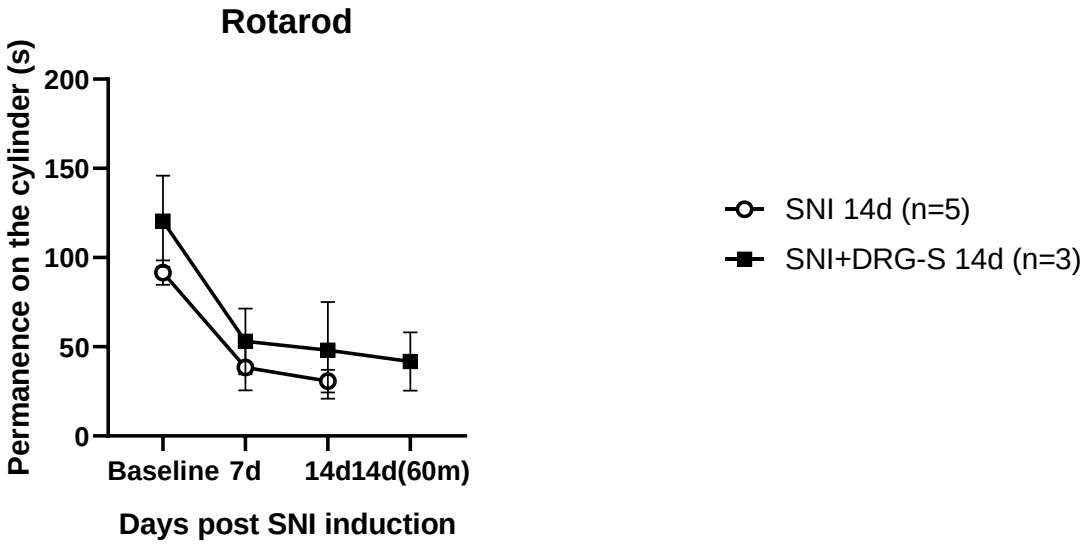

Figure 31: Effects of repeated dorsal root ganglion neurostimulation (DRG-S) on the time of permanence in the Rotarod, in seconds (s), on rats with SNI (SNI+DRG-S 14d group) in comparison to the SNI 14d animals. The DRG-S protocol did not change the motor coordination pattern of the SNI animals when this was evaluated 60 min after ending the stimulation protocol. Due to the low number of animals a statistical analysis was not performed.

Motor threshold for DRG-S electrodes

The threshold current to induce motor activity by DRG-S were also measured (Figure 32). These values were useful to indirectly evaluate the localization of the electrodes within the DRG, since the higher the threshold, the more distant are the electrodes from the DRG. The motor threshold showed a trend towards decreasing at 14 days after the surgery for the electrodes implantation, with mean values of 3 ± 1mA (at -7d) and 2 ± 0mA (at 7d). On day 9 after SNI induction, corresponding to the first day of conditioning for the CPP test, the mean value of motor threshold increased to 3 ± 1mA. However, on day 10 the mean values decreased to 1 ± 0mA and remained around this value (between 1 ± 0 and 2 ± 0 mA) until day 14. This suggests that the electrodes were not contacting close enough the DRG and during the healing process from the surgery they were probably brought closer, staying in that position until the end of the study. These values also indicate that the targeted neurons remained sensitive to the electrical stimulation throughout the whole duration of the experiments.

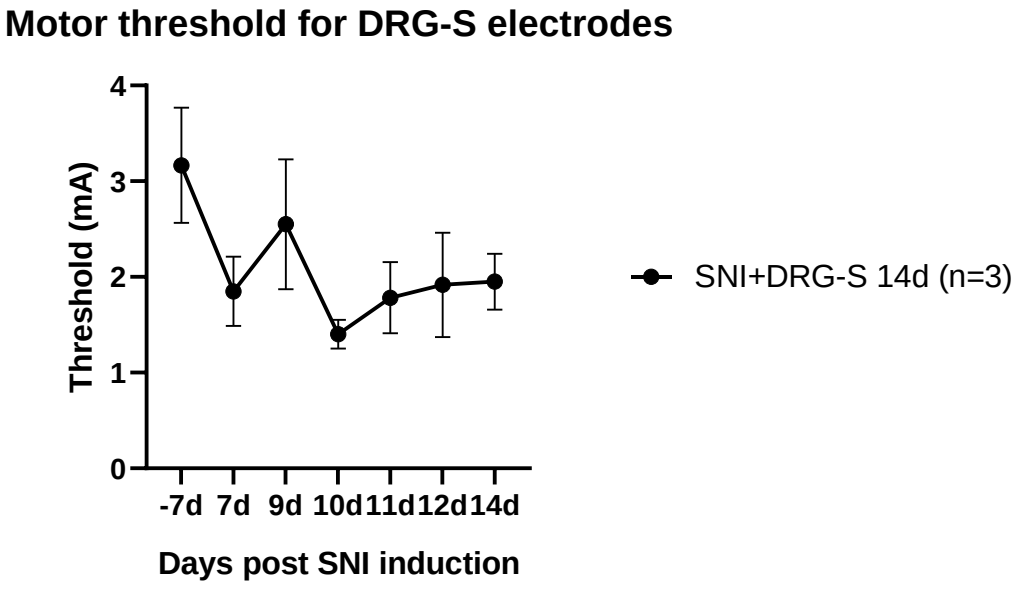

Figure 32: Motor threshold for the DRG-S electrodes. The time course shows a downward trend for the motor threshold on day 7, followed by an increase on day 9 and a decrease again in the

day after, remaining thereafter within similar lower values throughout the remaining experimental period, until the end of the study on day 14.

The Spontaneous Pain-related behavior of SNI animals was not affected by DRG-S

The ability of DRG-S to relieve spontaneous pain and the subsequent preference for the location where a conditional stimulus was administered was tested by the CPP test (Figure 33). Eight days after SNI, and 1 day after nociceptive behavioral evaluation, a preconditioning trial to confirm that animals had no prior preference towards one chamber was performed. As expected, the animals from both groups showed a percentage of time spent in one of the chambers close to 44% in the pretest (Figure 33), indicating no preference for any of the chambers. The pretest was followed by four sequential days of conditioning (30 minutes with 20-Hz of DRG-S in one chamber, 30 minutes with no DRG-S in the other). As control, the SNI 14d group also performed the conditioning period without any type of conditioning (no DRG-S was administered) in any of the chambers. On day 13, the day after the last conditioning day, testing of conditioned preference was performed by placing the animal in the central chamber. The SNI+DRG-S 14d group showed no preference for the chamber in which DRG-S has been delivered (30 ± 6%, Figure 33), which appears to indicate that the DRG-S has not produced a rewarding effect.

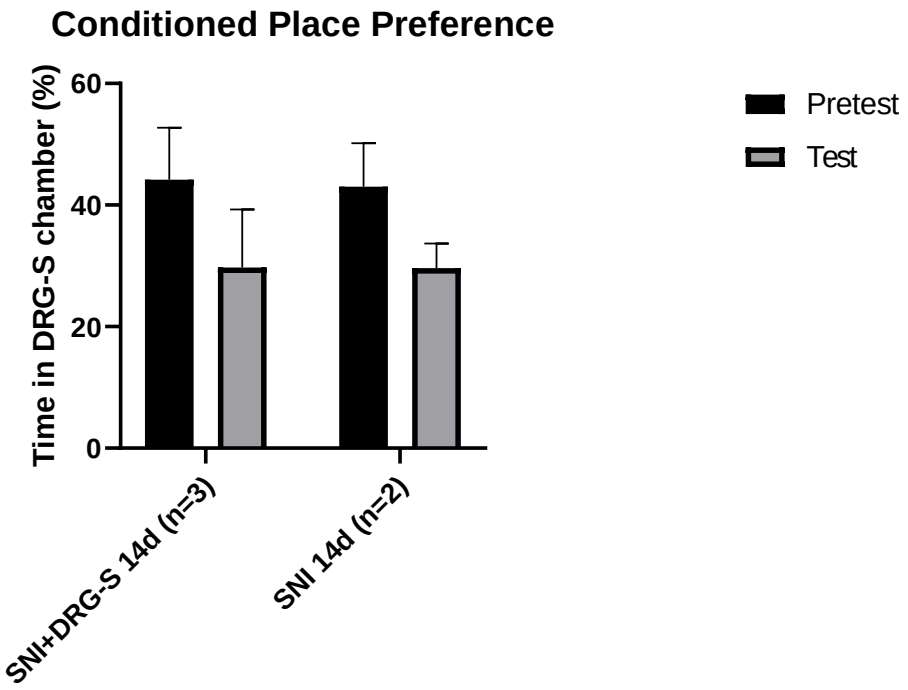

Figure 33: Conditioned Place Preference test. Before the four conditioning days, none of the two groups demonstrated a preference for one of the two chambers. After conditioning, during the test day, the SNI+DRG-S 14d rats continued to not show any obvious preference for the chamber where the DRG-S was delivered, neither in the test day nor in comparison with the pretest day. Mean ± SEM. Multiple unpaired t-tests. No significant differences were found between the pre-test and test days in the two groups evaluated.

Dorsal root ganglion stimulation (DRG-S) appears to reestablish the sensitivity when this is lost in neuropathic pain

One SNI animal was allowed to develop and maintain the neuropathic condition until 21 days after the model induction, and was submitted to behavioral evaluation and DRG-S on days 14 and 21, and to CPP conditioning by neurostimulation between days 16 and 19 (SNI+DRG-S 14d-21d). On the contrary to the other SNI rats, this animal developed an autotomy behavior and excessive grooming after SNI induction, which could be related with the lack of sensitivity to noxious and innocuous stimuli that was also

observed in the behavioral assessment. A feeling of numbness or a loss of sensation is also commonly observed in patients with neuropathic pain.

Mechanical allodynia – von-Frey test

At the baseline, the SNI animal showed a withdrawal threshold to mechanical innocuous stimulation with the von-Frey filaments at its maximum value, resulting in a cut-off established at the 60g filament, as expected (Figure 34). However, at 14 days of SNI the withdrawal threshold to von-Frey filaments remained high, on the 60g, on the contrary to what is described in this neuropathic pain model. After the DRG-S protocol on day 14, the withdrawal threshold to von Frey filaments remained high on the 60g, suggesting insensitivity to the stimuli. Interestingly, on day 21, before DRG-S protocol, the animal showed a lower threshold for withdrawal from the mechanical stimuli, that is characteristic of a neuropathic pain state, indicating a reestablishment of the sensitivity to mechanical innocuous stimuli. After the DRG-S protocol (30 minutes of duration), the animal showed an improvement in the withdrawal threshold at 21d(0m), and then a return to values similar to pre-DRG-S at 15 minutes after the stimulation (at 21d(15m)). At the 21d(30m) time-point the animal showed a higher increase in the withdrawal threshold, similar to the baseline values, probably as a result of the analgesia induced by the DRG-S. This improvement was maintained at the 21d(45m) and 21d(60m) time-points, since although the withdrawal thresholds were lower than at 21d(30m), they were higher than the values observed at 21 days pre-DRG-S. This suggests that the animal has recovered from the insensitivity found before initiating the DRG-S protocols.

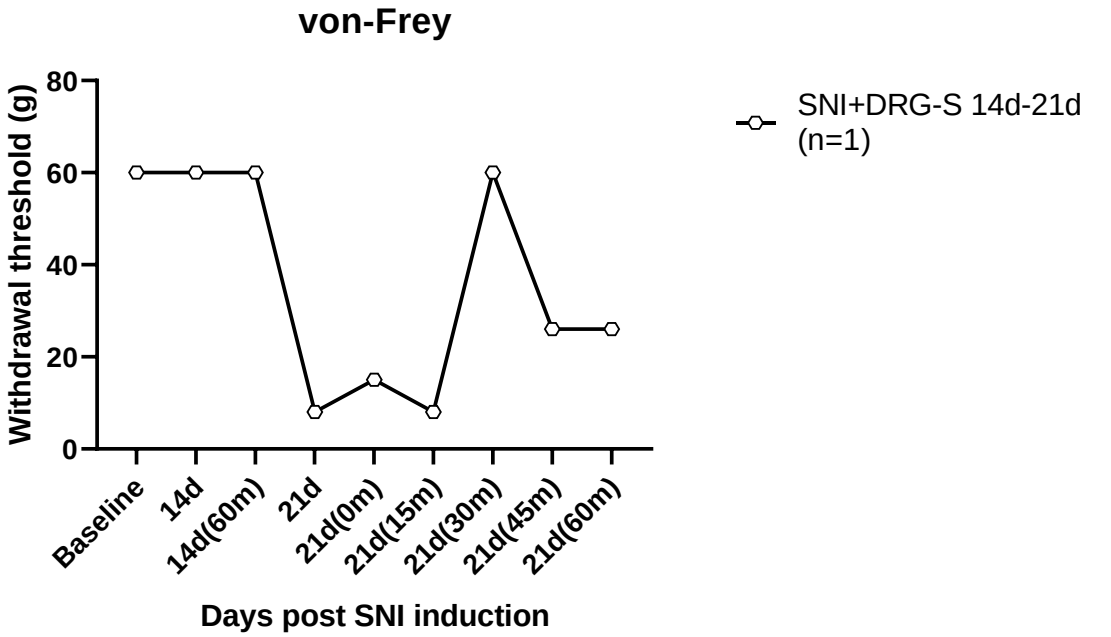

Figure 34: Ipsilateral paw withdrawal threshold, in grams (g), to von-Frey filaments of a SNI animal that developed the neuropathic condition for 21 days with signs of autotomy and insensitivity to sensory stimuli, and received the DRG-S protocol. The graph corresponds to the behavior of the animal during the 21 days of evolution of the neuropathic pain model and to the effect of DRG-S. After DRG-S, implemented between days 16 and 19 for the CPP test conditioning, the rat showed a recovery of the sensitivity with levels of mechanical sensitivity characteristic of the SNI model. Additionally, after the DRG-S protocol at 21 days, a transient improvement of mechanical allodynia was observed at 21(0m), 21d(30m), 21d(45m) and 21d(60m). Due to the low number of animals a statistical analysis was not performed.

Mechanical hyperalgesia – Pin-Prick test

As in the previous nociceptive test, the rat showed lack of sensitivity to the noxious mechanical stimuli of the Pin-Prick test at day 14 of SNI, which is uncommon in the SNI model (Figure 35). However, and on the contrary to the results in the von-Frey test, the animal showed a typical mechanical hypersensitivity at 1 hour after ending the DRG-S protocol (at 14d(60m)), which suggests a recovery of sensitivity to mechanical stimulation. On day 21, it was expected that the animal would respond to the

mechanical stimuli, but the response was very low, almost similar to the baseline values. After implementing the DRG-S protocol, the animal remained with low withdrawal responses throughout the whole experimental period, until 60 minutes after the electrostimulation.

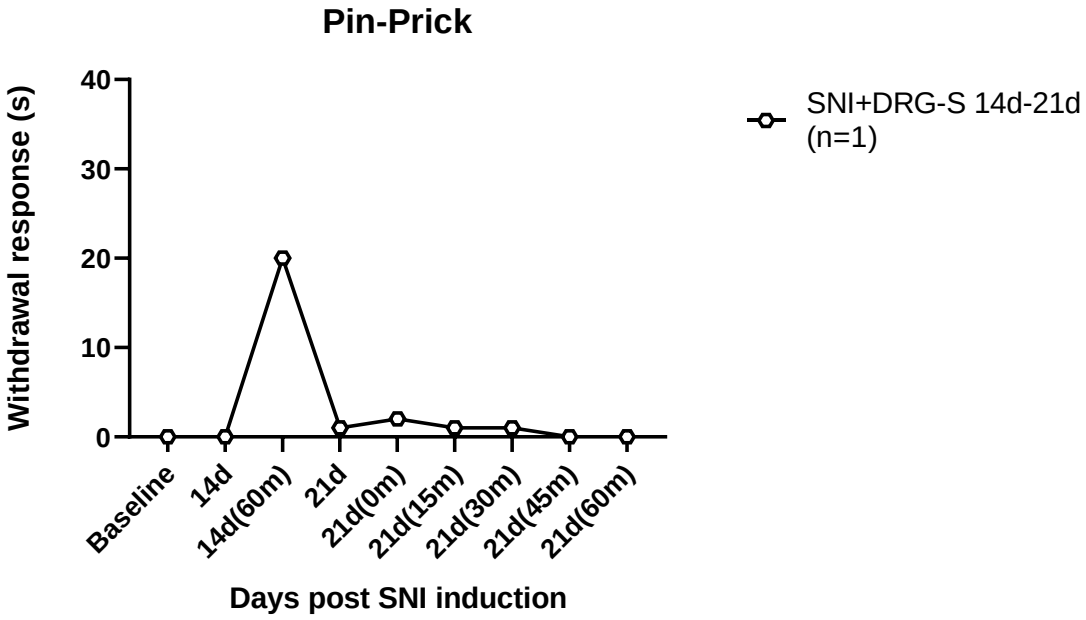

Figure 35: Ipsilateral paw withdrawal duration, in seconds (s), to mechanical noxious stimuli (Pin-Prick) of a SNI animal that developed the neuropathic condition for 21 days with signs of autotomy and insensitivity to sensory stimuli, and received the DRG-S protocol. The graph corresponds to the behavior of the animal during the 21 days of evolution of the neuropathic pain model and to the effect of DRG-S. One hour after the DRG-S implemented at day 14, the rat showed a recovery of the sensitivity with levels of mechanical sensitivity characteristic of the SNI model. The values were decreased again after the DRG-S that occurred between the days 16 and 19, for the CPP test conditioning, and remained low for the whole experimental period, after DRG-S at 21 days of SNI. Due to the low number of animals a statistical analysis was not performed.

Cold allodynia – Acetone test

As in the two previous nociceptive tests, the animal remained insensitive to the cold allodynic stimulus applied in the Acetone test at 14 days of SNI, thus showing withdrawal responses close to the baseline values (Figure 36). After implementing the DRG-S protocol at 21 days of SNI, the animal showed increased withdrawal responses at 15 and 30 minutes after DRG-S, which could be due to a recovery of the sensitivity to cold stimuli, a characteristic of neuropathic pain conditions.

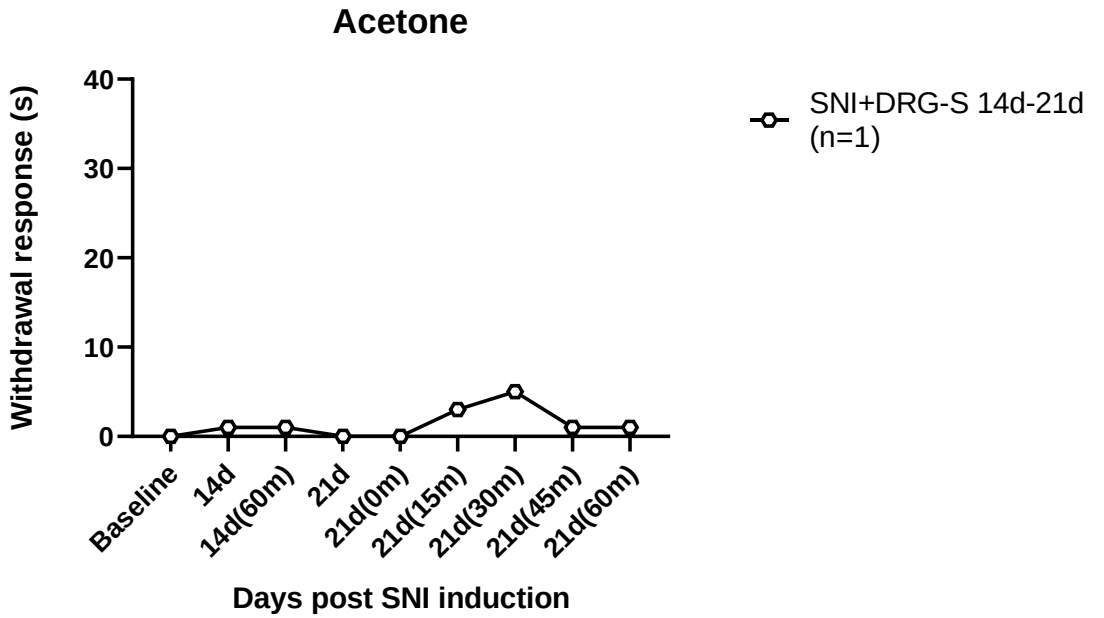

Figure 36: Ipsilateral paw withdrawal duration, in seconds (s), to cold stimuli (Acetone) of a SNI animal that developed the neuropathic condition for 21 days with signs of autotomy and insensitivity to sensory stimuli and received the DRG-S protocol. The graph corresponds to the behavior of the animal during the 21 days of evolution of the neuropathic pain model and to the effect of DRG-S. Only at 15 and 30 minutes after the DRG-S implemented at day 21, the rat showed a recovery of the sensitivity to cold stimuli characteristic of the SNI model. Due to the low number of animals a statistical analysis was not performed.

Motor coordination – Rotarod test

The animal showed a decrease in motor coordination after the SNI induction. This decrease was maintained until day 21 after SNI induction (figure 37). This suggests that the DRG-S protocol *per se* appears to not improve the motor coordination in this animal.

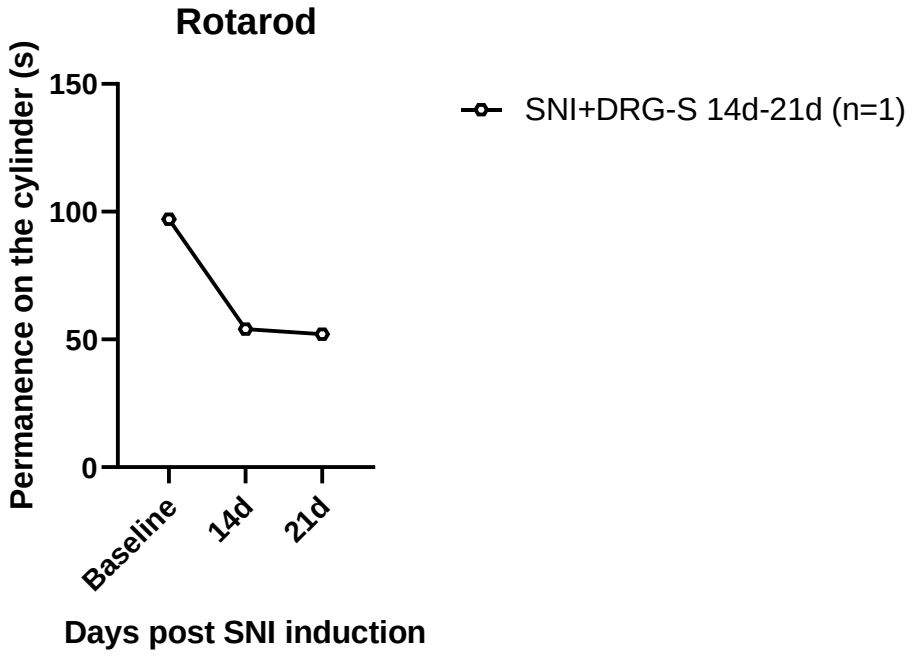

Figure 37: Time of permanence in the Rotarod, in seconds (s), of a SNI animal that developed the neuropathic condition for 21 days with signs of autotomy and insensitivity to sensory stimuli and received the DRG-S protocol. The graph corresponds to the motor coordination of the animal at the baseline, 14d, and 21d of SNI time-points. The implementation of the DRG-S protocol at 14 and 21 days, and between days 16 and 19 for the CPP test conditioning appears to not have affected the motor coordination, which was not improved. Due to the low number of animals a statistical analysis was not performed.

Motor threshold for DRG-S electrodes

The motor threshold showed a trend towards increasing during the first 7 days after the surgery for electrodes implantation, and remained constant throughout the first 2 weeks of SNI, until day 16 (Figure 38). At day 17 after SNI induction, there was a small decrease and the values remained quite stable from thereafter until the end of the experiment. This suggests that the electrodes remained stably located, that the tissue response at the electrode contacts was minimal, and that the targeted neurons remained sensitive to the electrical stimulation.

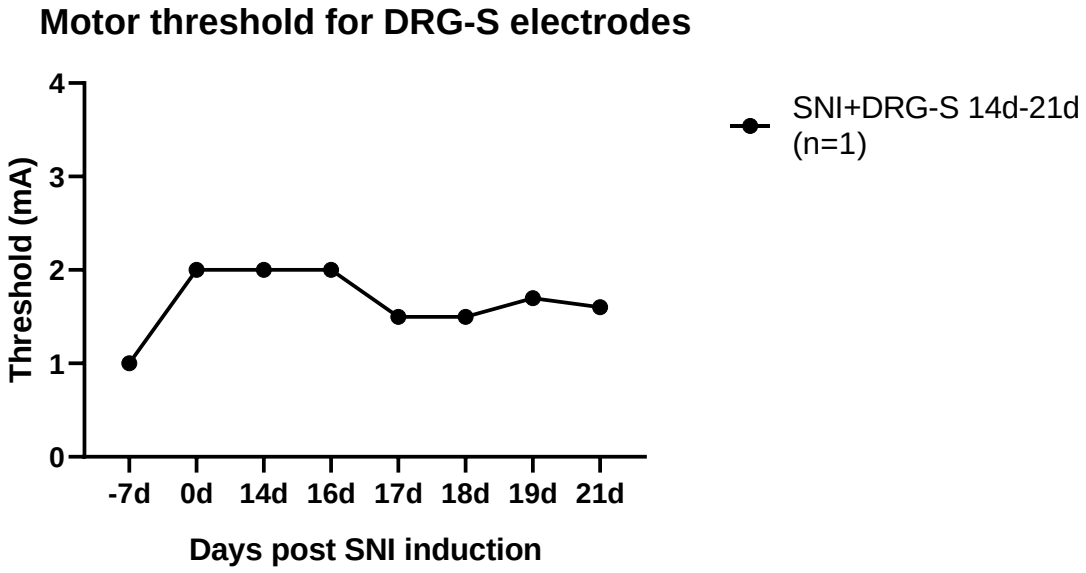

Figure 38: Motor threshold for DRG-S electrodes, in milliamperes (mA), of a SNI animal that developed the neuropathic condition for 21 days with signs of autotomy and insensitivity to sensory stimuli and received the DRG-S protocol. Time course shows an upward trend of the motor threshold over the first 7 days of measurement, after the surgery for electrodes implantation. The values remained quite constant in the first two weeks of SNI, and until the end of the study on day 21. Due to the low number of animals a statistical analysis was not performed.

Evaluation of protein expression

Immunofluorescence for ATF3

To determine the effects of the DRG-S electrodes placement and maintenance (Sham-DRGS), and the effect of neurostimulation on the neuronal stress of DRG neurons in SNI animals, we studied the DRG tissue harvested at different time-points (7d, 14d, and 21d) after SNI induction. For the three SNI+DRG-S groups, only the L5 DRG was analyzed, since the L4 DRG did not present well-preserved cells. The ATF3 immunoreactivity in the ipsilateral L4 and/or L5 DRGs appears to show an increase in the SNI 14d group, with a mean of 56± 6% ATF3-IR cells, as expected, as well as in two of the three groups that received neurostimulation protocols, with means of 52 ± 13%, and of 51 ± 2%, in the SNI+DRGS 7d and SNI+DRG-S 14d, respectively. On the other hand, the SNI+DRG-S 14d-21d rat showed a lower increase in the percentage of ATF3-IR cells with a value of 37%. The Sham-DRGS animal showed only a small percentage of ATF3-IR cells, with a value of 16%, probably due to the electrode's implantation (Figure 39). In the contralateral ganglia no ATF3-IR cells were detected (data not shown).

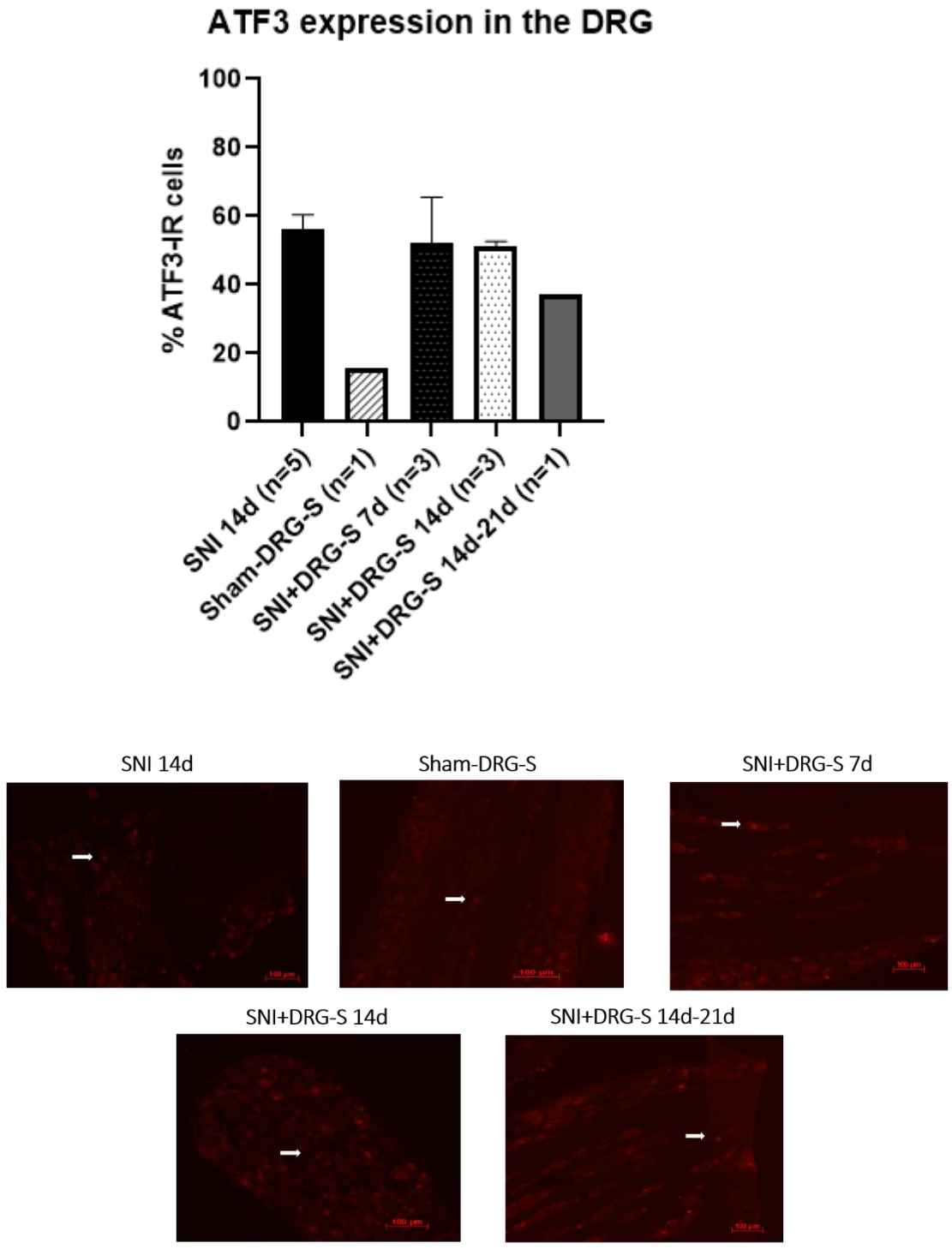

Figure 39: Immunoreaction for ATF3 in L4 and/or L5 DRG. The graph on the top shows that the labelling for the neuronal injury marker was minimally affected by the procedure for electrodes implantation and their maintenance on the L4 DRG (Sham-DRG-S animal), when compared to SNI animals, or to SNI animals with implanted electrodes and which were subjected to

neurostimulation protocols. In the SNI 14d group, and SNI+DRG-S (7d/14d/14d-21d) groups the exposure, ligation and transection of the tibial and peroneal nerves resulted in increased neuronal expression of ATF3. The images below are representative photomicrographs of DRG sections immunoreacted against ATF3. Arrows indicate ATF3-positive cells (red, labeling the nucleus of the neuronal cell bodies). The scale is uniform for all fields; scale bar = 100 μm (10x magnification).

Immunofluorescence for NPY

Immunoreaction against NPY detected expression of the neuronal injury marker in ipsilateral ganglia (L4/L5). For the three SNI+DRG-S groups, only the L5 DRG was analyzed, since the L4 DRG did not present well-preserved cells. Data shows a tendency towards an increase in the SNI 14d group with a mean of 34 ± 2% of NPY-IR cells, as well as in the SNI+DRG-S 7d (39 ± 8 % NPY-IR) and SNI+DRG-S 14d-21d groups (47% NPY-IR). On the other hand, in the Sham-DRG-S and SNI+DRG-S 14d rats, the expression of the neuronal marker was lower, with values of 18% and 22 ± 3 % of NPY-IR cells, respectively (figure 40). In the cells of the contralateral DRGs, the labeling for NPY was very low in all the four groups, with almost negligible NPY expression (data not shown).

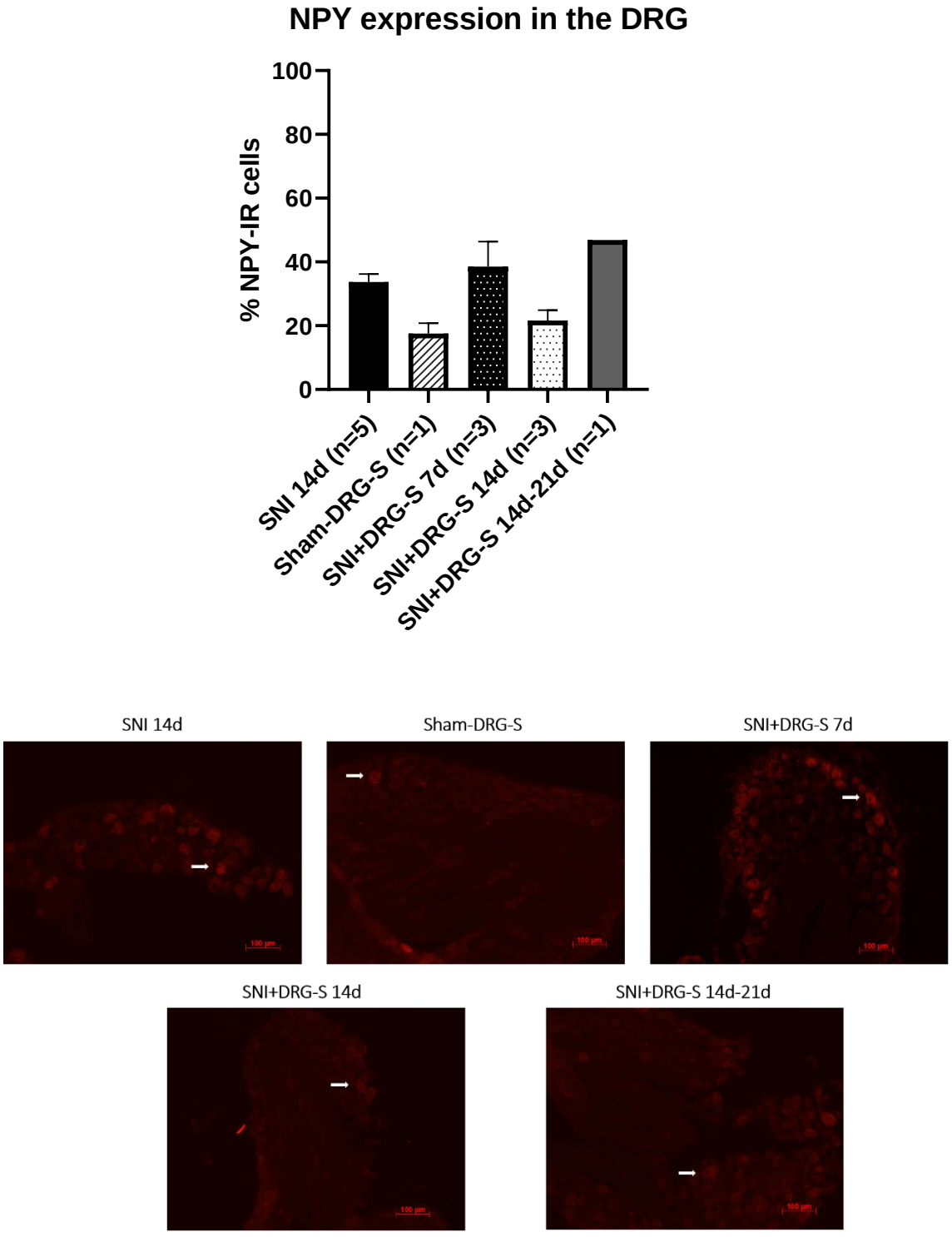

Figure 40: Immunoreaction against NPY in L4 and/or L5 DRG. The graph on the top shows that the labelling for NPY was minimally affected by the procedure for electrodes implantation and their maintenance on the L4 DRG (Sham-DRG-S animal), when compared to SNI animals, or to

SNI animals with implanted electrodes and which were subjected to neurostimulation protocols, except for the SNI+DRG-S 14d group, which also showed lower numbers of NPY-IR cells. The images below are representative photomicrographs of DRG sections immunoreacted against NPY. Arrows indicate NPY-positive cells (red, labeling the cytoplasm of the neuronal cell bodies). The scale is uniform for all fields; scale bar = 100 μm (10x magnification).

Discussion

In the present study, we implemented, for the first time in our lab, a surgical protocol for implanting electrodes underneath the L4 in rats and evaluated the effects of acute and repeated neurostimulation protocols at different time-points of evolution of SNI, a well validated model of peripheral neuropathic pain. Although the representative number of animals in each experimental group is still low, and some technical problems were not completely solved, our data appears to suggest that the neurostimulation protocol has analgesic effects on the nociceptive behavior of the animals, particularly affecting mechanical allodynia, as inferred by the data from the von Frey test. The expression of the neuronal injury marker ATF3 in the L5 DRG, which are in the neighborhood of the DRG receiving electrical stimulation (the L4), was not significantly affected by neurostimulation, as compared with the SNI, in contrast with that of NPY. This suggests that ATF3 may not be primarily involved in the electrical stimulation effects on nociception but that NPY may be possibly implicated in the mechanisms of DRG-S induced analgesia.

The pre-clinical and clinical concern in neuropathic pain treatment has been renewed in the search for new and effective strategies, and for that, the conventional therapies have been substituted by modulatory approaches, like the SCS and DRG-S (Guha and Shamji 2016; Liem et al. 2016). DRG stimulation (DRG-S) appeared as an alternative approach in the clinical context, since the DRG have many features that make them appealing targets for the application of neuromodulation. Indeed, some clinical studies, like the ACCURATE trial, published in 2017, reported that the DRG-S provides a decrease in the pain sensation in >50% when compared to the SCS, being also superior in a high degree of statistical significance in many others parameters, like postural stability, paresthesia precision and mood improvement (T. R. Deer et al. 2017). Basic research studies that have also been done throughout the years, mainly *in vitro,* demonstrated that neuromodulation evoked the increase of Ca^{2+} channels, and drove a decrease in the multiple actions potentials and in the conduction velocity of the sensory neurons (Koopmeiners et al. 2013b). However, in spite of these findings and of the fact that

neuromodulation has been used in the clinical practice for many years, there is still much to know since the mechanisms behind the analgesic effects following neurostimulation of DRG remain largely unknown (Vuka et al. 2018). Altogether, this encouraged us to implement and improve a neurostimulation protocol in the DRG of SNI rats, in order to evaluate the effect of electrical field stimulation on the nociceptive behavior and on the expression of molecules, such as neuronal stress/damage markers, which are known to be altered in DRG cells in neuropathic pain models.

To our best knowledge, up till now DRG-S has been used in pre-clinical research in animal models by only two research groups, worldwide. The first group is from the Medical College of Wisconsin, in USA, and has evaluated the DRG-S effect on nociception in the tibial nerve injury (TNI) rat model of neuropathic pain (Pan et al. 2016). The second group is from the Netherlands and has been focusing the research on evaluating similar outcomes in a rat model of diabetic polyneuropathy, having published only last year their first article on this topic (Koetsier et al. 2019). In our investigation, we decided to use a different rat model of neuropathic pain, namely the SNI model, since in the clinical context the DRG-S has proved to be more effective on the foot region (Koopmeiners et gal. 2013b). *Pan et al.* and *Koetsier et al.* research groups performed the surgery for the electrode's implantation by using an approach similar to the used in clinical settings, thereby inserting the electrode into the intervertebral foramen dorsolateral to the DRG, and leaving it in a juxtaposition with the DRG. In our surgical approach, we decided to improve the method used in our group for the intra-ganglionic injection of rAAV vectors (Alonso, Neto, and Ferreira-Gomes 2017), in such a way that the L4 DRG nerve root was completely exposed by removing the intravertebral foramen instead of using it as support. To assess the effect of the electrodes on the ganglia, we performed the surgery for implantation of the electrodes in a naïve animal. Our results showed that the surgery *per se*, as well as the maintenance of the electrodes (without performing a DRG-S protocol) in contact with the L4 DRG, during 21 days, had no effects on the animal's behavior. Additionally, histological evidence of minimal injury to the DRG was found, as inferred from the low expression of the neuronal injury markers ATF3 and NPY, which was in line with the findings of Pan et al. 2016.

Until having an animal with a successful surgery and post-surgery recovery, many technical problems were overcome, not only with the surgical approach itself, but also with the animal recovery and the maintenance of the electrodes in the initial position throughout the needed time to complete the whole experimental period. Two of the problems associated with the surgery were to develop an effective method to hold the electrodes to the ganglion, and to also hold them across the body until tunneling them to the head of the rats. We managed to solve these issues, but in some animals their electrodes broke in the region where they were subcutaneously tunneled and in others the electrodes broke at halfway of the tunneling. In addition to these, some animals developed a green subcutaneous lump the day after the surgery that progressed into a wound the next day. The wound healed with the help of antibiotic administration but we were not able to disclose their cause since they appeared in different sites in different animals and they were not related with the electrodes tunneling region, nor with the injection sites. In addition to the problems associated with the surgery for electrodes implantation, we had also many SNI rats that developed excessive grooming and, in extreme cases, autotomy. The animals having those behaviors were housed, manipulated and behaviorally evaluated in the same weeks when the animal facilities (Biotério of FMUP) were undergoing improvement construction works. For that reason, we have attributed these behaviors to the stress evoked altogether by the model induction and the construction works.

The development of a preclinical model is useful to generate insights into the potential effectiveness of new clinical applications, providing the tools for testing mechanistic hypotheses. In our SNI animals, the mechanical and thermal hypersensitivity characteristic of neuropathic pain were evident in the von-Frey test, by a decrease in the mechanical force necessary to produce paw withdrawal, and in the Pin-Prick and Acetone tests, by an increase in the withdrawal response time after the application of the stimuli, which were similar to those described by Decosterd and Woolf 2000. Also, the rotarod test showed an impairment in the motor coordination of the animals that was sustained until the end of the protocol. In the SNI animals subjected to the electrode implantation surgery and the DRG-S protocol (SNI+DRG-S 7d), the baseline evaluation

was similar to the SNI animals. However, after applying the neurostimulation protocol an analgesic effect was observed, as inferred by the attenuation of mechanical and thermal allodynia, as well as of mechanical hyperalgesia. Our findings of attenuation of nociceptive behaviors were similar to those observed in a previous study of DRG-S in a TNI model (Pan et al. 2016). However, the results cannot be directly compared since the neuropathic pain models used were different, and mostly because Pan and colleagues evaluated the nociceptive behavior during the execution of the DRG-S protocol and 15 and 30 minutes after discontinuing it, while we have only evaluated the behavior at the cessation of the neurostimulation protocol, at 0, 15, 30, 45 and 60 minutes. Therefore, only three-time points are comparable between our results and the study of Pan et al. 2016. In the comparable time-points, the DRG-S effect was similar, so that 15 minutes after the end of the protocol (45 minutes in the study of Pan et al. 2016) the analgesic effect was higher in our study, showing a possible higher washout effect. On the other hand, at 30 minutes after DRG-S (60 minutes in Pan et al. 2016) the results were also similar, since in both studies the withdrawal response was similar to the pre-DRG-S results. We additionally tested the thermal allodynia using the Acetone test, which showed an increase in the withdrawal response time in pre-DRG-S. After neurostimulation, there was a partial reversal of the SNI-induced increase in the response to cold, which was sustained until 60 minutes after the end of the protocol, since the withdrawal response time no longer regressed to pre-DRG-S values. In comparison to the Pan et al. 2016 study, we found that just after we discontinued the DRG-S, our data were similar to those they found at 30 minutes. However, while in our study there was a decrease in the response to cold at 15 minutes after DRG-S, Pan et al. 2016 reported an increase of the animals' responses, which have returned to pre-DRG-S values at 60 minutes. Finally, we also examined the sustained nociceptive behaviors induced by the application of a Pin-Prick, a noxious mechanical force. Before the DRG-S, the animals showed an increase in the duration of response to the stimulus. After DRG-S, this period of time was decreased to values similar to those at baseline (pre-DRG-S), and these were sustained until the end of the evaluation. Looking at the results of the TNI animals in Pan et al. 2016 study, they presented similar outcomes at 30 minutes

after DRG-S (corresponding to 0 min in our study), but the values tended to reverse to those at baseline at timepoints over the 30 minutes of washout effect. Together, our results appear to indicate that DRG-S may affect the plasticity events of the sensory system, which appears to result on an antihyperalgesic effect. The works published by Pan and colleagues (Pan et al. 2016; Yu et al. 2020) have the limitation of only having evaluated the nociceptive behavior at one session of DRG-S administration. To overcome this and try to understand the effect of neuromodulation in a more sustained protocol of administration, we decided to test the SNI animals between the 7 and 14 days of disease evolution (SNI+DRG-S 14d). Our findings showed that DRG-S relieved the nociceptive behaviors immediately after discontinuing the neuromodulation protocol. In the von-Frey test, the analgesic effect was demonstrated by an attenuation of the withdrawal response, which was sustained until 15 minutes after DRG-S cessation. These results were seen both at 7 and 14 days after SNI induction, but the analgesic effect was higher on the first day of DRG-S. This was not expected, because on day 14 the animals had already been subjected to four consecutive days of a 30 minutes daily session of DRG-S (conditioning days of the CPP test). In the Pin-Prick test, used to evaluate the noxious mechanical hyperalgesia, there was an analgesic effect after implementing the DRG-S protocol that was sustained until 15 minutes after it ended. However, the nociceptive evaluation of these animals before the DRG-S did not reflect the described behavior for the SNI animals on day 7 of disease evolution. The DRG-S appears to have increased the nociception in these animals, since the withdrawal response was higher at 30 minutes after DRG-S than at the pre-DRG-S. On day 14, the pre-DRG-S values were higher but they were not in line with what is described for SNI animals. This may possibly be due to the conditioning days, when the animals received neurostimulation. After DRG-S the withdrawal responses were decreased to baseline values right after it ended, and they did not return to the pre-DRG-S withdrawal values until the end of the nociceptive evaluation. We also showed that the DRG-S partially reversed the SNI-induced increase in acetone responses. Once again, the pre-DRG-S values were very low when compared to the SNI animals. On day 14, the pre-DRG-S values were more similar to those observed in SNI animals, and therefore the analgesic

effect after DRG-S was more evident. In what concerns cold allodynia, there was a decrease that was sustained until the end of the evaluation, since the animals did not present withdrawal responses similar to those of pre-DRG-S values. The lower pre-DRG-S values for the Pin-Prick and the Acetone tests, which were not in accordance with what is usually reported in SNI conditions, could be explained by the construction works that were occurring in the animal facilities at FMUP, which certainly exposed the SNI+DRG-S 14d group of animals to unnecessary stress during the 2 weeks of nociceptive evaluation, possibly triggering some sort of stress-induced analgesia-related events. These could explain the fact that the effect of DRG-S was not so sustained as it was in the SNI+DRG-S 7d group. In addition to the nociceptive behavior, we have also decided to evaluate the motor coordination. The animals showed no improvement after DRG-S, neither on the 7^{th} nor on the 14^{th} day of evaluation. These results were not expected since the animals showed an improvement in weight bearing on the ipsilateral paw as well as on its positioning on the floor during the DRG-S protocol, in comparison to what is normally observed in SNI animals, which exhibit the paw always raised and in closed position.

The limitation of relying exclusively on the assessment of evoked pain to evaluate pain in animals is becoming increasingly evident. Moreover, the standard nociceptive behavior tests fail to identify and evaluate the spontaneous pain component, which is a common feature of the neuropathic pain described by patients that can reveal the affective aspects of pain. To overcome these problems, we also performed the Conditioned Place Preference test in the SNI+DRG-S 14d animals. The CPP test was able to identify the motivational aspects of pain (King et al. 2009). Thirty minutes daily sessions were performed on four consecutive days, and no differences were detected in the preference for the DRG-S chamber, between the pre-test and the test days. So, our results do not reflect the manifestation of DRG-S-induced pain relief behaviors associated with the affective component. However, the number of animals evaluated was very low (3 SNI+DRG-S 14d animals; and 2 SNI 14d animals), which might be the reason why our data is not in accordance with Pan and colleagues' studies (Pan et al.

2016; Yu et al. 2020). In fact, their data strongly suggest that DRG-S relieves spontaneous pain, since their animals showed a preferential choice for the DRG-S chamber.

To evaluate the effects of DRG-S at later time-points of disease evolution, and because our results were promising in the SNI+DRG-S 14d group, we decided to test the effect of DRG-S on analgesia between the 14 and 21 days of SNI evolution. We chose these time-points as all the manifestations of a proper SNI development, with associated sensory hypersensitivity, were already evident on day 14. Unfortunately, due to the many experimental problems we have already discussed, only one animal was able to survive with the electrodes long enough to perform the nociceptive evaluation at these time-points. Nonetheless, the data obtained with this animal were clinically relevant, since they reproduce, in part, the features that are typical of neuropathic condition in the clinical context. The animal (SNI+DRG-S 14d-21d) presented an absence of withdrawal responses to the three nociceptive tests, on day 14, which may reflect an inability of the animal to feel and respond to innocuous and noxious stimuli. This loss of sensation and numbness is also commonly observed in patients with neuropathic pain (Marchettini et al. 2006).

Even though the animal was not exhibiting the typical hypersensitivity-like behaviors described for the SNI model, we decided to go along with the experimental protocol and, thus, we performed the four consecutive days of 30 minutes DRG-S sessions, but we did not evaluate the CPP-related behaviors. Our findings of mechanical allodynia on day 21 showed a withdrawal response in the von-Frey test similar to the expected for SNI animals, suggesting a recovery of sensation for the mechanical stimuli. Thirty minutes after DRG-S cessation, the animal showed a withdrawal response similar to the baseline values, and this improvement was sustained until the end of the protocol since the values did not return to those found at pre-DRG-S. In the Pin-Prick test, the animal showed an increase in the withdrawal responses to noxious mechanical stimuli at 60 minutes after DRG-S, on day 14, with levels of mechanical hyperalgesia characteristic of the SNI model. Indeed, as the values observed before the DRG-S protocol were already very low, it is impossible to infer if they resulted from insensitivity or analgesia. On day 21 (after 4 consecutive days of 30 minutes DRG-S sessions), the mechanical thresholds

remained low for the whole experimental period, which again could be related with either of the above-mentioned explanations. Surprisingly, this animal did not present the expected responses for the Acetone test, neither at pre-DRG-S nor after DRG-S, on day 14. On day 21, the animal showed a slight increase in the withdrawal response at 15 and 30 minutes after DRG-S, which could possibly represent a recovery of sensation to cold stimuli triggered by neuromodulation, and then a return to baseline values at 45 minutes after-DRG-S. In what regards motor coordination, the animal showed a decrease in the time spent on the rotarod apparatus after DRG-S, which is in line with the observed in the SNI+DRG-S 14d group. Data of this animal suggest that DRG-S was able to recover the sensitivity to innocuous stimuli that the animal had lost due to the neuropathic condition.

Although neuromodulation, both at the spinal cord and DRG levels, is becoming widely used in the clinical context to alleviate neuropathic pain, the mechanisms causing analgesia remain unknown. As Pan et al. 2016, we tested the intensities of stimulation used in patients in our study, to have more direct translational results. The evaluation of the motor threshold enabled to infer the position of the electrode within the DRG throughout the 7, 14, or/and 21 days. These assessments gave information about the distance between the electrodes and DRG, because the higher the energy required to have a motor response, the more distant is the electrode. The oscillation of the mA seen in the SNI+DRG-S 14d group was not in agreement with what was expected (Pan et al. 2016) since the mA after surgery was high and only on day 10 after SNI induction came to mA values near to the ones described in the clinical studies (Eldabe et al. 2015; Van Bussel, Stronks, and Huygen 2015). Besides, the electrodes were probably implanted deflected from the DRG center or even distant from the DRG, a situation that was reverted. For both the SNI+DRG-S 14d and SNI+DRG-S 14d-21d groups, the mA were stable after the healing process, which is in line with the described in the clinical context (Eldabe et al. 2015; Van Bussel, Stronks, and Huygen 2015).

The nociceptive behavior that presented the most robust analgesic effect after DRG-S in the three experimental groups was mechanical allodynia, which was evaluated through the von-Frey test. The innocuous stimulus elicited by the von-Frey filaments is perceived

as low-threshold and for that reason is conveyed by the Aβ fibers. The activation of Aβ LTMRs fibers is assumed to have an important role both in the inhibition of noxious inputs processing in the DRG as well as in the physiologic pain inhibition at the spinal cord (Graham et al. 2019). In a computational prediction model for the neuromodulation of the L5 DRG it was deduced that, regardless of the distance between the electrode and the DRG as well as of the pulse width and/or the pulse frequency used, the Aβ LTMRs fibers are always activated in a higher percentage when compared to the Aα and the Aδ fibers (Graham et al. 2019). The Aδ fibers are the ones conveying the stimuli associated with the Pin-Prick and Acetone tests and may be also differentiated into LTMRs or HTMRs. As suggested by the computational model, the Aδ LTMRs fibers are more activated than the HTMRs, although in a very low percentage, since the distance between the electrode and the DRG appears to influence the number of fibers being activated (Graham et al. 2019). Although one has to take into account that computational modelling considers the DRG in an isolated environment, without the real conditions that occur in an *in vivo* model, the conclusions of Graham et al. 2019 are in agreement with the lack of lower analgesic effect efficacy found in the Pin-Prick and Acetone tests, and the higher pain relief observed in response to the von-Frey filaments. The same computational modelling study predicts that pulse widths between 100 μs and 200 μs are preferred for maximizing the activation of innocuous neurons while minimizing activation of potential nociceptors neurons (Graham et al. 2019). In accordance to this data, we decided to use a lower pulse width (150 μs) than the one used by physicians (200-300 μs) in their clinical practice.

In our study we evaluated, for the first time, the effects of DRG-S on the expression of neuronal stress markers. Immunohistochemistry was performed on the L5 DRG and not on the L4 ganglia, which received the neuromodulation protocol, because the L4 DRG did not have well-preserved cells, probably as a consequence of the high pulse frequency used (20Hz). In fact, we used the same pulse frequency as that used in the clinical context, but it is important to acknowledge that human DRG are bigger structures than the rat's DRG, and for that reason more robust to support higher pulse frequencies. In the L5 DRG, the number of ATF3-IR cells was similar between the SNI and

SNI+DGR-S groups. The number of ATF3-IR cells found in the SNI group was in agreement with what was expected, since the ATF3 is considered as a marker of neuronal injury, and SNI has been shown to induce high expression levels of this marker in the DRGs (Tsujino et al. 2000b). Additionally, in the SNI+DGR-S groups, the electrodes were in direct contact with the DRG, and although they are biologically compatible, they delivered an electrical stimulus. This did not apparently worsen the neuronal stress when compared with the SNI condition *per se*, as judged by ATF3 expression. On the other hand, the Sham-DRG-S animal, which had electrodes in contact with the L4 DRG during 21 days but did not receive the DRG-S protocol, showed a low percentage of ATF3-IR cells, suggesting that the electrode implantation *per se* appears to produce minimal neuronal stress and that it is well tolerated. This in accordance with the results of Pan et al. 2016, in what concerns their analysis of ATF3 immunolabeling in the DRG of Sham-TNI animals, although a direct comparison is hindered by differences in the neuropathic pain models used (TNI *vs* SNI) and the fact that they did not analyze the relative percentage of the immunoreactive cells, as we did, and rather only published representative images.

In the SNI as well as in the SNI+DRG-S 7d and SNI+DRG-S 14d-21d animals, there was an upregulation of the percentage of NPY-IR cells. In contrast, in the Sham-DRG-S animal and the SNI+DRG-S 14d group, the percentage of NPY-IR cells was lower. NPY is a regulator molecule that is implicated in pain mechanisms, particularly in the dorsal horn of the spinal cord (P. R. Brumovsky et al. 2002; Magnussen, Hung, and Ribeiro-da-Silva 2015; Malet et al. 2017b; Intondi et al. 2008). After nerve injury an upregulation of the NPY expression was found, particularly in large neurons, and its expression was correlated with the nerve lesion extension (Magnussen, Hung, and Ribeiro-da-Silva 2015), which is in agreement with our data in the SNI group of rats. Moreover, NPY was described as a molecule that remains available to grant a long-term inhibition of the pronociceptive transmission (Solway et al. 2011). Taking this into account, one could expect that the DRG-S would decrease the levels of NPY-IR cells, since the neuromodulation appears to had an analgesic effect, as inferred by the behavioral assessment. In the SNI+DRG-S 7d group this did not happen maybe because the time of

DRG-S was insufficient to alter the molecular paradigm of the cells. In the SNI+DRG-S 14d-21d the scenario was different, since the animal appears to have recovered the ability to perceive innocuous sensory stimuli, which could possibly be associated with the higher expression of NPY. In contrast, in the SNI+DRG-S 14d group, which had repeated DRG-S with an evident analgesic effect, at least in the von Frey test, the NPY values were similar to those of Sham-DRG-S, suggesting that repeated electrical stimulation had an effect on NPY expression. This may possibly indicate that NPY is implicated in the mechanisms of DRG-S induced analgesia, but of course more studies are needed to disclose this.

Conclusions

Dorsal root ganglion stimulation (DRG-S), both acute and repeated, of the L4 DRG, appears to reduce the neuropathy-induced behavior in SNI animals, in different time-points of disease evolution.

Behavioral Evidences

The surgery for electrodes implantation and the maintenance of the electrodes are secure procedures

Although the surgical approach for implanting the electrodes near the DRG is not similar to the one used in the clinical context, the procedure that we developed in rats appears to be secure and have minimal drawbacks in what regards to nociceptive behavior as well as neuronal injury. We need, however, a higher number of animals to have robust data.

DRG-S reduces the neuropathy-induced behavior of SNI animals in different time-points of disease evolution

DRG-S seems to have an antinociceptive potential in SNI animals at different time-points of disease evolution. However, the number of animals *per* group is low, and more robust groups are needed to achieve consistent results.

DRG-S did not reverse the spontaneous pain of SNI animals

Despite the behavioral results, and the qualitative analysis done throughout the experimental time, the Conditioned Place Preference test results did not reflect a decrease in the spontaneous pain (which also reflects the emotional and affective components of pain).

Molecular Evidences

DRG-S showed a direct relationship with NPY but not with ATF3

ATF3 protein expression had the same levels in ipsilateral DRG of SNI and SNI+DRG-S animals, while in the Sham-DRG-S animal was lower. On the other hand, in the SNI+DRG-S 14d group the NPY expression levels were similar to those in Sham-DRG-S, and thus lower than in the SNI group, which may be related with the decrease of the nociceptive behavior observed in the animals receiving DRG-S.

Future Perspectives

We are aware that additional improvements are necessary to have better success rates in the surgical approach for electrodes implantation and in DRG-S protocol.

In the surgical approach, we need to grant the stability of the electrodes across the dorsal part of the rats' body where the electrodes where tunneled. We think this could be achieved with the insertion of the electrodes inside a catheter along the subcutaneous tunneling region. We also need to improve the connection of the extremities of the electrodes to the external neurostimulator. To better achieve this goal, a more multi-disciplinary team, comprising an electrical engineer, would be helpful to assist in some technical questions, like with the breakage of the electrodes problems and the construction of a plug-in connection, which would reduce the time that animals need to be under volatile anesthesia.

In terms of the DRG-S protocol, we think that a lower pulse-frequency (<20Hz) would yield successful behavioral results and would provide the opportunity to study the mechanism behind the analgesic effect in the DRG subjected to neuromodulation, since lower pulse-frequency would probably increase the preservation of DRG cells.

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Appendix

Solutions preparation-general reagents

Phosphate buffer 0.4M (stock solution)- 26.2g of hydrated sodium dihydrogen phosphate (NaH2PO4.H2O), 140g of potassium hydrogen phosphate (K2HPO4) diluted in distilled water up to the final volume of 2.5L (pH between 7.2 and 7.4).

Phosphate buffer saline 1M (PBS 10X) – 80g sodium chloride (NaCl), 2g potassium chloride (KCl), 11.4g sodium phosphate (Na_2HPO_4,) 2.7g potassium dihydrogen phosphate (KH_2PO4), distilled water up to 1L.

Phosphate buffer saline 0.1M (PBS) – 100mL of PBS 10X and distilled water up to 1L.

Phosphate buffer saline 0.1M with Triton X-100 (PBS-T) – 100mL of PBS 10X in distilled water up to 1L. Add 3 ml of Triton-X-100.

Tyrode´s solution- 6.8g of sodium chloride (NaCl), 0.40g of potassium chloride (KCL), 0.32g of hydrated magnesium chloride (MgCl2.6H2O), 0.1g of hydrated magnesium sulphate (MgSO4.7H2O), 0.17g of hydrated sodium dihydrogen phosphate (NaH2PO4.H2O), 1.0g of glucose and 2.2g of sodium hydrogen carbonate (NaHCO3); add distilled water up to the final volume of 1L.

PFA 4% - 800mL of PBS, 40g of paraformaldehyde. Adjust the volume with PBS up to 1L.

Sucrose solution 30% in PBS- 30g of sucrose diluted in phosphate buffer 0.1M to final volume of 1L.