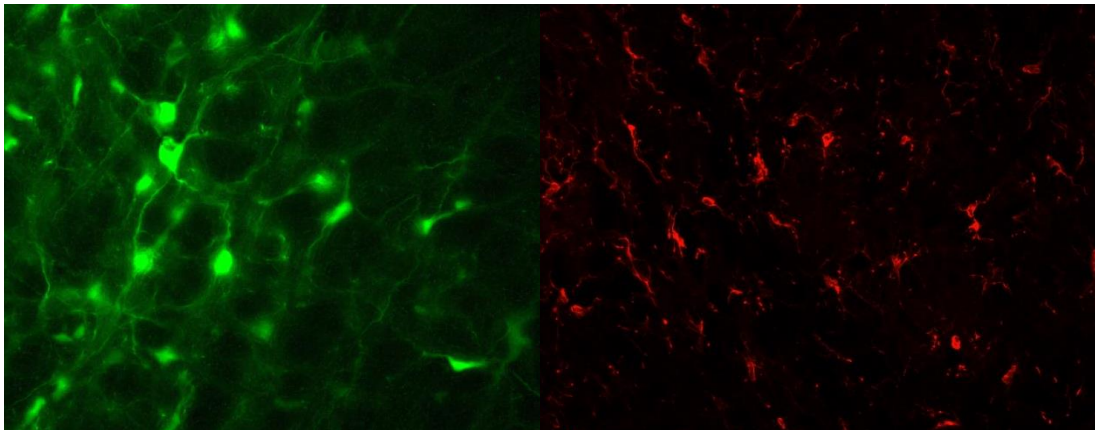

Unravelling the role of descending pain facilitation during opioid-induced hyperalgesia.

-Studies in a model of chronic neuropathic pain-

Integrated Master on Bioengineering

Molecular Biotechnology



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Porto, 23rd of June 2014

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Abstract

Chronic pain afflicts a large number of patients worldwide and opioids represent the most commonly used drugs to treat this maladaptative pain. The chronic treatment with opioids may induce, paradoxically, an increase of pain also known as **opioid-induced hyperalgesia** (OIH). OIH has been reported both in clinical studies and in several animal models of pain, however its mechanisms are still poorly understood. One of the mechanisms thought to be involved in OIH is mediated by descending facilitation. The **dorsal reticular nucleus** (DRt) is a medullary area that exerts a unique role in descending facilitation and its activity is modulated by opioids. The studies included in the present thesis aimed at determining whether chronic morphine administration induces OIH in the spared nerve injury (SNI) model of chronic neuropathic pain and evaluate the involvement of the DRt in the mediation of OIH.

To determine the effects of chronic morphine administration, two to three weeks after SNI induction, the animals were implanted with osmotic mini-pumps filled with morphine ($45 \mu\text{g}\cdot\mu\text{l}^{-1}\cdot\text{h}^{-1}$) or saline, which released their content continuously for 7 days. Evoked pain was tested before and at 2, 4 and 7 days after the mini-pump implantation by the von-Frey, pin-prick and acetone tests which evaluate mechanical allodynia, mechanical hyperalgesia and cold allodynia, respectively. We show for the first time, that chronic morphine administration induces OIH in animals suffering from neuropathic pain. Spontaneous pain was also assessed using the conditioned place preference test (CPP). In animals chronically treated with morphine, the acute administration of morphine failed to induce CPP, unlike in control animals, which indicates a loss of the analgesic effect of morphine.

To study the involvement of DRt in the mediation of OIH, we performed a lentiviral-mediated knock-down of the expression of the μ -opioid receptor (MOR) at the DRt. For that, the animals were injected with lentiviral vectors and implanted with osmotic mini-pumps containing saline or morphine ($45 \mu\text{g}\cdot\mu\text{l}^{-1}\cdot\text{h}^{-1}$). The animals were tested before and at 2, 4 and 7 days after the stereotaxic injections and mini-pump implantation by the evoked pain tests mentioned above. The knock-down of MOR in control animals showed an increase of pain behaviours, confirming thus the inhibitory effects of the opioidergic modulation at the DRt. In animals chronically treated with

morphine, the knock-down of MOR prevented the development of OIH. This indicates that chronic morphine exposure induces plastic changes at the DRt that result in increased DRt facilitation. We also studied the expression of glial markers, at the DRt, by immunohistochemistry. We show that chronic morphine treatment induces an increase in the expression of microglia (Iba-1) and astrocyte (GFAP) markers.

Our results indicate that chronic morphine exposure induces OIH in a model of chronic neuropathic pain and that the DRt is involved in the mediation of OIH. The mechanisms underlying an increase of the DRt facilitation during chronic morphine exposure are likely triggered by MOR activation. Given the increase in the expression of glial markers, it would be very interesting to explore the involvement of these cells in pain transmission from the DRt, during opioid-induced hyperalgesia.

Acknowledgments

I would like to express my deepest appreciation to all of those who provided me the possibility to complete my master thesis. I am sure that the success of this project depended largely on the encouragement and guidance of many people.

First and foremost, I would like to express my special appreciation to my supervisor, Professor Isabel Martins. You have been incredible during this work. Without your cheerfulness, assistance, support, patience and encouragement this thesis would not have been possible. Thank you for allowing me to grow as a research scientist and for all your help while I was writing this thesis.

To Professor Isaura Tavares, I cannot thank you enough for allowing me to be a part of this research group and for all precious suggestions and debate during these last months. I would also like to show my gratitude to Professor Carla Morgado and Marisa Oliveira for all the interesting discussion during this thesis.

To Rita, my right arm and left leg during this thesis, you have been instrumental in the successful completion of this project. Thank you for backing me up through this work. For all members of “Pain and friends” group. The fun and nice environment in the lab is all thanks to you. Mariana Mesquita, Marta Alves, Marta Louçano and José Tiago Pereira, it has been a pleasure. Paulina, even if for not long enough, your help was immeasurable, I should also thank you for helping me to become part of this group. Maybe we will all meet again in this crazy world of scientific research. Elisa, I am deeply thankful for all your help during this thesis.

To my mother and brother, since my first year at university, we overcame a lot and we grow together as a family. Thank you for the support and for allowing me to be successful in my journey.

To Ana Moço, Ana Sofia Silva, Ana Pimentel and Tiago Santos, the family with whom I chose to share the most beautiful and amusing memories of these last years. Thank you for being there for me every time I needed you and for understanding me even when I was unable to do so.

To my “biotecos”. You almost made long days of studying seem fun. Thank you for all the help and for cheering me up and encouraging me to work harder.

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Abbreviations

AC: Adenylate cyclase

ACC: Anterior cingulate cortex

AMY: Amygdala

AP: Anterior-posterior

Ca²⁺-CaM: Calcium-calmodulin

cAMP: Cyclic adenosine monophosphate

cDNA: Complementary DNA

CNS: Central nervous system

COX: Cyclooxygenase

CPP: Conditioned place preference

CREB: cAMP response element-binding protein

Cu: Nucleus cuneate

DAB: 3,3'-diaminobenzidine tetrahydrochloride

DLPT: Dorsolateral pontine tegmentum

DOR: δ -opioid receptor

DRt: Dorsal reticular nucleus

DV: Dorso-ventral

EGFP: Enhanced green fluorescent protein

GAD: Glutamate decarboxylase

GFAP: Glial fibrillary acidic protein

G-protein: Guanine-nucleotide binding protein

H Height

HSV-1: Herpes-simplex virus-1

hSYN-1p: Human synapsin promoter

Hyp: Hypothalamus

i.p: Intraperitoneal injection

IASP: International Association for the study of Pain

Iba-1: Allograft inflammatory factor-1

Ins: Insular cortex
IR: Immunoreactive
IRES: Internal ribosome entry site
KOR: κ -opioid receptor
L: length
LM: Latero-medial
LTR: Long terminal repeated sequences
LV: Lentivirus
LV-Control: Lentiviral- Control
MOR: μ -opioid receptor
MOR-1: μ -opioid receptor 1
MOR-2: μ -opioid receptor 2
MOR-R: μ -opioid receptor in reverse orientation
Mot: Motor cortex
NMDA: N-methyl-D-aspartate
NO: Nitric oxide
NTS: Nucleus tractus solitaries
OIH: Opioid-induced hyperalgesia
ORL-1: Opioid Receptor-Like -1
PAG: Periaqueductal grey matter
PB: Phosphate buffer
PBS: Phosphate buffer saline
PBS-T: Phosphate buffer saline with Triton X-100
PCR: Polymerase chain reaction
PKC: Protein kinase C
PNS: Peripheral nervous system
RNAi: small RNA interference
RVM: Rostral ventromedial medulla
s.c: Subcutaneous injection

SNI: Spared nerve injury

Som: Somatosensory cortex

SP5C: Spinal trigeminal nucleus, pars caudalis

TU: Transducing units

VLM: Caudal ventromedial medulla

VRt: Ventral reticular nucleus

W: Width

WAH: Withdrawl-associated hyperalgesia

WPRE: Woodchuck hepatitis virus post-transcriptional regulatory element

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Introduction

1 PAIN

1.1 PAIN DEFINITION

According to the International Association for the study of Pain (IASP), pain can be defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage [1]. Experiencing pain is a warning sign of imminent or actual tissue damage, causing coordinated reflex and behavioural responses to minimize negative outcomes. When the tissue is affected, a profound but reversible hypersensitivity in the inflamed and surrounding area is established by a set of excitability changes in the peripheral nervous system (PNS) and in the central nervous system (CNS) leading to wound repair by avoiding contact with the damaged part, until healing has occurred [2, 3].

Pain is definitely not a static process. When pain is unrelieved, a set of plastic changes happens in the neural structure and some of those changes are so drastic that pain, specially chronic pain, cannot be considered just a symptom but, instead, it should be seen as a disease itself [4, 5]. Chronic pain offers no biological advantage and causes suffering and distress [2, 3]. This maladaptive pain afflicts 20% of the adult population in Europe [2, 5] and 30% in Portugal [6]. Many other diseases can be the triggering point of chronic pain, including autoimmune diseases (e.g., multiple sclerosis), metabolic diseases (e.g., diabetes), infections (e.g., shingles and the sequel, postherpetic neuralgia), vascular diseases (e.g., stroke), trauma and cancer. Although many diseases may be the culprit, it seems that, in order to initiate chronic pain, the leading lesion must directly involve the nociceptive pathways [7]. Regarding treatment, there have been some progresses, but chronic pain remains resilient to treatment. The use of non-steroidal anti-inflammatory agents (e.g., aspirin) and opioids (e.g., morphine) is the most usual treatment, but these drugs are associated with adverse dose-limiting side-effects, dependence and tolerance. The lack of effective treatment with controlled side effects reflects how poorly the molecular pathophysiology underlying these pain states is understood [8].

Chronic pain may be inflammatory, neuropathic or functional and all forms share some common characteristics [4]. When tissue injury happens, the most likely pain to rise is inflammatory pain, occurring mainly after trauma, surgery or during chronic inflammatory diseases, having damaged and inflammatory cells recruited to the injured tissue in order to release activators of peripheral nociceptors [4, 9]. Neuropathic pain is defined by IASP as pain arising as a direct consequence of a lesion or disease affecting the somatosensory system [1], in other words, it is classified as a combination of spontaneous pain and hypersensitivity with pathological changes in the PNS or in the CNS [4]. Functional pain is a relatively new concept and is defined as pain sensitivity caused by an abnormal processing or function of the CNS response to normal stimuli and may occur in fibromyalgia and irritable bowel syndrome [4].

1.2 PAIN TRANSMISSION

Primary afferent neurons innervate cutaneous tissues, bone, muscle, connective tissues, vessels and viscera and nociception occurs when these neurons are activated by noxious stimuli [4, 10]. These sensory neurons can be distinguished in several fibers based on anatomical, neurochemical and functional attributes. C-fibers are characterized as small-diameter, unmyelinated fibers and they conduct action potentials slowly; A δ -fibers have medium-diameter and are thinly myelinated, conducting action potentials quickly; heavily myelinated A β -fibers have a larger diameter [4, 10]. C- and A δ -fibers are able to encode noxious mechanical, thermal and chemical stimuli and, for this reason, are considered the main nociceptive afferents signalling pain [4, 7, 10]. Primary afferent neurons (Figure 1 – item 1) convert the stimuli into electrical activity in the peripheral terminals, causing depolarization of the neuronal membrane. If the stimuli is translated into a sufficiently strong electrical signal, voltage gated sodium channels will be activated and the signal will be transmitted into central terminals of nociceptors, in the spinal cord. Subsequently, these signals are conveyed by second order neurons, which constitute the ascending pathways (Figure 1 – item 2), to the thalamus and brainstem where the information is evaluated resulting in an appropriate response, conveyed by descending pathways (Figure 1 – item 3) to the spinal dorsal horn (Figure 1 – item 4) [4, 11].

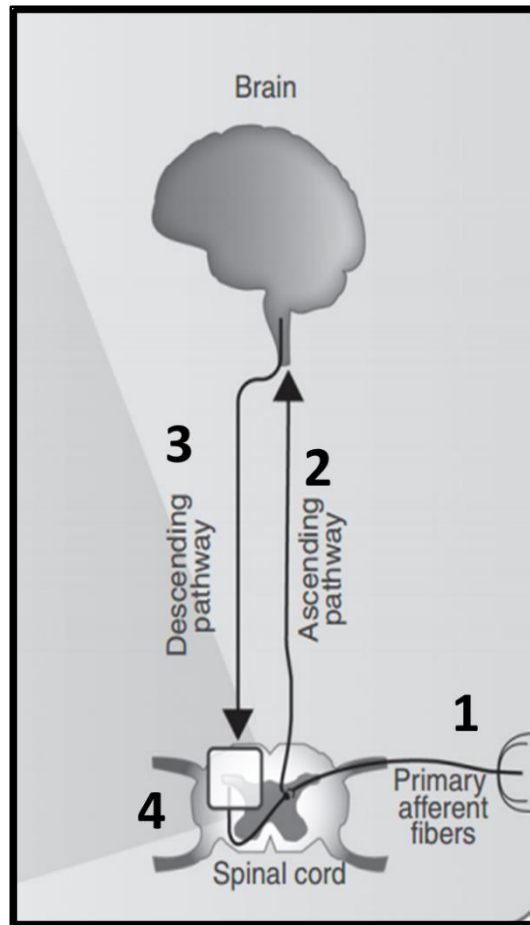


Figure 1: Transmission of nociceptive information. Nociceptive information is conveyed by primary afferent neurons (1) from the periphery to the spinal cord and then reaches the brainstem through ascending pathways (2) constituted by second order neurons. In the brain the nociceptive information is evaluated and an appropriate response is generated and conveyed by the descending pathway (3) to the dorsal horn of spinal cord (4). Adapted from Argoff, C [12].

More recently, attention has focused on spinal cord projections to the parabrachial region of the dorsolateral pons, because the output of this region provides for a rapid connection with the amygdala (AMY), a region generally considered to process information relevant to the aversive properties of pain experience. From these brainstem and thalamic loci, information reaches cortical structures. [13]. There is no single brain area essential for pain. Rather, pain results from activation of a distributed group of structures, some of which are more associated with the sensory-discriminative properties, such as the somatosensory cortex, and others with the emotional aspects, such as the anterior cingulate gyrus and insular cortex. Imaging studies demonstrated activation of prefrontal cortical areas, as well as regions not generally associated with pain processing, such as the basal ganglia and cerebellum, but the contribution of the activation of these areas to pain perception is not clear [10].

1.3 DESCENDING PAIN

1.3.1 The endogenous pain control system

The endogenous pain control system is a complex web of brain areas responsible for the regulation of pain transmission at the spinal cord. Its role in pain inhibition is long known but, more recently, it has also been associated with pain facilitation [14, 15].

The mesencephalic periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM) have been extensively described as important role players in pain modulation (Figure 2) [16]. The PAG is directly connected with the hypothalamus and limbic forebrain structures, including the amygdala, and also receives direct spinomesencephalic input. This area is also connected with several brainstem areas, including the RVM, and largely exerts modulatory nociceptive transmission to the spinal cord, using the RVM as a relay (Figure 2 and 4) [11, 15].

The RVM can be considered the output of the midline pain-modulation system. This area is constituted by the nucleus raphe magnus and adjacent reticular formation and projects to the superficial layers of dorsal horn laminae and to deep dorsal horn (Figure 2 and 4) [15]. In the RVM, there are distinct populations of neurons classified as ON- and OFF-cells, which exert facilitatory and inhibitory effects of nociception, respectively. OFF- and ON-cells project to the dorsal horn and μ -agonists affect these two types of cells by direct inhibition of ON-cells and by disinhibition of OFF-cells [11, 17-19].

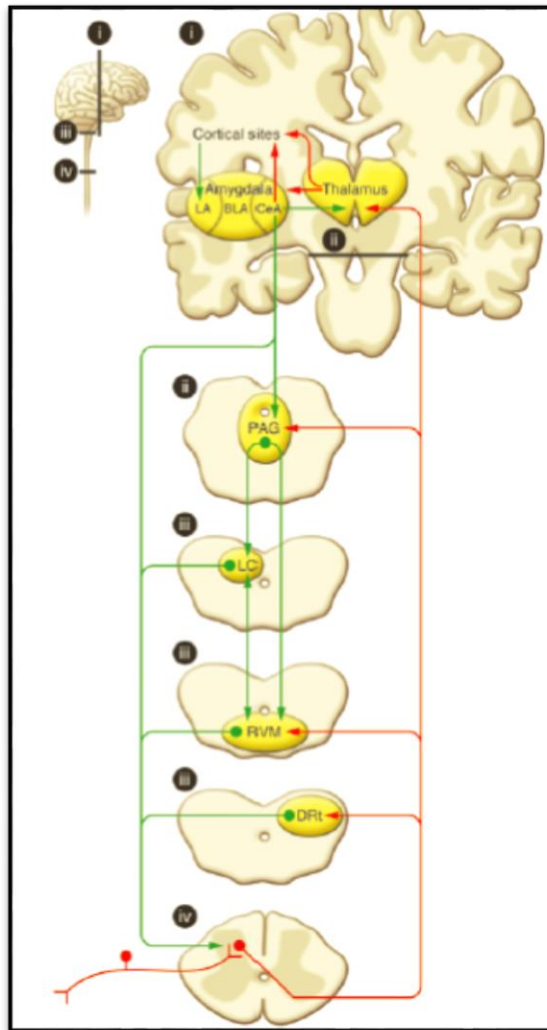


Figure 2: Schematic representation of the pain modularity circuitry. Primary afferent neurons convey nociceptive inputs to the spinal dorsal horn. From the dorsal horn there are ascending projections (labelled in red) targeting the thalamus, the DRt, the RVM and the PAG. The thalamus is connected to some cortical sites and to the amygdala. Descending pain modulation is mediated through projections (labelled in green) from these cortical areas to the PAG, which communicates with the RVM and the LC, and send descending projections to the spinal dorsal horn. Areas labelled “i–iv” in the small diagram correspond to labelled details of the larger diagram. Abbreviations – DRt – dorsal reticular nucleus; LC- locus coeruleus. Adapted from Ossipov et al [14].

1.3.2 The dorsal reticular nucleus

The dorsal reticular nucleus (DRt) deserves special attention since this area will be the focus of the present thesis.

The DRt is located in the most caudal portion of the medullary dorsolateral reticular formation, more specifically, in the dorsolateral quadrant of the medulla oblongata [20]. It is located medially to the spinal trigeminal nucleus, pars caudalis (Sp5C), laterally to

the nucleus tractus solitaries (NTS), ventral to the nucleus cuneate (Cu) and dorsal to the ventral reticular nucleus (VRt) [21] (Figure 3).

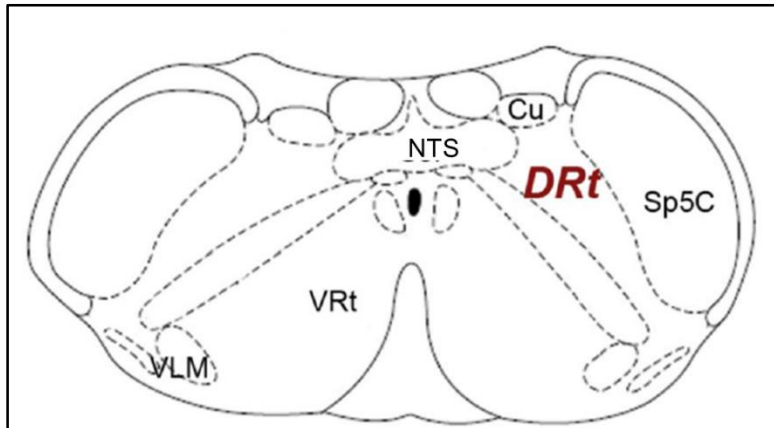


Figure 3: Diagram of a coronal section of the caudal medulla oblongata. Abbreviations: DRt-Dorsal reticular nucleus; Cu-Nucleus cuneate; NTS-Nucleus tractus solitaries; Sp5C-Spinal trigeminal nucleus, pars caudalis; VLM-Caudal ventromedial medulla; VRt-Ventral reticular nucleus. Adapted from Paxinos and Watson [22].

The DRt receives bilateral projections from the spinal cord laminae I, IV–VII and X, with a clear ipsilateral predominance of those originated in the dorsal horn and the connections between lamina I and the DRt are characterized by excitatory synaptic contacts at both sites, which indicates that this reciprocal connection exerts excitatory actions at both spinal and DRt levels functioning thus as a reverberating system that leads to signal amplification [23]. This area has connections with brainstem areas such the ventrolateral medulla (VLM), PAG, RVM, locus coeruleus and the A5 and A7 noradrenergic cell groups [16, 23, 24]. The DRt also projects to the medial thalamus and the limbic system, which suggests an integration of the DRt activity with the emotional aspects of pain processing [16, 24]. Additionally, the DRt is connected with the extrapyramidal and orofacial motor system, which suggests an involvement of the DRt in motor reactions associated with pain [24].

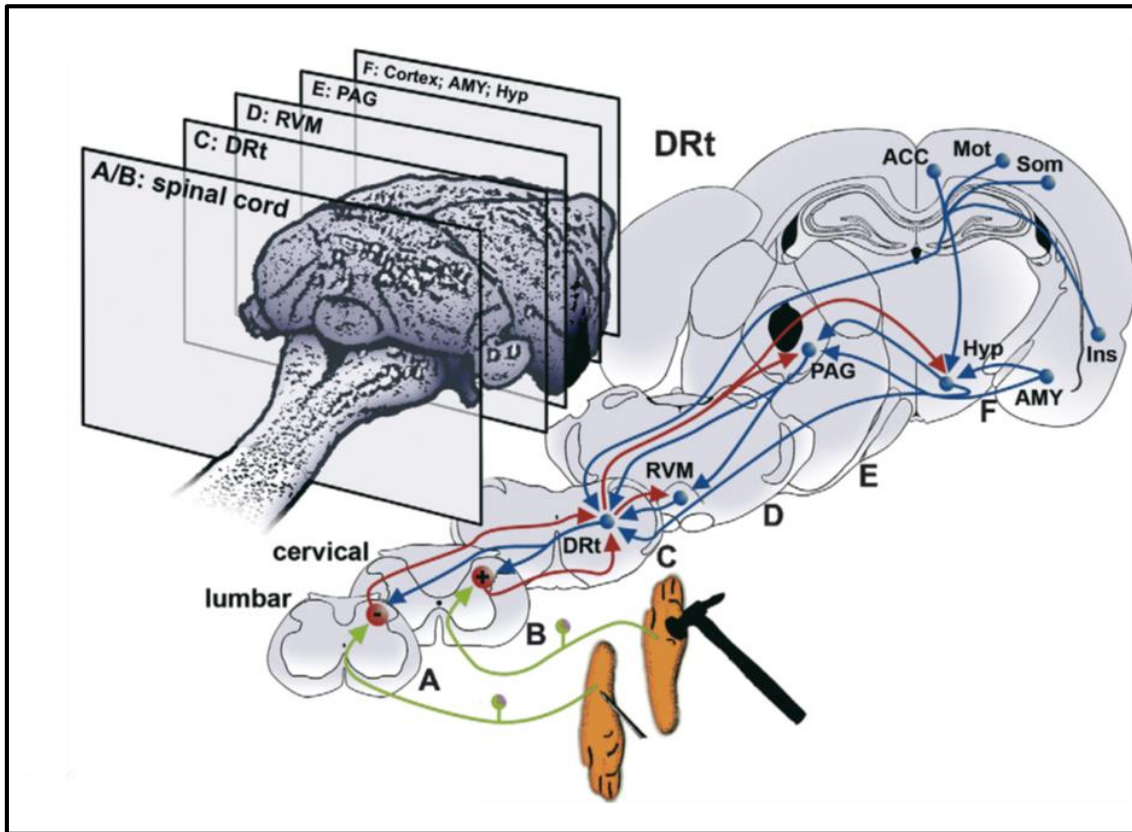


Figure 4: DRt involvement in pain modulating circuitries. Ascending connections are represented in red, descending projections are in blue and nociceptors are depicted in green. A–D are central nervous system sections and represent the spinal dorsal horn (A), the medulla oblongata and pons (B), the mesencephalon (C) and the forebrain (diencephalon and telencephalon, D). Abbreviations: ACC, anterior cingulate cortex; Ins, insular cortex; Mot, Motor cortex; Som, somatosensory cortex; Hyp, hypothalamus. Adapted from Almeida et al. [16].

DRt neurons are exclusively activated by cutaneous or visceral noxious stimulation conveyed by A δ - and C-fibers from the entire body [16, 25]. Glutamate administration in the DRt induces a long-lasting increase in the responsiveness of spinal nociceptive neurons [26], while lidocaine administration in the DRt results in the suppression of responsiveness [23]. At the behavioural level, the DRt was shown to be involved in pain facilitation both in acute and chronic pain models [27-29]. Recently, it was found that the facilitatory effects of the DRt, during chronic neuropathic pain, were mediated by noradrenaline release at the DRt [30].

2 OPIOIDS AND PAIN

2.1 OPIOIDS IN PAIN TREATMENT

Clay tablets from around Early Bronze Age prove that, at least since the Ancient Sumerian civilization, opioids have been used to treat pain and to “ease the harshness

of life” [31]. Nowadays, opioids are the most common treatment for acute and cancer-related pain. More recently, they arose as a therapy for comforting patients with chronic nonmalignant pain [17, 31, 32], as consequence, opioids are now the most often prescribed drugs for chronic pain [8, 32].

2.2 OPIOID RECEPTORS

Opioids activate peripheral, spinal and supraspinal opioid receptors. To date, three different groups of opioid receptors have been identified (μ - MOR; δ - DOR and κ - KOR) which are further divided into different sub-families [11, 18, 33]. MOR-1 mediate opioids' analgesic and euphoric effects as well as physical dependence and MOR-2 mediate bradycardia and respiratory depressant effects. DOR, with two subtypes identified so far, mediate spinal analgesic effects and it is involved in tolerance. KOR mediate spinal analgesia, miosis, sedation and diuresis [34]. A fourth related receptor, the orphanin-receptor like 1 (ORL-1) was described. The opioid receptors mediate their effects via activation of guanine-nucleotide binding proteins (G-proteins) [11, 18, 33]. The MOR presents the widest distribution in the brain and spinal cord, while the DOR and the KOR have a more restricted distribution. In peripheral tissues, opioid receptors are responsible for the modulation of several physiological functions [33].

The most powerful analgesics act on MOR and so those are the most deeply studied receptors [11]. These receptors are expressed both on pre- and post-synaptic neurons in the CNS [11, 33] and exert a major inhibitory influence in pain transmission at the spinal level, exerting their actions via MOR expression in pre-synaptic primary sensory neurons and in post-synaptic secondary neurons [4, 11, 33]. Furthermore, MOR is expressed in several supraspinal areas related to pain modulation, such as the insular cortex, amygdala, hypothalamus, PAG, RVM [11, 33] and the DRt [35] and are abundantly expressed in the limbic system inhibiting the emotional perception of pain [33].

2.3 EFFECTS OF OPIOIDS IN PAIN MODULATION

There are several neurochemical systems involved in the pain control and opioids display an important role in this matter since they are included in the most relevant and ancient therapeutic approaches for pain [33]. Endogenous opioids are proteins naturally existing in the organism [34]. β -endorphin, met-and leu-enkephalin, dynorphins and β -

endorphins, respectively result from three known precursors: proenkephalin, prodynorphin and pro-opiomelanocortin [33, 36]. These endogenous opioid peptides have different affinities for opioid receptors. For instance, the enkephalins activate mainly the DOR having some effects on the MOR, the dynorphins activate mainly the KOR and β -endorphins act on the MOR and the DOR [33, 36]. Two additional peptides endomorphin-1 and -2, have no precursor for endogenous synthesis identified so far, but it is known that they bind with high affinity to MOR [33, 34]. In addition, an endogenous opioid-like substance, nociceptin, is the product of a novel gene distinct from the gene families from which the endogenous opioids are derived [34].

At the periphery, endogenous opioids are synthesized by the neuroimmune system and bind to opioid receptors in the peripheral nerve terminals, which causes a decrease in nerve excitability and the release of inflammatory mediators. In the CNS, opioids regulate nociceptive pathways both at spinal and supraspinal levels. At the spinal level, opioids inhibit nociceptive transmission conveyed by A δ - and C-fibers [33]. The opioid peptides β -endorphin, dynorphins and enkephalins are widely distributed in the brain, whereas in the spinal cord dynorphins are mainly present in interneurons. Spinal enkephalins are found primarily in long descending pathways from midbrain to the dorsal horn [36].

The opioids are also involved in pronociceptive effects in the PNS and in the CNS. An increase in spinal dynorphins has been related with the development of hyperalgesia and allodynia since it increases the release of excitatory neurotransmitters, which contribute to amplify pain transmission [17, 33]. At the supraspinal level, opioids inhibit ON-cells and activate OFF-cells [11]. Serotonergic neurons from the RVM are activated by enkephalinergic neurons from the PAG and project to the spinal cord, inducing the release of enkephalins which inhibit the activity of A δ - and C-fibers entering the spinal cord. Noradrenergic neurons from the locus coeruleus projecting to the spinal dorsal horn are also regulated by opioids [11]. The MOR exert direct and indirect influence in descending facilitation from the DRt, based on the expression of MOR on local and spinally-projecting neurons [37]. In a model of inflammatory pain, a decrease in the expression of DOR and MOR was reported at the DRt [35, 38]. Based on the location of these receptors on the DRt-spinal pathway, their decrease is likely to amplify the DRt-spinal facilitatory effects [23].

3 OPIOID-INDUCED HYPERALGESIA

3.1 DEFINITION

Opioid-induced hyperalgesia (OIH) is an opioid use related problem that arose in the past years [31]. In some scenarios, treating patients and animals with opioids elicits, paradoxically, increased perception of pre-existing pain and, in some cases, it is associated with a modification of pain characteristics and an extension of the painful area [17, 39]. Stated otherwise, opioids may directly facilitate pro-nociceptive pathways such that patients are overly affected by noxious stimuli compared with opioid naïve patients [17, 31, 32].

OIH definition is often confused with opioid tolerance and withdrawal-associated hyperalgesia (WAH). These syndromes can manifest similar symptoms, but are clinically differentiated from OIH due to differing effective interventions [40].

Tolerance occurs when the patient seeks pain relief and increasing doses of opioid are necessary to maintain appropriate analgesia (Figure 5 B) [31, 40] . This definition could be confused with OIH, however, in opposition to tolerance, increasing doses of opioids will only worsen pain (Figure 5 A) [39].

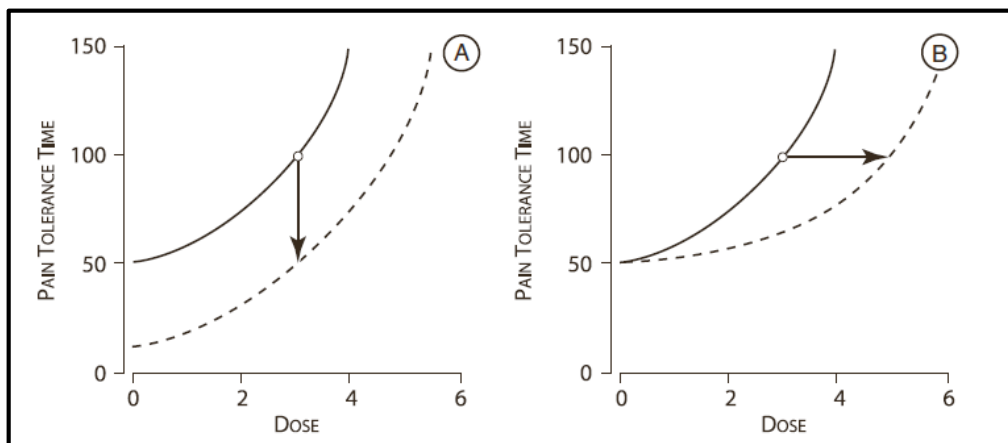


Figure 5: Alterations in opioid dose-response relationship with chronic opioid administration. It is a hypothetical experience, where an acute opioid infusion is used to detect changes in the analgesic dose-experimental pain response curve that occur as a result of chronic opioid exposure. The responses of opioid naïve patients are shown as a solid line. A, In OIH, the dose-response curve of the chronic opioid user (dashed line) is shifted downward. B, In analgesic tolerance, the slope of the dose-response curve of the chronic opioid user (dashed line) becomes attenuated and rightward shifted, but, there is no significant change in pain sensitivity at baseline Adapted from Chu et al. [31].

WAH is a time limited reaction, translated as a diffuse joint pain and body aches taking place along with detoxification from chronic opioid use or if scheduled doses are skipped [40].

3.2 CLINICAL AND ANIMAL EVIDENCE

Several studies suggest that OIH can develop in humans and may have important clinical implications [31, 32]. Studies have been conducted using several distinct methodologies.

In the mid-1960s, methadone was firstly applied as a substitution treatment for former opioid addicts and, in the past years, it became a basic therapy for these cases, preventing abstinence symptoms [41]. There are several studies where observations in this set of patients are compatible with the hypothesis that OIH, when diagnosed, is caused by chronic opioid exposure [32, 42].

Regarding diagnosis of OIH in the scenery of acute intraoperative opioid exposure, several clinical studies reported increased postoperative pain in patients that received higher doses of intraoperative opioids, in the context of major abdominal surgery [32, 43-45].

There are also studies describing OIH in patients with acute physical opioid dependence and, in this case, OIH was paired with aggravation of the induced hyperalgesic skin lesions, enlargement of the area of mechanical hyperalgesia induced by transdermal electrical stimulation, aggravation of pressure-evoked pain and/or increased sensitivity to cold pressor pain in healthy human volunteers following precipitated opioid withdrawal [31, 32].

A few prospective clinical studies also show evidence for the development of OIH. A small prospective study showed OIH in 6 patients with chronic back pain, after one month of oral morphine treatment, when compared to baseline values [46, 47]. Another study, involving a bigger sample population, showed a significant negative correlation between experimental OIH and all clinical pain measures, in a dose dependent manner [47, 48]. Additional indirect evidence for OIH comes from another prospective study of patients with chronic pain receiving intermediate-term opioid treatment who attended a pain rehabilitation program, which included the cessation of opioid use. Heat pain thresholds were increased, at the end of the program, compared to their levels prior to enrolment [47].

Kayan et al. [49] first described the phenomenon of hyperalgesia after acute morphine injection in rats in 1971 and there are now more than 90 publications available

describing OIH in a wide range of animal models [31]. For more than three decades, it has been recognized that rodents show hyperalgesic responses after the administration of an opioid antagonist which causes withdrawal or during spontaneous withdrawal after cessation of opioid administration [32]. Chronically administered opioids are now also shown to cause a sustained pronociceptive response, related both to the opioid dose administered and to the experimental pain model used (i.e., thermal, mechanical, electrical or chemical) [40].

Two fundamental patterns characterizing the onset and resolution of OIH in animals can be distinguished. The first is observed after acute administration, that is, the systemic administration of one to four relatively high opioid doses within one hour, evoking brief hyperalgesia which lasts for hours or, exceptionally, for days in a dose dependent manner [32]. The second and most usual pattern is observed after animals are exposed to opioids for three to twelve days. In this case, if opioids are continuously administered an antinociceptive response is usually reported in the first day and then a loss of this effect is observed or along with the induction of a hyperalgesic state during ongoing drug administration. Alternatively, if animals are given repeated systemic or intrathecal boluses of opioids for several days, they gradually develop hyperalgesia to thermal or mechanical stimuli. When studied, it was also possible to directly correlate the time course of resolution of OIH with the time course of its development [32].

3.3 MOLECULAR MECHANISMS

OIH is thought to result from neuroplastic changes in the PNS and in the CNS resulting in the sensitization of pronociceptive pathways and it can be described based on the site of the plasticity [19], where the relevant mechanisms are probably unique [31].

Spinal cord plasticity underlying OIH has been demonstrated after both intrathecal and systemic administration of opioids [19, 31, 32] and the most obvious consequence of spinal sensitization is the higher transmission of noxious inputs to supraspinal sites [17]. The major role players involved in spinal sensitization described until now include N-methyl-D-aspartate (NMDA) receptors, non-NMDA excitatory amino acid receptors, [50], protein kinase C (PKC) [17, 19, 31, 32], spinal dynorphin, spinal prostaglandins [32] and spinal cyclooxygenase (COX) [19]. The spinal dorsal horn is vital to many

mechanisms supporting OIH [19], as the correlation between OIH and spinal cord plasticity is consonant with the emerging appreciation of spinal inflammation as participating in many abnormal pain syndromes [17, 19, 31].

Regarding the molecular mechanisms underlying OIH, there are evidences suggesting that after binding morphine to MOR, on a post-signalling neuron, there are the activation of G-protein mediated PKC translocation and the removal of the NMDA receptor Mg^{2+} plug (Figure 6 – item 1). Glutamate is released from pre-synaptic cells inducing the ionotropic NMDA receptor to allow Ca^{2+} influx, resulting in augmented intracellular Ca^{2+} which leads to several downstream effects, including activation of calcium-calmodulin (Ca^{2+} -CaM), changes in gene expression and further activation of PKC (Figure 6 – items a-b-c). Ca^{2+} -CaM in turn initiates the conversion of L-arginine into nitric oxide (NO) by NO synthesis. NO may then act as a retrograde messenger to enhance glutamate release from the pre-synaptic neuron. With continual activation of these pathways, by opioid receptor occupation, PKC may uncouple the G-protein from MOR preventing any downstream signalling upon ligand binding [50].

There are also evidence suggesting that this process is not limited to neuronal cells and that glial cells also play an important part in OIH. Indeed, inhibition of glial cells may be enough to re-establish opioids therapeutic effects. Chronic opioid administration may act through the MOR on glial cells increasing the production and release of cytokines and chemokines or act directly on glial and neuronal glutamate transporters to alter synaptic glutamate levels (Figure 6 - items 2 and 3). Once released, cytokines may then act on the pre- or post-synaptic neurons to induce hyperalgesia or on other glial cells to promote further neuroimmune activation [50].

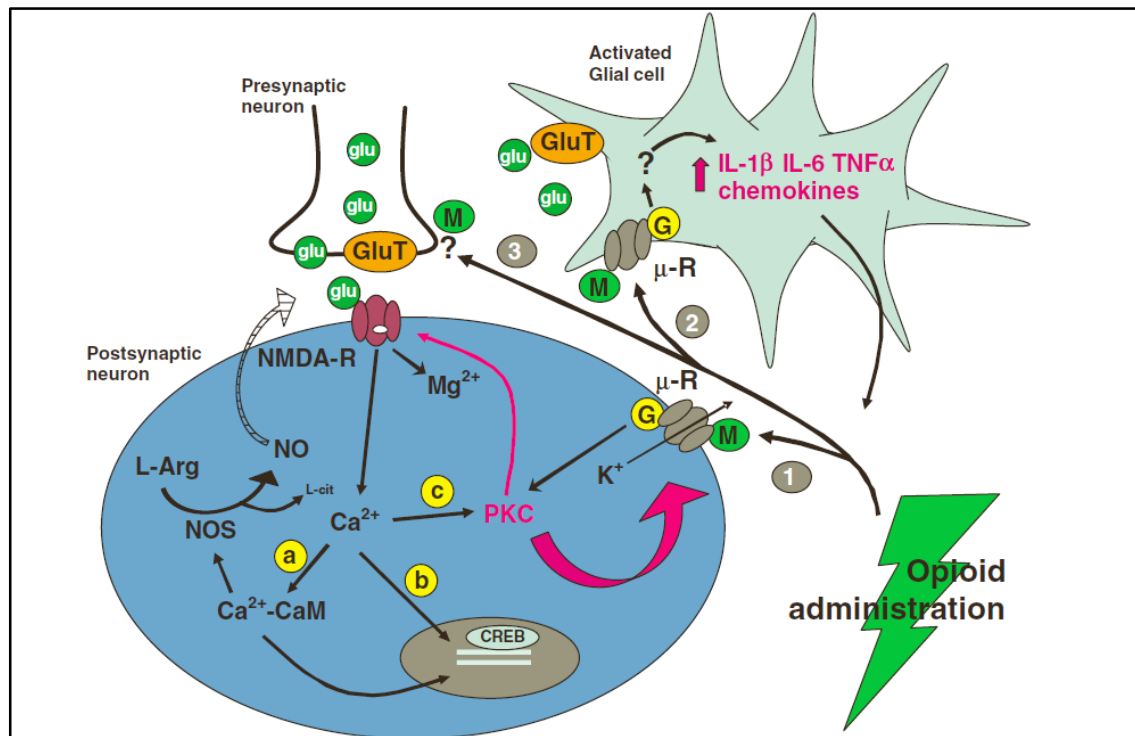


Figure 6: Cellular mechanisms of morphine hyperalgesia. Morphine (represented by M, as a representative opioid) may act on the post-synaptic neuron (1), on the glial cells (2) or on the pre-synaptic neuron (3). Regarding the post-synaptic neuron (1), it binds to the μ -receptor (μ -R) activates G-protein mediated protein kinase C (PKC) translocation and activation promoting removal of the Mg^{2+} plug from the NMDA receptor (NMDA-R). Glutamate (glu) released from the pre-synaptic cells induces NMDA-R to allow Ca^{2+} influx, increasing intracellular Ca^{2+} which will result in the activation of Ca^{2+} -CaM (calcium-calmodulin) (a), changes in gene expression (b) and further activation of PKC (c). Ca^{2+} -CaM in turn initiates the conversion of L-arginine to NO mediated by nitric oxide synthase (NOS), which will enhance glutamate release from the pre-synaptic neuron. Chronic opioid administration also affects the μ -R on glial cells (2), causing an augmented production and secretion of cytokines and chemokines and the neuronal glutamate transporters (GluT) to alter synaptic glutamate levels (3). Abbreviations in figure not presented in text: G = G-protein coupled to receptor; NO = nitric oxide; NMDA = N-methyl- D-aspartate Adapted from Deleo et al. [50].

The influence of higher CNS centres in OIH is yet poorly studied, however, there has been an increase in proven influence of supraspinal sites through enhanced descending facilitation to the spinal cord dorsal horn. So far, only the involvement of RVM was more deeply studied for OIH [17, 19, 31].

The underlying mechanisms of OIH are quite similar to the ones underlying neuropathic pain, which can become an obstacle for treating this syndrome, since, if opioids are able to activate the same pathways as neuropathic pain, administration of opioids during or after nerve injury may facilitate, instead of preempt, the development of neuropathic pain [39].

3.4 GLIAL CELLS

Since the end of last century, there is an emergence of the importance of the neuroimmune interface, meaning, the bidirectional signalling between neurons and

immune-competent cells from the CNS, including glia, endothelial cells, perivascular macrophages and infiltrating T cells [3, 51, 52].

Not that long ago, information about glial functions was limited to their role providing structural support for neurons and immune surveillance, aiding the maintenance of CNS homeostasis [51, 53, 54]. In recent years, experimental and clinical studies have confirmed the neuroimmune interface as fundamental for the development of pain [51, 52].

The lack of axons and their yet-to-be-discovered part in cell-to-cell communication delayed the attention glia deserved in pain facilitation until the early 1990s. From such decade, an intriguing feature of glial cells has emerged since it is now known they can act in areas of CNS quite remote from the focus of injury or disease [51]. This research was accompanied by a growing appreciation that the behavioural effects observed in pain could also be due to the local release of cytokines by glia residing within the CNS [3, 52]. With this discovery, it was possible to postulate the existence of a crosstalk between glia and neurons of the CNS where CNS neurons can activate glial cells and, when recruited, these glial cells can, in turn, regulate neuronal function [51].

3.4.1 Glial cell types

Glial cells account for 70% of CNS total cells and can be divided into two main groups: microglia and macroglia. Macroglia can be further divided into astrocytes, oligodendrocytes and radial cells like Bergmann cells, located in cerebellar cortex, and Müller cells, located in retina. Besides neurons, these cells are the second main element of neural tissue and although they cannot transmit nerve impulses, they are central in the synthesis, release and uptake of neurotransmitters [52, 54].

Microglial cells represent the immune system at the spinal cord level and represent as little as 5–12% of all cells in the CNS [54]. This is however altered during ongoing pathological states, when microglia undergoes strong proliferation and dynamically modulates neuronal functions [52]. These cells have distinct morphological and functional properties that are developed under the influence of nearby astrocytes, which produce colony stimulating factors, and neurons, which can modulate microglial function via the release of neurotrophins [51]. Under a basal surveillance state, the cytoarchitecture of microglia allows them to continuously sample the extracellular space

for perturbations [3, 54]. Nevertheless, microglia plays an important role in several diseases, for instance, in the case of neuropathic pain, microglial cells are the first to become activated following peripheral nerve injury and remain active for several weeks [52]. In normal conditions, microglia exhibit ramified processes that are highly motile and express receptors for complement components and low levels of cell-surface immune molecules. This status changes dramatically following injury to the CNS or to peripheral nerves or peripheral tissues [51, 54]. It is already known that microglia presents pleomorphic responses to distresses elicited in the nervous system, with a premise that activated microglia emerges from a resting state (Figure 7 – item a) and undergoes phenotypic alterations in a variety of morphological forms (Figure 7), including, for example, the amoeboid (the most activated morphology - Figure 7 – items e and f) [55].

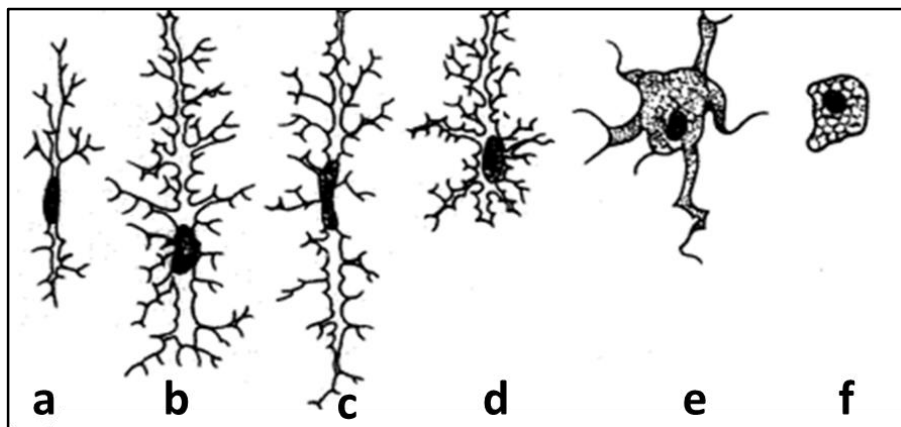


Figure 7: Progressive stages of microglial activation, from a (the inactive state) to f (the most active state). Adapted from Kreutzberg, G.W. [56].

Morphologic alterations of microglia are accompanied by modifications in gene expression, including the upregulation of receptors and the production of a repertoire of cytokine and chemokine mediators [51, 54]. Microgliosis is often correlated with increased expression of CD11b and allograft inflammatory factor 1 (AIF-1; also known as Iba-1) which are regularly used as markers of microglial cells [3].

Astrocytes are the most abundant cells in the CNS [3, 52, 54]. In addition to providing structural support, promoting formation of the blood–brain barrier and regulating cerebral blood flow, astrocytes contribute to synaptic transmission, provide trophic support and promote repair of neuronal systems [3]. Through the expression of numerous transport proteins, astrocytes are capable of maintaining homeostasis by

means of regulation of extracellular levels of ions, proteins and neurotransmitters in their surrounding environment [3, 52, 54]. Astrocytes are intimately associated with neuronal synapses because a single astrocyte can make contact with several neurons [54]. As with microglia, damage to peripheral nerves and peripheral tissues alters the resting state of astrocytes, most prominently near the central terminals of damaged sensory neurons and around motor neurons [51]. This altered state is referred to as activation and it is marked by an increased expression of the glial fibrillary acidic protein (GFAP) [3, 51, 52, 54].

3.4.2 Involvement of glial cells in OIH

Although the adverse effects from opioids use can be partly explained by neuronal mechanisms, the emerging role of central immune signalling is revolutionizing opioid pharmacology [3]. Glia now have a well-established role in initiating and maintaining increased nociception in response to peripheral nerve injury. Several research groups have documented that glia can powerfully modulate the analgesic actions of chronically administered opioids [53]. There are now several studies suggesting that CNS glia, in concert with proinflammatory cytokines and chemokines [19, 31], contribute to hypersensitivity and the decreased efficacy of opioids in chronic neuropathic pain states, in the spinal cord [50, 52, 57, 58]. The understanding of glial involvement in neuropathic pain and opioid pharmacodynamics has grown together, such that they are now indelibly intertwined. These pain-relevant topics are of interest primarily because clinical pain syndromes occur in epidemic proportions worldwide, including chronic pain states that last for years to a lifetime of unremitting pain [54].

The notion that glia can modulate opioid actions was first postulated in the late 1980s. Several studies showed that chronic morphine administration causes glial activation in spinal cord [53, 54], and co-administration of morphine with a glial activation inhibitor resulted in the maintenance of analgesic efficacy and in a corresponding reduction of glial activation. Some studies conducted in neuropathic pain models, led to the conclusion that at least some products of activated glia that enhance neuropathic pain may also oppose morphine analgesia [53].

It appears that morphine acts not only at classical opioid receptors on nociceptive neurons, but also that glial activation signal produces a cascade of events that results in

increased nociception. The sum of morphine's neuronal antinociceptive activity and its pronociceptive glial activation results in a net reduction in analgesia. Moreover, since glial activation increases with chronicity of opioid treatment, it seems that opioid-induced glial activation contributes significantly to the atypical allodynia and hyperalgesia that results from chronic opioid administration [53].

4 GENETIC MANIPULATION OF THE NOCICEPTIVE SYSTEM

In recent years, the development of selective genetic manipulation has largely enriched the understanding of molecular mechanisms of the descending pain modulatory system [21, 59]. Pre-clinical trials of gene therapy for pain control reporting promising results, related to safety and efficacy, along with an early clinical trial with exciting outcomes show the potential of the genetic manipulation of nociceptive system [30, 60-62].

Due to the deeper knowledge achieved regarding mechanisms of pain, it is now possible to engineer vectors carrying transgenes with specific promoters directed to targets of the CNS and of the PNS deeply involved in facilitation of pain and somatosensory system areas [59]. Thereby, gene therapy allows the delivery or manipulation of genes, increasing the specificity, avoiding, thus, side effects and off-target toxicity, mediating gene expression for a controlled and extended period of time [63]. The greater advantage of gene therapy is that this system is readily controllable. There are three main components that can be manipulated: the vector, the transgene and the promoter [59].

The vector is the carrier of the transcriptional cassette and its main function is to deliver its content to specific cell targets. Vectors can be derived from viral or nonviral systems, however, viral vectors have greater efficiency and specificity for the delivery of exogenous genes to cells, therefore they are the most used and studied delivery vectors [59, 60, 64]. Some of the viral vectors have the ability to be transported retrogradely, which allows the vector to be uptaken at the nerve terminal and then migrate to the nucleus, often located in remote areas, surgically difficult to access [59]. Briefly, the ideal viral vector, should be able to express external genes for a long period and should be non-pathogenic and nontoxic. In this sense, viral vectors are created by deleting and

replacing nonessential and pathological genes from the virus, by exogenous coding sequences, and by maintaining structural motifs needed to infect cells and transfer their content. The most commonly used viral vectors are derived from the herpes-simplex virus (HSV-1), adeno-associated virus, adenovirus and lentivirus [60, 64]. Lentiviral vectors have the advantage of allowing long term transgene expression. They belong to a subclass of retroviruses capable of inserting DNA into the cell genome. They are therefore interesting vectors for non-dividing cells as neurons and they have also been used for gene delivery in neural stem cells and progenitor cells [64].

The transgene is a coding sequence of a gene which can be fused with tags (small unrelated sequences) or even expressed under the same promoter with fluorescent proteins, so cells transfected with the transgene can be easily detected [63]. These coding sequences generally express neurotransmitters and receptors involved in pain transmission, neurotrophic factors and anti-inflammatory substances [59]. However, it is also possible to insert antisense sequences or RNAi molecules in order to promote down-regulation of the expression of a gene [60, 63]. As for the promoter, cell-type specific promoters are preferred in order to restrict gene expression to a specific cell type or even to a neurochemical neuronal population. Synapsin I, calcium/calmodulin-dependent protein kinase II, tubulin alpha I and neuron-specific enolase are some of the promoters specifically targeting only neurons [59].

Targeting brain circuits of pain is definitely challenging mainly because the access to brainstem areas is a huge hurdle and the complex neuronal circuits are also greatly difficult to manipulate. Gene transfer in the endogenous pain control system has been mainly achieved with HSV-1 vectors to express opioid peptides [35, 62], glutamate decarboxylase (GAD) [65] and tyrosine hydroxylase [30], inducing analgesia in several pain models [35, 59, 61, 62].

Aims and methodology

The analgesic role of opioids for treating chronic pain are of extreme importance, since chronic pain afflicts a large amount of people worldwide. Nonetheless, chronic opioid administration may lead to several side effects, including a paradoxical hyperalgesic effect, also known as opioid-induced hyperalgesia (OIH). Several lines of evidence suggest that descending facilitatory pathways are involved in the modulation of OIH. The dorsal reticular nucleus (DRt) exerts a unique role in descending pain facilitation and its activity is modulated by opioids.

One of the goals of the present thesis was to determine the behavioural effects of chronic morphine administration in a chronic pain model, the spared nerve injury (SNI) model. The SNI model presents substantial and prolonged changes in mechanical sensitivity and thermal responsiveness that closely mimic many features of clinical neuropathic pain [66]. We first assessed the effects of chronic administration of morphine in evoked pain by three behavioural tests: the von-Frey test to assess mechanical allodynia [67], the pin-prick test to verify changes in mechanical hyperalgesia [68] and the acetone test to study cold allodynia [69]. Then, we used the conditioned place preference (CPP) test to evaluate the effect of chronic morphine in spontaneous pain. Additionally, we evaluated the effect of chronic morphine in the basal expression of the proto-oncogene c-Fos, a marker of neuronal activation, in the spinal dorsal horn [70].

The second aim of this thesis consisted on studying the involvement of the DRt during OIH, induced in the SNI model. For that, first we evaluated the effects of μ -opioid receptors (MOR) knock-down at the DRt. Then we determined whether glial cells were activated in the DRt.

To knock-down the expression of MOR at the DRt we used a lentiviral vector. This vector was chosen since it does not undergo retrograde expression and so it was possible to specifically target DRt neurons and control MOR expression in these neurons [71]. We determined the effects of MOR knock-down during chronic morphine administration in evoked pain by the behavioural tests described above.

The effects of chronic morphine treatment on glial activation were determined by the evaluation of the expression of the microglia marker Iba-1 and the astrocyte marker GFAP. Additionally, we performed a morphological analysis of microglia to ascertain its activation.

Materials and Methods

1 ANIMALS

Pathogen-free adult male Wistar rats (Charles River colony, France) were pair-housed in standard Plexiglas cages with *ad libitum* food and water. After stereotaxic injections, the animals were housed individually. The colony room was maintained at $22 \pm 2^\circ\text{C}$ on a standard 12/12 h light/dark cycle. All experiments were conducted during the light phase. Upon arrival, rats were allowed one week of acclimation before any procedure. All procedures were performed in accordance with the European Community Council Directive (86/609/EEC) and the ethical guidelines for pain investigation [72].

2 LENTIVIRAL VECTORS

The lentiviral vectors used in this study were kindly provided by Professor Steven Wilson from the University of South Carolina (Dpt. of Pharmacology, Physiology and Neurosciences). We used a lentiviral vector that knocks down MOR expression (MOR-R, Figure 8) carrying the human synapsin promoter (hSYN-1p), MOR cDNA in reverse orientation and enhanced green fluorescent protein (EGFP). The control vector (LV-Control; Figure 8) only carries the EGFP transgene. The vectors further carry the encephalomyocarditis virus internal ribosome entry site (IRES) and the transcriptional regulatory element (WPRE).

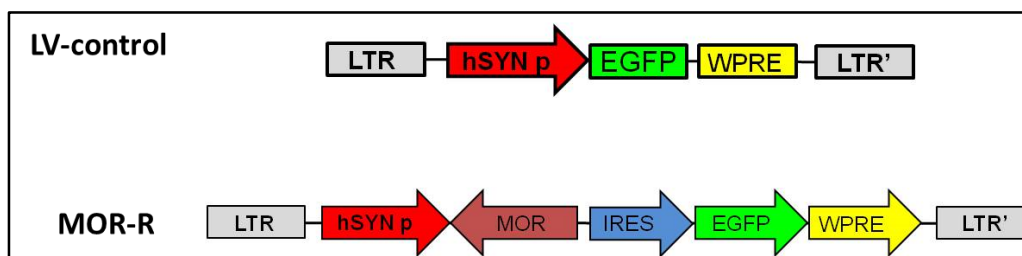


Figure 8: Schematic diagrams of the vectors. Abbreviations: LTR- long terminal repeat.

The lentiviral particles were produced by co-transfection of human embryonic kidney 293T cells with the lentiviral vectors, a packaging plasmid (pCMV Δ R8.92), a plasmid encoding the rev protein (pRSV-Rev) and a plasmid encoding the vesicular

stomatitis virus G glycoprotein (pMD.G). The vectors were titrated by quantitative real-time PCR and stored in 10% sucrose in PBS (Appendix A).

3 SURGICAL PROCEDURES

3.1 NEUROPATHIC PAIN INDUCTION

The neuropathic pain model of Spared Nerve Injury (SNI) was induced as described by Decosterd and Woolf [66]. First, rats were administered intraperitoneally (i.p.) with a mixture of ketamine hydrochloride (Imalgene 1000[®] - 0.06 g/Kg) and medetomidine (Medetor[®] - 0.25 g/Kg). Then, animals' left thigh was shaved and disinfected with iodopovidine (Betadine[®]), the skin was incised vertically approximately 3,5 cm and, using a blunt-pointed scissors, a section was made directly through the biceps femoris muscle exposing the sciatic nerve and its three terminal branches: the common peroneal (1), tibial (2) and sural (3) nerves (Figure 9).

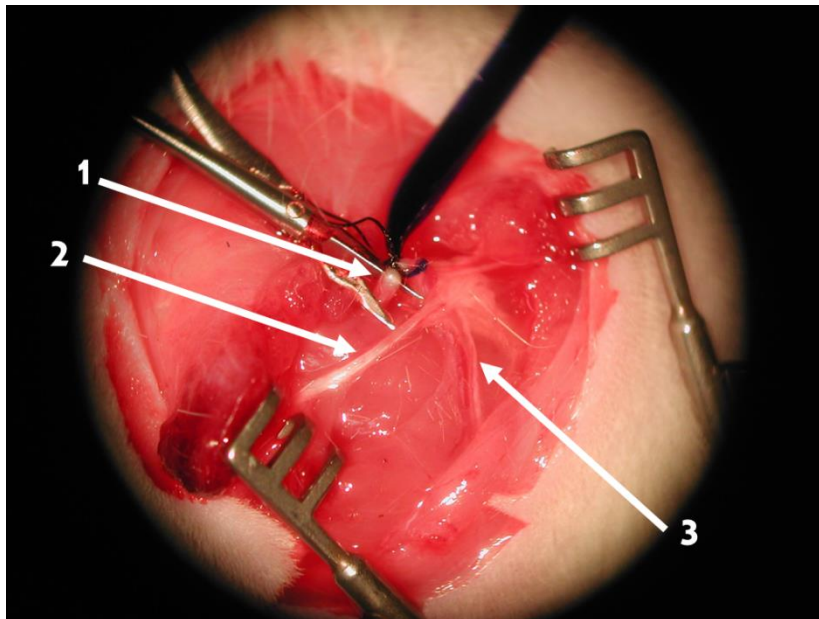


Figure 9: Exemplificative picture of the terminal branches of sciatic nerve. (1) Common peroneal, (2) tibial (3) sural nerves.

The SNI procedure comprised the axotomy and ligation of the tibial and common peroneal nerves leaving the sural nerve intact. The common peroneal and the tibial nerves were isolated from the sciatic nerve, tight-ligated with 4.0 silk and sectioned distal to the ligation, removing 2 ± 4 mm of the distal nerve stump, preventing nerve regeneration. During the procedure, the sural nerve was carefully maintained intact.

After the procedure, the muscle was sutured with absorbable line 4-0 and the skin was sutured with staples (Stoelting®, U.S.A.).

All animals were rehydrated by subcutaneous (s.c) administration of saline solution 0,9% and the anaesthesia was reverted with an s.c injection of atipamezole hydrochloride (Revertor®- 0,5 g/Kg). The staples suturing the skin were removed approximately 7 days after surgery.

3.2 OSMOTIC MINI PUMP IMPLANTATION

Mini-osmotic pumps (ALZET- model 2001- 200 µL; Figure 10 A), were filled with saline (n=6) or morphine ($45 \mu\text{g}^{-1}\cdot\mu\text{L}^{-1}\cdot\text{h}^{-1}$; n=6) and immersed in a 0,9% saline solution at 37 °C for at least 4 hours before implantation, for stabilization purposes. Animals were anesthetized with isoflurane (IsoFlo®) and their dorsum was shaved and cleaned with Betadine® solution. A midline incision was made to the skin and with a blunt-pointed scissors the skin was separated from the fascia and pumps were implanted subcutaneously (Figure 10 B). The incision was closed with surgery staples (Stoelting®, U.S.A.) and animals returned to their home cage.

Mini-pumps were implanted 2-3 weeks after SNI induction, the animals were monitored daily to evaluate body weight and to detect withdrawal signs caused by incorrect functioning of the mini-pumps such as teeth chattering, diarrhea, rhinorrhoea, ptosis, irritability, lacrimation, escaping, penile erection and abnormal posture [73]

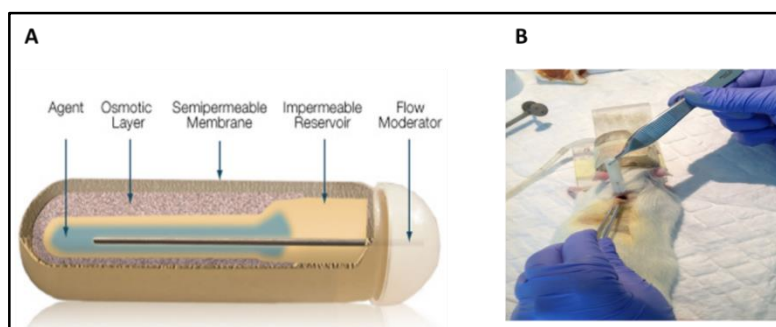


Figure 10: Implant of osmotic morphine/saline mini-pumps. (A) Schematic representation of osmotic mini-pumps. These mini-pumps act by osmotic pressure differences between their interior side (osmotic layer) and the tissue where the mini-pump is implanted. The higher concentration of osmotic layer constituents triggers the entrance of water to the mini-pump through a semi-permeable membrane covering the surface of the mini-pump. As water comes in, the osmotic layer compresses the flexible compartment releasing the agent previously packaged inside the reservoir. The flow is determined by the exterior membrane permeability, by temperature (which should be around 37 °C) and by osmolality. (B) Implant of the mini-pumps in animals' dorsum.

3.3 STEREOTAXIC INJECTIONS

Rats weighing 285-315g were deeply anesthetized by an i.p injection of a mixture of ketamine hydrochloride (0.06 g/Kg) and medetomidine (0.25 g/Kg). The rats were placed on a stereotaxic frame (David Kopf Instruments, U.S.A.) by positioning their head in the incisor bar (Figure 11 – item B) and insertion of the earbars (Figure 11 – item C) into each ear canal. Once each earbar was inserted, verified by a blink reflex usually induced by the contact of the earbar with the ear canal, the rat was placed into the holder and fixed (Figure 11). The head of the animal was cleaned with Betadine® solution and using a scalpel, a midline incision was made to separate the muscle and fascia, to expose the bone. Then a small burr hole was drilled over the targeted area and, with a blunt needle, the dura was carefully pierced.

Using a Hamilton syringe (Figure 11- item A), the rats received two injections of the lentiviral vector (0.6 µl each injection at $5 \cdot 10^6$ TU¹/µl) in two different rostrocaudal locations of the DRt following the stereotaxic coordinates shown in Table 1. The interaural line was used as a reference to calculate the coordinates (Figure 12). The suspensions were injected at the slow rate (0.2 µl/2 min) and, at the completion of the injections, the needle was left in place for 10 min to avoid reflow, before being slowly removed. After stereotaxic surgery, all animals were rehydrated by subcutaneous (s.c) administration of saline solution 0,9% and the anaesthesia was reverted with an s.c injection of atipamezole hydrochloride (Revertor®- 0,5 g/Kg) and then the animals were individually housed and monitored daily to evaluate body weight, any visible motor deficit and sedation.

¹ TU- Transducing units

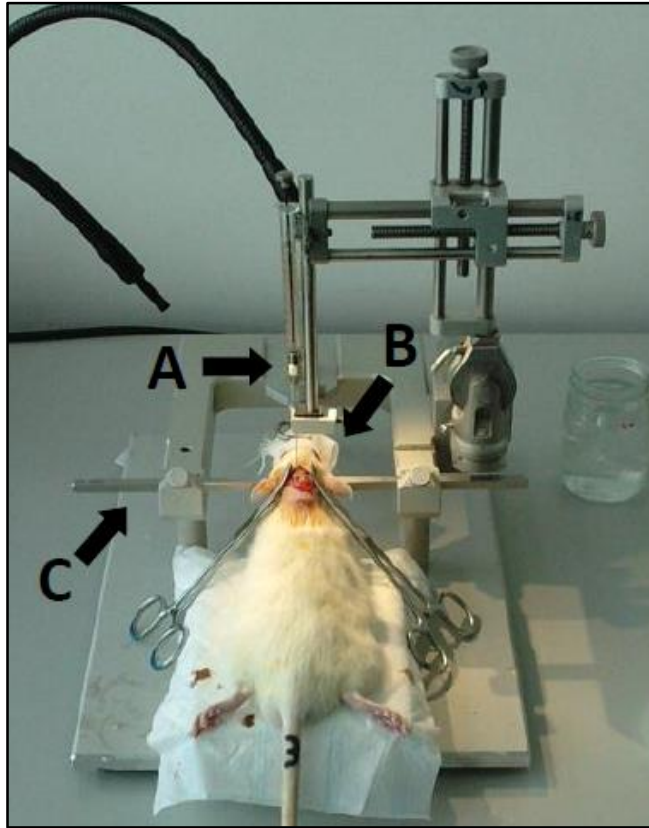


Figure 11: The stereotaxic frame. (A) Hamilton syringe; (B) Incisor bar and (C) Earbars

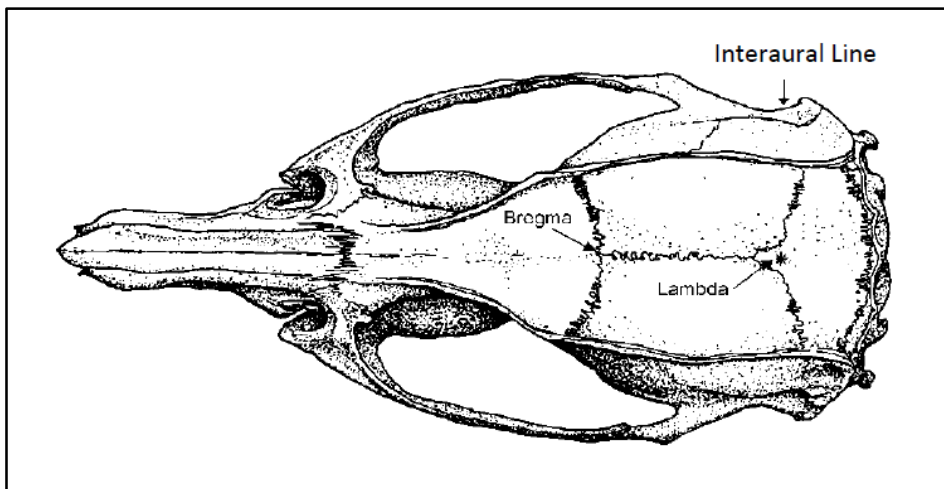


Figure 12: Dorsal view of the rat skull. Position of the interaural line used as a reference to calculate the coordinates to target the DRt. The positions of bregma and lambda points are also shown in the diagram. Bregma and lambda are used as references to calculate coordinates to target rostral brain regions. Adapted from Paxinos and Watson [22].

Table 1: Stereotaxic coordinates to target the left DRt

Coordinates (mm)	Left DRt (1st injection)	Left DRt (2nd injection)
AP (Anterior-posterior)	-6.0	-6.4
LM (Latero-medial)	+1.4	+1.3
DV (Dorso-ventral)	-1.5	-1.7

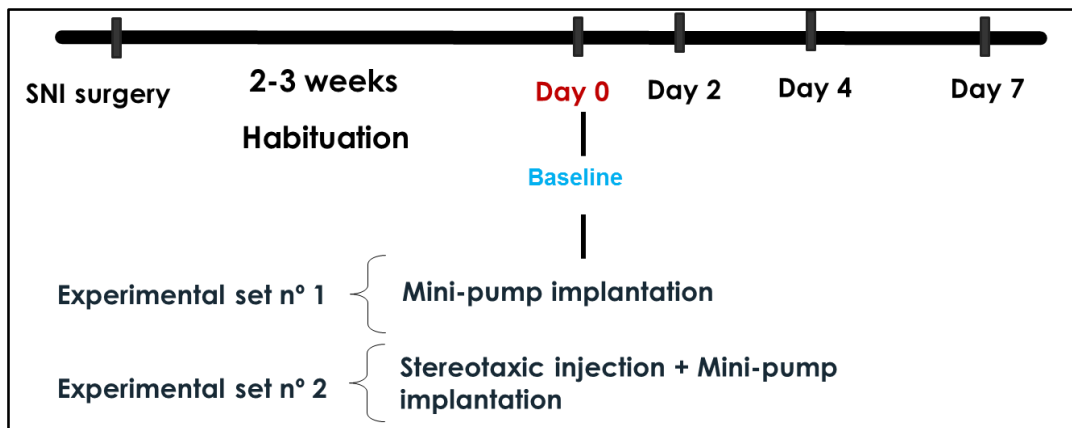
The stereotaxic surgeries were performed between 2-3 weeks after SNI induction. At the completion of the stereotaxic procedure, the animals were implanted with osmotic mini-pumps filled with morphine ($45 \mu\text{g}^{-1} \cdot \mu\text{L}^{-1} \cdot \text{h}^{-1}$) or saline and the animals were assigned to different experimental groups. In one group, the animals were injected with LV-Control and implanted with osmotic mini-pumps filled with morphine (n=5) or saline (n=5); in the other group, the animals were injected with MOR-R and implanted with osmotic mini-pumps filled with morphine (n=6) or saline (n=4)

4 BEHAVIOURAL ANALYSIS

4.1 EVOKED PAIN

The behavioural evaluation of evoked pain was performed after a period of habituation of one week, during which the animals were handled in the behavioural test room for 30 min every day and placed in the testing apparatus for another 30 min (Figure 13 A). The criteria for adequate habituation were that animals did not freeze or defecate when placed in the test apparatus.

After SNI induction, the animals typically develop mechanical allodynia, mechanical hyperalgesia and cold allodynia in the injured paw [66] which are ascertained by von-Frey, pin-prick and acetone tests, respectively. To evaluate the development of these pain behaviours, the rats were placed on an elevated transparent cage with a mesh wire bottom allowing the stimulation of the lateral plantar surface of the injured hindpaw.



Scheme 1: Time course of nociceptive behavioural evaluation after mini-pump implantation and/or stereotaxic surgery + mini-pump implantation. After 2-3 weeks of SNI surgery, animals were divided into 2 experimental settings. Animals from experimental set n° 1 were implanted with osmotic mini-pumps containing saline or morphine ($45 \mu\text{g}^{-1} \cdot \mu\text{L}^{-1} \cdot \text{h}^{-1}$). Animals from experimental set n°2 were stereotaxically injected with LV-Control or MOR-R and osmotic mini-pumps of either saline or morphine were implanted in each group. Animals were tested at Day 0, before the surgical procedures, and at days 2, 4 and 7.

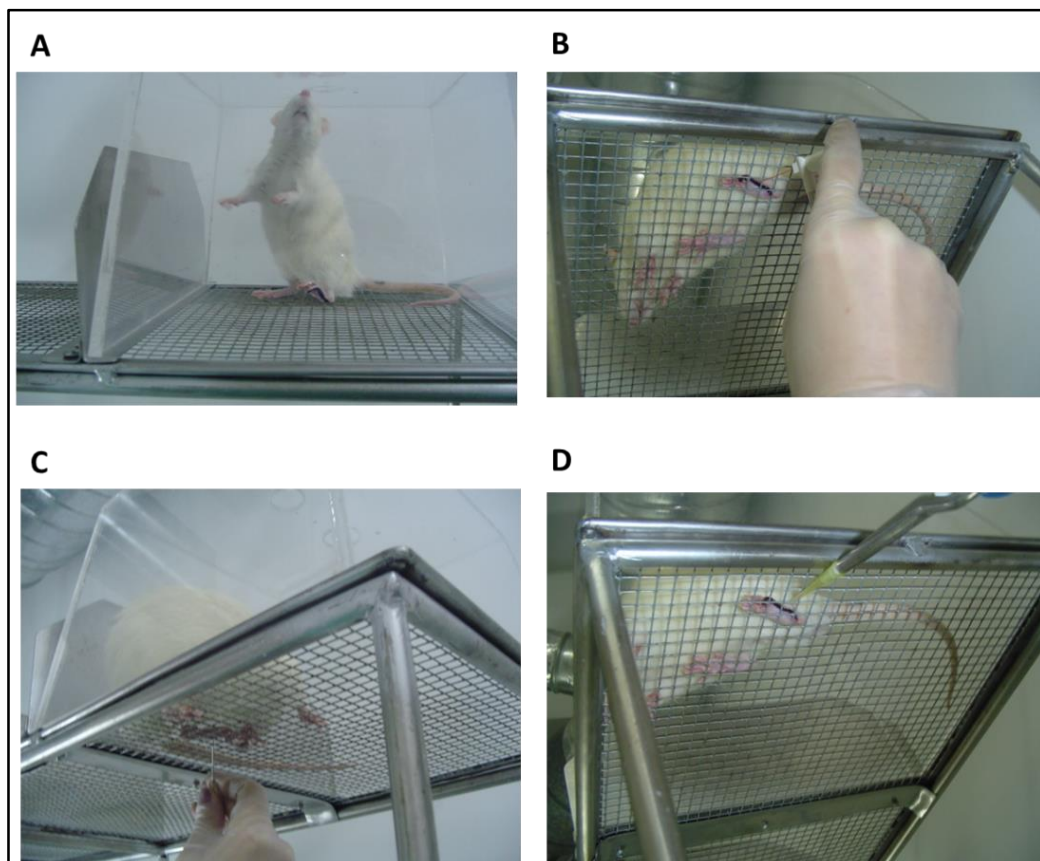


Figure 13: Behavioural tests apparatus. (A) Animal placed in the individual Plexiglas container in the wire grid; (B) von-Frey test; (C) Pin-prick test; (D) Acetone test.

The von-Frey test was performed by applying von-Frey monofilaments (Stoeling, U.S.A.) in a sequence of increasing stiffness for 5 seconds (Figure 13 B) [67]. The

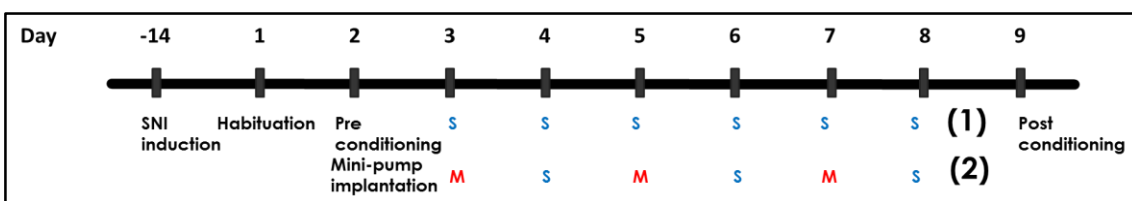
threshold was taken as the lowest force that evoked a brisk withdrawal response to one of five repetitive applications. The pin-prick test was performed by the application of a brief stimulation with a safety pin (Figure 13 C) at an intensity sufficient to produce a reflex withdrawal response but not penetrate the skin and the duration of paw withdrawal was clocked [68]. The acetone test was performed by application of 40 μ L of acetone using a micropipette tip connected to a micropipette without touching the skin (Figure 13 D) and the duration of the withdrawal was timed [69].

Two experimental sets of animals were used. One set of animals was used to evaluate the time course effect of morphine administration (Experimental set n^o 1, Scheme 1). The animals from this set were first submitted to SNI surgery and 2-3 weeks later they were implanted with a mini-pump filled with saline or morphine (n=6, each group; Scheme 1). The second set of animals was used to evaluate the time course effect of MOR expression knock down during chronic morphine exposure (Experimental set n^o 2, Scheme 1). The animals from both experimental sets were behaviourally evaluated by von-Frey, pin-prick and acetone tests before and at 2, 4 and 7 days after mini-pump implantation and/or stereotaxic injections with mini-pump implantation (Scheme 1).

4.2 SPONTANEOUS PAIN

Spontaneous pain was evaluated by the conditioned place preference (CPP) test. CPP was performed in a Plexiglas apparatus measuring 100 [length (L)] 40 [width (W)] X 40 [height (H)] cm and comprising two distinct conditioning environments separated by a neutral space. Each conditioning environment measured 40 (L) X 40 (W) X 40 (H) cm. Each environment contained a visual and a tactile clue. One environment had a floor consisting of 0,5 cm metal bars spaced 2 cm apart (edge-to-edge) and walls with alternating 3 cm wide black and white horizontal stripes. The floor of the second environment was a mesh wire with 0,5 cm holes, and walls with alternating wide black and white vertical stripes. The neutral area measured 20 X 40 X 40 cm, with black Plexiglas walls and floor. During the conditioning phase, Plexiglas partitions matching their respective environments were inserted to restrict the rats to a specific designated environment. The behaviour of each rat was recorded using a camera and the videos were analyzed by Ethlog 2.2.

An unbiased protocol was used starting 14 days after SNI induction. All rats underwent a 2 day habituation period during which they were exposed to the environment with full access to all chambers for 15 min. On day 2 (pre-conditioning; Scheme 2), behaviour was recorded for 15 min and analyzed to verify no pre-conditioning chamber preference. Rats spending less than 20% or more than 80% of the entire time in one of the chambers were excluded. Each rat was then randomly assigned to a treatment group and a conditioning environment in a counterbalanced fashion. After recording their behaviour, the rats were implanted with osmotic mini-pumps filled with morphine or saline (Scheme 2). On days 3-8 (conditioning; Scheme 2 - item 1) the animals were subcutaneously injected with saline once a day or morphine (10 mg/Kg; Scheme 2 – item 2) on alternate days and, immediately after the injection, the animals were placed into the designated conditioning chamber for 1 hour. On day 9 (post-conditioning; Scheme 2) rats were placed in the CPP box in a drug-free state, with access to all chambers, their behaviour was recorded for 15 min and the difference between post- and pre-conditioning time spent in each compartment was calculated.



Scheme 2: Time course for spontaneous pain evaluation in the CPP test. Animals were allowed a 15 min period of habituation at day 1 and were recorded day 2 to ascertain preferences. Mini-pumps were implanted and one group (1) received saline (S) s.c every day or (2) morphine (M, 10 mg/Kg) every other day for 6 days and placed in a specific compartment for 1 hour each day. On day 9 animals were allowed to explore the apparatus in a drug free state for 15 min and were recorded to ascertain preferences.

5 VASCULAR PERFUSION AND MATERIAL PROCESSING FOR IMMUNOHISTOCHEMICAL ANALYSIS

After the behavioural evaluation, the animals were given an overdose of sodium pentobarbital (65 mg/Kg of body weight), placed in the supine position, the abdomen and the thorax were opened to expose the heart and 0,2 mL of heparine (Braun Medical, Portugal) were injected into the left ventricle. A catheter was then introduced into the ascending aorta for perfusion with 200 mL of calcium-free Tyrode's solution (Appendix A), followed by 800 mL of fixative solution containing 4% paraformaldehyde in 0.1 M PB, pH 7.2 (Appendix A).

The animals implanted with mini-pumps and stereotaxically injected with the lentiviral vectors (Experimental set nº 2; Scheme 1) were perfused using a set up that

allowed the perfusion of animals by gravity. After perfusion, the brain was removed and immersed in the fixative solution for 4h followed by 30% sucrose in 0,1M PB, pH 7,2 overnight at 4°C. The brainstem was serially cut in a freezing microtome at 40 µm, collected in 4 sets and stored in a cryoprotector solution (Appendix A) at -20°C.

The animals implanted with mini-pumps (Experimental set n° 1; Scheme 1) and without any further surgical manipulation were perfused using a perfusion pump at a 30 mL/min rate. After perfusion, the brain and the L₄ spinal segment were removed and immersed in a fixative overnight and 4 hours, respectively followed by 30% sucrose, in a 0,1 PB, pH 7,2, as above. The brainstem sections were processed as above and used for the immunohistochemical analysis of microglia and astrocytes. The L₄ spinal segment was serially cut at 40 µm and the sections were collected in 3 sets and stored in a cryoprotector solution at -20°C. One set of the spinal L₄ sections was used to evaluate Fos expression. The material from this last experimental set of animals was processed with these modifications because our preliminary experiments showed that under these conditions the immunolabelling of glial cells was more consistent compared to the material processed by gravity and post-fixed for 4h.

6 TRANSDUCTION PATTERN ANALYSIS AND HISTOLOGICAL VERIFICATION OF INJECTION SITES

To analyse the transduction patterns of the lentiviral vectors, one set of brainstem sections from animals injected with LV-Control were mounted on gelatine-coated slides, cover slipped with a solution of glycerol diluted in PB (1vol/3vol) and analyzed in an ApoTome Slider (Zeiss®) fluorescence microscope with an excitation length of 488 nm. EFGP positive cells were plotted on diagrams of medulla oblongata sections.

MOR-R vectors also carry the EFGP transgene (Figure 8) but its expression levels are undetectable, likely due to the RNA interference reaction induced by antisense RNA of MOR which degrades EGFP RNA placed in the second position of the bicistronic construct. Therefore the location of the injection sites of MOR-R-injected-animals was determined by checking the position of the needle tract after counterstaining the medullary sections with thionin (Appendix A) [74]. Only animals with vector injections centred in the DRt were included in data analysis.

7 IMMUNOHISTOCHEMICAL ANALYSIS OF MICROGLIA

7.1 DENSITOMETRIC ANALYSIS

Brainstem sections were incubated with a rabbit polyclonal anti-Iba-1 antibody (Wako, Japan) in 0,1 M PBS containing 0,3% Triton X-100 (PBS-T) at 1:2500 for 48 hours at 4 °C. After washing with PBS-T the sections were incubated for 1h with a swine biotinylated anti-rabbit serum (Dako, Denmark) diluted in PBS-T containing 2% normal swine serum. Sections were washed again and incubated for 1h in PBS-T containing the avidin-biotin complex (1:200; ABC; Vector Laboratories, U.S.A). After washing in 0,1 M Tris-HCl, pH 7,6, bound peroxidase was revealed using 0,0125% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich, U.S.A.) and 0,025% H₂O₂ in the same buffer. The sections were mounted on gelatine-coated slides, cleared in xylol and cover slipped with Eukitt (Sigma, U.S.A.). Five sections encompassing the rostro-caudal extent of the DRt were taken from each animal and photomicrographs of the left DRt (ipsilateral to SNI surgery) and right DRt (contralateral to the SNI surgery) were taken using a Zeiss® light microscope with a high-resolution digital camera.

7.2 MORPHOLOGICAL ANALYSIS

The second set of brainstem sections encompassing the DRt was incubated with a rabbit polyclonal anti-Iba-1 antibody (Wako, Japan) in PBS-T at 1:1000 for 48 hours at 4°C. After washing with PBS-T the sections were incubated for 1h with a donkey anti-rabbit IgG 594 (Alexa Fluor®, U.S.A.) diluted in PBS-T containing 2% normal swine serum. The sections were mounted on gelatine-coated slides and cover slipped with a solution of glycerol diluted in PB (1vol/3vol). Z-stack images from ipsilateral and contralateral DRt from each animal, with an optimal distance of 0,720 µm, were acquired with an ApoTome Slider (Zeiss®) fluorescence microscope, with an excitation length of 594 nm (20x objective) and the maximum intensity projection of the Iba-1 channel was obtained in order to visualize all microglial processes. Using the Image J® software, for one representative DRt contralateral and ipsilateral to SNI surgery per animal, these maximum projections (Figure 14 A) were converted to a binary image (Figure 14 B) and then skeletonized (Figure 14 C). The AnalyzeSkeleton plugin (<http://imagejdocu.tudor.lu/>) was then applied to all skeletonized images to collect data

on the number of endpoints per frame, as an indicator of total number of microglial processes, (Figure 14 D1, blue) and process length (Figure 14 D1, orange), which were then normalized dividing by total cell number. The number of amoeboid cells was also counted to obtain the rate of these cells in the total cell number per section.

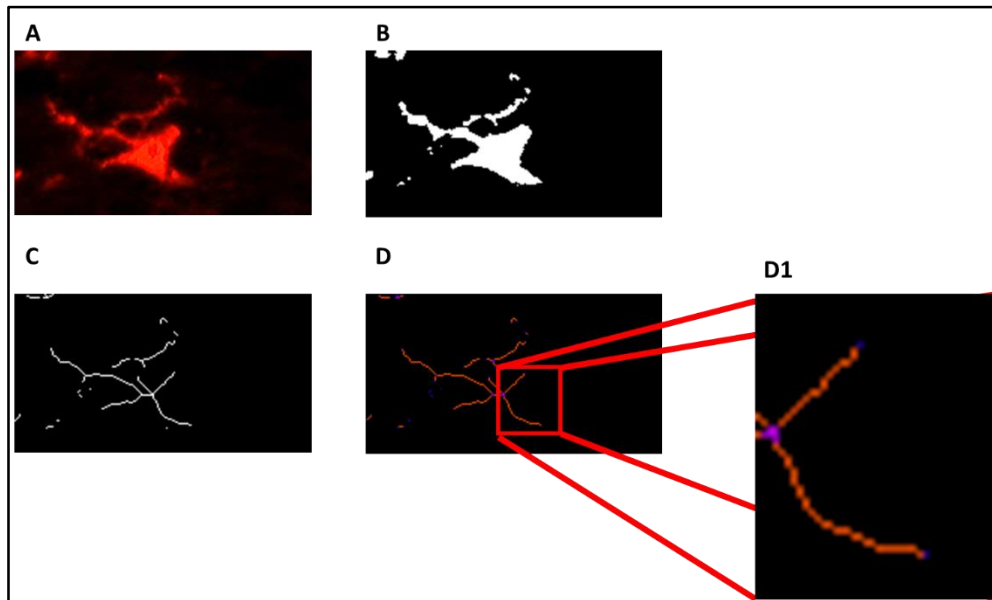


Figure 14: Skeleton analysis of microglia morphology. (A) Maximum intensity projections of fluorescence images were converted to (B) binary images and then (C) skeletonized. The number of microglia (D1) process endpoints (blue) and process length (orange) were summarized for statistical comparisons from Analyse Skeleton plugin by Image J®.

8 IMMUNOHISTOCHEMICAL ANALYSIS OF ASTROCYTES

Brainstem sections were incubated with a mouse polyclonal anti-GFAP antibody (Sigma, U.S.A.) in PBS-T at 1:1000 for 48 hours at 4°C. After washing with PBS-T, the sections were incubated for 1h with a horse biotinylated anti-rabbit serum (Dako, Denmark) diluted in PBS-T containing 2% normal horse serum. Sections were washed again and the detection of the immunoreaction was performed using the ABC solution (1:200; ABC; Vector Laboratories, U.S.A) as above. The sections were mounted on gelatine-coated slides, cleared in xylol and cover slipped with Eukitt. Five sections encompassing the rostro-caudal extent of the DRt were taken from each animal and photographed. The densitometric analysis was performed as described above for Iba-1.

9 IMMUNOHISTOCHEMICAL ANALYSIS OF SPINAL FOS

The spinal cord sections were incubated with a polyclonal anti-Fos antibody raised in rabbit (Oncogene, U.K.), diluted at 1:10000 in PBS-T and 2% normal swine serum, for 48 hours at 4°C. After washing with PBS-T, the sections were incubated for 1h with a swine biotinylated anti-rabbit serum (Dako, Denmark) diluted in PBS-T containing 2% normal swine serum. Sections were washed again and the detection of the immunoreaction was performed using the ABC solution (1:200; ABC; Vector Laboratories, U.S.A) as above. The sections were cleared in xylol and coverslipped with Eukitt. Ten sections were randomly photographed from each rat using a Zeiss® light microscope with a high resolution camera and the number of Fos-immunoreactive (IR) neurons was counted in the spinal dorsal horn (laminae I–II and laminae III–VI) using the cell counter plugin from Image J® software.

10 STATISTICAL ANALYSIS

Evoked pain scores were analyzed by two way ANOVA for repeated measures followed by Bonferroni post-hoc tests. The spontaneous pain behaviour (CPP test) was analyzed by one-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons.

The number of Fos-IR neurons, the densitometric analysis of glial cells and the morphological analysis of microglia were analyzed by using two-way ANOVA followed by Bonferroni post-hoc tests for multiple comparisons. The statistical analysis was performed by Graphpad Prism 6®. The significance level was set at 0,05.

Results

1 GENERAL CONDITIONS OF THE ANIMALS

The weight of the animals was taken as a measure of their well-being, so animals were weighted during the whole period of behavioural assessment.

After the implantation of the mini-pumps all animals gained weight throughout the entire period of behavioural testing (Figure 15 A). No significant differences between animals chronically administered with morphine or saline were observed. At day 7, animals administered with morphine (n=6) gained $23 \pm 4,69$ g and animals receiving mini-pumps filled with saline (n=6) gained $24,5 \pm 6,77$ g.

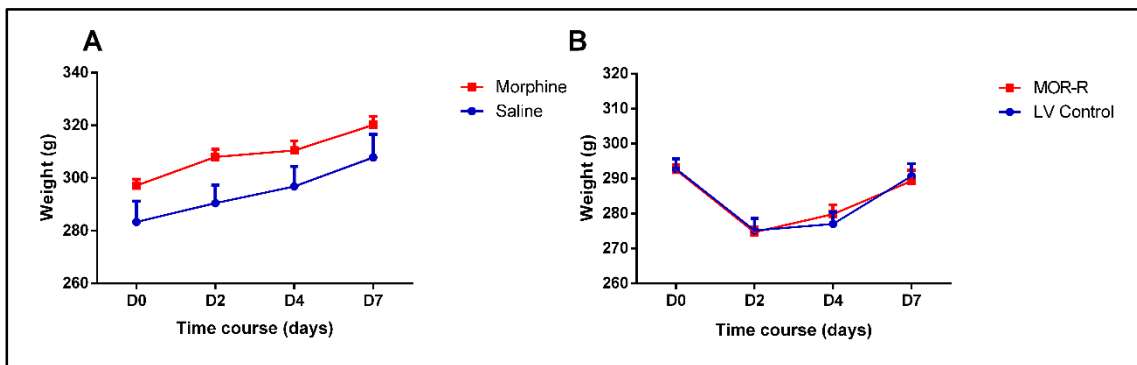


Figure 15: Animals' weight evolution after (A) mini-pumps implantation (saline – blue line n=6; morphine – red line n=6) and (B) after stereotaxic injection of lentiviral vectors into the DRt (LV-Control – blue line n = 10; MOR-R – red line n = 10).

After stereotaxic surgery, weight evolution was similar both in animals injected with the control vector (LV-Control) and MOR-R. Animals injected with LV-Control lost $17,6 \pm 10$ g and animals injected with MOR-R lost $18 \pm 5,6$ g during the first 2 days (LV-Control n = 10; MOR-R n = 10) and then progressively regained weight (Figure 15 B). Additionally, all animals showed normal exploratory activity and did not exhibit any visible behavioural abnormality until the end of the experiments.

2 EFFECTS OF CHRONIC MORPHINE ADMINISTRATION

2.1 BEHAVIOURAL EFFECTS

2.1.1 Effects on evoked pain behaviour

The effects of the chronic morphine administration were tested before and at 2, 4 and 7 days after mini-pumps implantation (Scheme 1).

In pin-prick test, animals chronically administered with morphine showed a significant increase of withdrawal duration at day 4 ($p=0,0027$; Figure 16 A) and day 7 ($p=0,0001$; Figure 16 A) compared to baseline. At day 7, the withdrawal duration of the morphine group was also higher than in the saline group ($p=0,0159$; Figure 16 A). No significant differences were observed in the saline group compared to baseline.

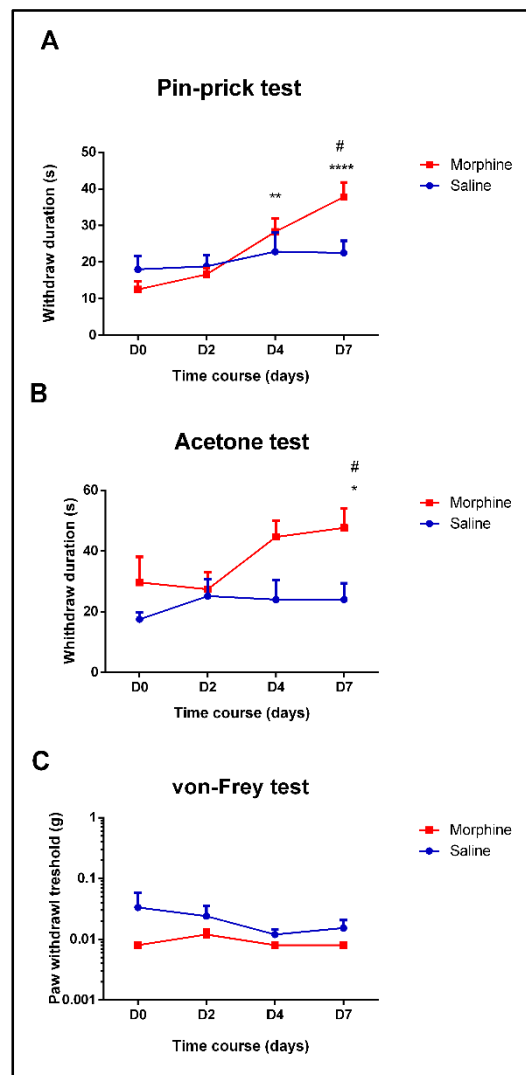


Figure 16: Time course effects of morphine administration on (A) mechanical hyperalgesia (B) cold allodynia and (C) mechanical allodynia. Implant of osmotic mini-pumps was performed on day 0. Data are presented as mean \pm SEM. (saline – blue line $n=6$; morphine – red line ($n=6$)) * $p<0,05$; ** $p<0,01$; *** $p<0,001$ vs.D0; # $p<0,05$ morphine vs saline.

In the acetone test, animals treated with morphine showed a significant increase of the withdrawal duration at day 7 compared to day 0 ($p=0,0287$; Figure 16 B) and compared to saline ($p=0,0289$; Figure 16 B). No significant differences were observed in the saline group compared to baseline.

In the von-Frey test, no significant differences were observed within each group and between both groups (Figure 16 C).

2.1.2 Effects on spontaneous pain behaviour

A CPP was performed on animals chronically administered with saline ($n=6$) and morphine ($n=6$).

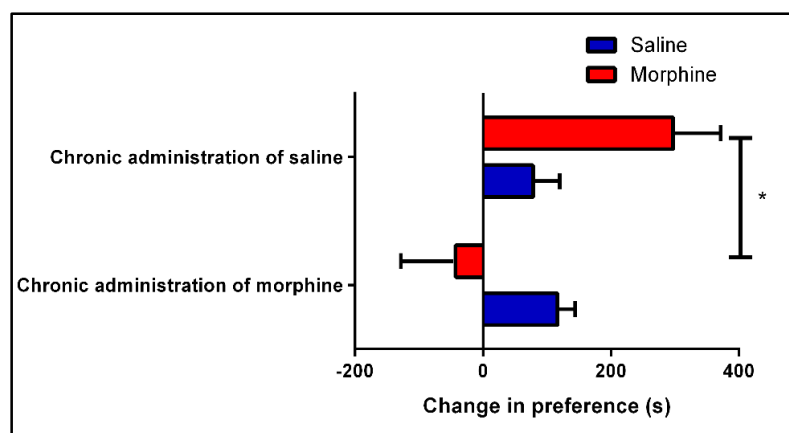


Figure 17: Effects of chronic administration of morphine on the conditioned place preference test. During the conditioning, morphine (red bars - 10 mg/kg, s.c.) was injected in alternate days in animals chronically administered with saline ($n=3$) or morphine ($n=3$). On a separate group of animals saline (blue bars) was injected every day in animals chronically administered with morphine ($n=3$) or saline ($n=3$). Data are means \pm SEM, * $p=0,0277$, $n=3$ /group.

Animals chronically treated with saline and receiving morphine in alternate days ($n=3$) spent longer time in the morphine-paired chamber compared to control animals receiving saline every day ($n=3$), although this comparison did not reach statistical significance (Figure 17). Animals chronically treated with morphine and receiving morphine in alternate days ($n=3$) spent significantly less time in the morphine paired chamber compared to animals chronically treated with saline and receiving morphine in alternate days ($p=0,0277$; Figure 17). No differences were found between animals

chronically treated with saline (n=3) or morphine (n=3) and paired with saline (Figure 17).

2.2 EFFECTS ON THE EXPRESSION OF SPINAL FOS

Chronic administration of morphine showed no significant changes in the number of Fos-IR cells compared to saline, both in the left (ipsilateral to SNI) and right (contralateral to SNI) spinal dorsal horn (Figure 18).

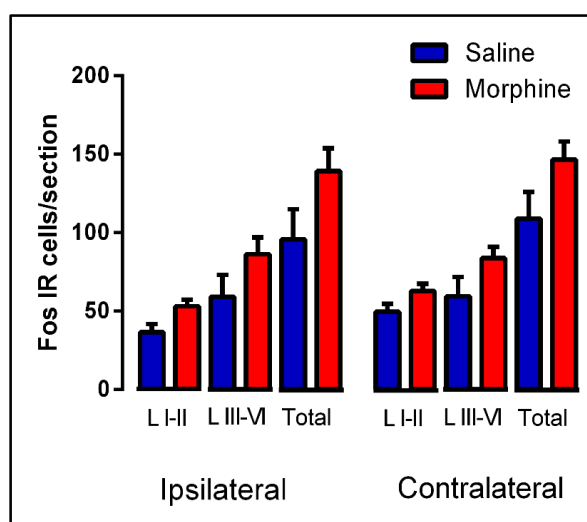


Figure 18: Effects of chronic administration of morphine on the expression of Fos at the spinal dorsal horn. Number of Fos-IR cells in laminae I-II, laminae III-VI and total per section. Data are presented as means \pm SEM (morphine n=6; saline n = 6).

3 EFFECTS OF MOR-KNOCK DOWN EXPRESSION AT THE DRT DURING CHRONIC MORPHINE ADMINISTRATION

3.1 PATTERN OF LENTIVIRAL TRANSDUCTION AND INJECTION SITE ANALYSIS

The pattern of lentiviral transduction was analyzed in animals injected with LV-Control and with the injection site centred at the DRt (Figure 19 A, B). In those animals, the injection site was constituted by a central dark zone corresponding to the needle tract with numerous EGFP⁺ neurons (Figure 19 B) around this central region. A total of ten animals out of eleven were successfully injected with the LV-Control vector at the DRt. All EGFP⁺ neurons were located within the boundaries of the DRt which indicates that injections correctly placed at the DRt show a pattern of lentiviral transduction restricted to the DRt.

The injection site of MOR-R-injected animals was analyzed in medullary sections stained with thionin, because EGFP expression from these constructs (Figure 8) was almost undetectable. The injection site was identified by the presence of the needle tract in the DRt. In those animals the injection site was constituted by a central dark core corresponding to the needle tract surrounded by a peripheral zone lightly stained by thionin (Figure 19 C). Only animals with the injection site placed at the DRt (MOR-R n = 10 out of 12) were included in data analysis.

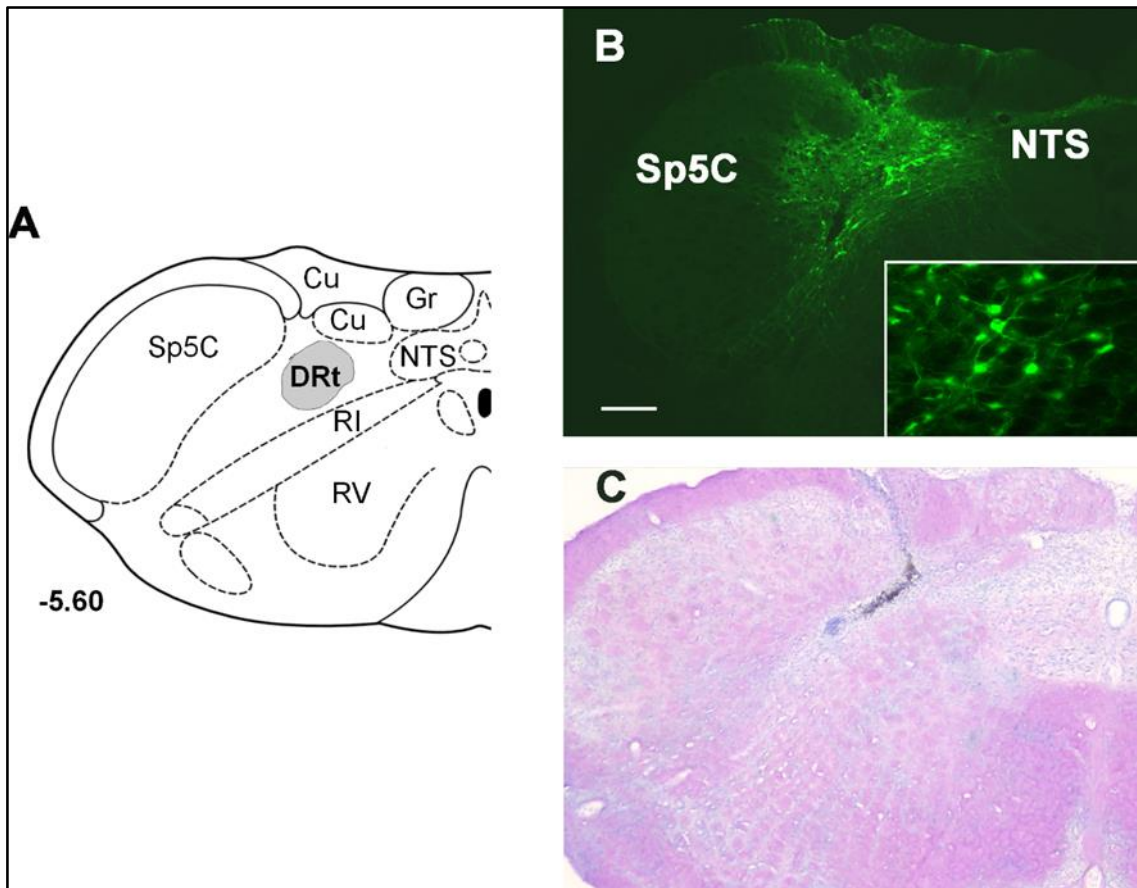


Figure 19: Localization of the injection site in the DRt. (A) Diagram depicting the location of the DRt, at 5.60 mm caudal to the interaural line adapted from the Paxinos and Watson [22]. (B) Fluorescence photomicrograph of the injection site at the DRt showing EGFP neurons better depicted in the insert. (C) Representative photomicrograph of a thionin-stained section illustrating a correct vector injection at the DRt. Scale bar in B: 200 μ m (C is at the same magnification).

3.2 BEHAVIOURAL EFFECTS

The effect of the MOR knock-down at the DRt during chronic treatment with morphine was evaluated before and at 2, 4 and 7 days after the mini-pumps implantation and stereotaxic injections.

In the pin-prick test, animals chronically treated with morphine and injected with the control vector show a significant augmented withdrawal duration at all times tested (Day 2, $p=0,0316$; Day 4, $p=0,0182$; Day 7, $p=0,0022$; Figure 20 A) compared to baseline.

Animals injected with MOR-R and chronically exposed to morphine did not present significant alterations compared to baseline. At day 7, the withdrawal responses of MOR-R animals were significantly lower than in the LV-Control group ($p=0,0109$; Figure 20 A).

In animals chronically treated with saline, the injection of LV-Control did not induce significant alterations compared to baseline. MOR-R caused a significant increase of the withdrawal duration at day 2 ($p=0,0137$; Figure 20 B) and at day 7 ($p=0,0002$; Figure 20 B). At day 7, the withdrawal duration of the MOR-R group was also higher than in the LV-Control group ($p= 0,0053$; Figure 20 B).

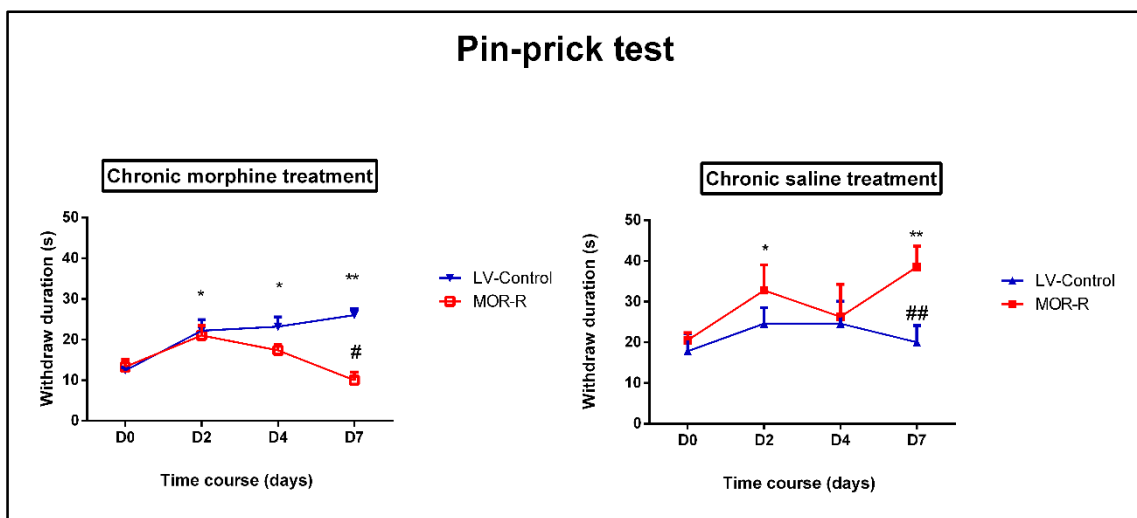


Figure 20: Time course effects of MOR knock-down at the DRt in mechanical hyperalgesia during chronic morphine administration (A) and chronic saline administration (B). Stereotaxic injection + implant of osmotic mini-pumps were performed at D0. Data are presented as mean \pm SEM. (chronic morphine treatment: LV-Control-injected $n=5$, MOR-R-injected $n=6$; chronic saline treatment: LV-Control-injected $n=5$; MOR-R-injected $n=4$). * $p<0,05$; ** $p<0,01$ vs.D0; # $p<0,05$; ## $p<0,01$ MOR-R vs LV-Control.

In the acetone test, animals chronically treated with morphine and injected with LV-Control showed a significant increase in the withdrawal duration, at day 7 ($p=0,0393$; Figure 21 A) compared to baseline. Animals injected with MOR-R showed no significant differences compared to baseline. At days 4 ($p=0,0227$; Figure 21 A) and 7 ($p=0,0007$; Figure 21 A) animals injected with the MOR-R vector displayed lower withdrawal responses compared to the LV-Control group.

Animals chronically administered with saline and injected with MOR-R showed an increase of withdraw responses at all times (day 2, $p=0,0024$; day 4, $p= 0,0103$; day 7, $p=0,0001$; Figure 21 B) compared to baseline. Animals injected with the LV-Control showed an increase in withdrawal duration at day 2 ($p=0,001$; Figure 21 B) and at day 4 and 7 the withdrawal responses decreased to values similar to baseline. At day 7 ($p=$

0,0255; Figure 21 B) the animals injected with MOR-R display higher withdrawal duration compared to the LV-Control group.

No significant differences were observed in the von-Frey test within each group and between both groups (Figure 22).

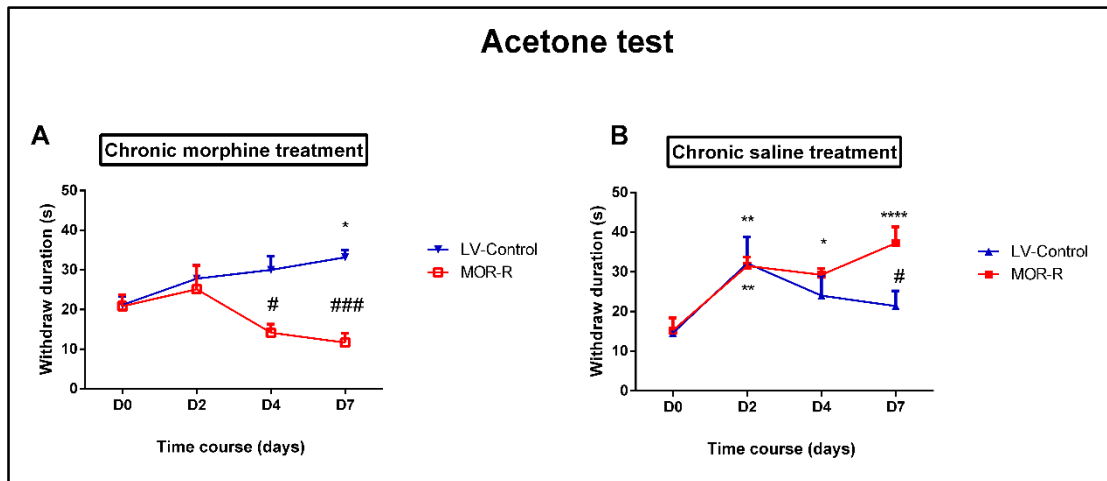


Figure 22: Time course effects of MOR knock-down at the DRt in cold allodynia during chronic morphine administration (A) and chronic saline administration (B). Stereotaxic injection + implant of osmotic mini-pumps were performed at D0. Data are presented as mean \pm SEM. (chronic morphine treatment: LV-Control-injected n=5, MOR-R-injected n=6; chronic saline treatment: LV-Control-injected n=5; MOR-R-injected n=4). * $p < 0,05$; ** $p < 0,01$; *** $p < 0,0001$ vs. D0; # $p < 0,05$; ### $p < 0,001$ MOR-R vs LV-Control.

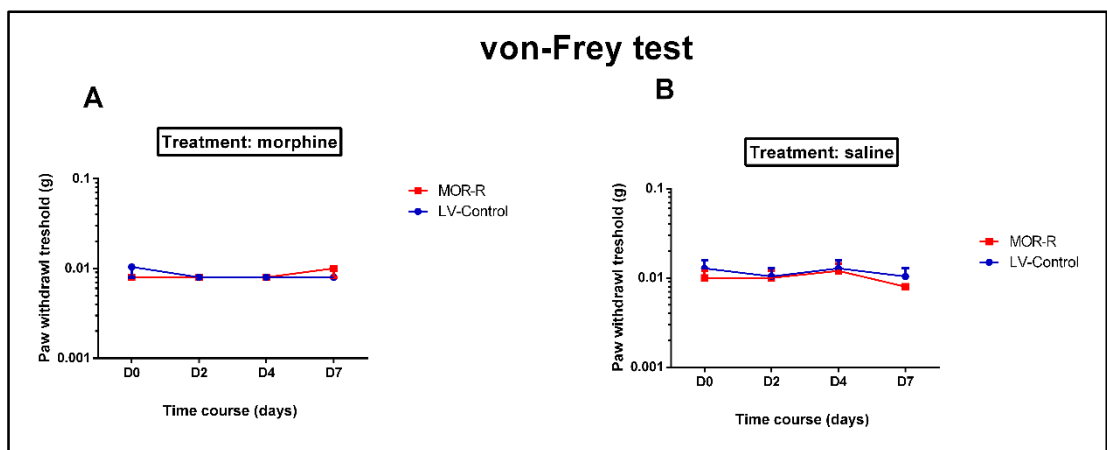


Figure 21: Time course effects of MOR knock-down at the DRt in mechanical allodynia during chronic morphine administration (A) and chronic saline administration (B). Stereotaxic injection + implant of osmotic mini-pumps were performed at D0. Data are presented as mean \pm SEM. (chronic morphine treatment: LV-Control-injected n=5, MOR-R-injected n=6; chronic saline treatment: LV-Control-injected n=5; MOR-R-injected n=4). Data are presented as mean \pm SEM.

4 EFFECTS OF CHRONIC MORPHINE ADMINISTRATION ON GLIAL EXPRESSION

4.1 EFFECTS ON MICROGLIA

4.1.1 Densitometric analysis

The evaluation of Iba-1 expression in the DRt was performed using DRt sections from animals, chronically administered with morphine or saline.

Iba-1 expression in the DRt quantified by densitometry was significantly higher in animals treated with morphine compared to animals treated with saline both in left (ipsilateral to SNI; Figure 23 A, B, C) and the right DRt (contralateral to SNI; Figure 23 C).

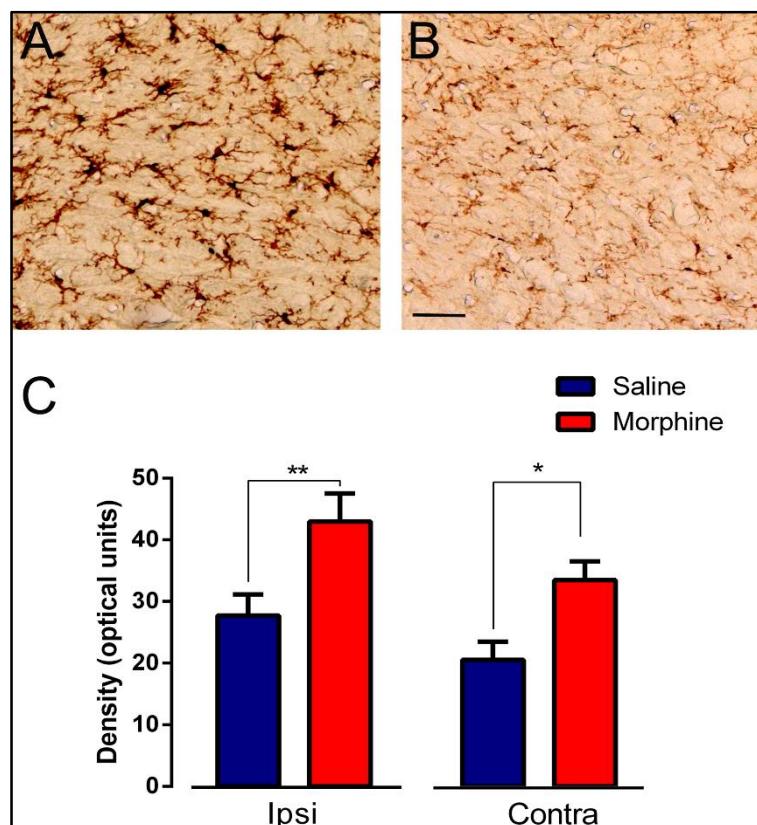


Figure 23: Effects of chronic administration of morphine on the expression of Iba-1 at the DRt. Representative photomicrographs of Iba-1-IR labeling on the left DRt (ipsilateral to SNI) of animals chronically administered with morphine (A) and in animals chronically administered with saline (B). Data in C shows the expression of Iba-1 at the left (ipsilateral to SNI, Ipsi) and right (contralateral to SNI, Contra) DRt of saline and morphine treated animals. Data are presented as mean \pm SEM (saline n=6; morphine n=6) * p <0,05; ** p <0,01. Scale in B: 100 μ m (A is at the same magnification)

4.1.2 Morphological analysis

The morphological analysis of microglia in the DRt was performed to assess if there were differences in total branch number per cell, process length per cell and rate of amoeboid cells in microglia in the left DRt (ipsilateral to SNI) and right (contralateral to SNI) between animals chronically administered with morphine and chronically administered with saline.

No significant differences were observed in the number of branches and their length per cell between saline- and morphine- treated animals. An increase in the percentage of amoeboid cells was observed in the left DRt (ipsilateral to SNI) of morphine-treated animals compared to saline ($p=0,0300$; Figure 24 C).

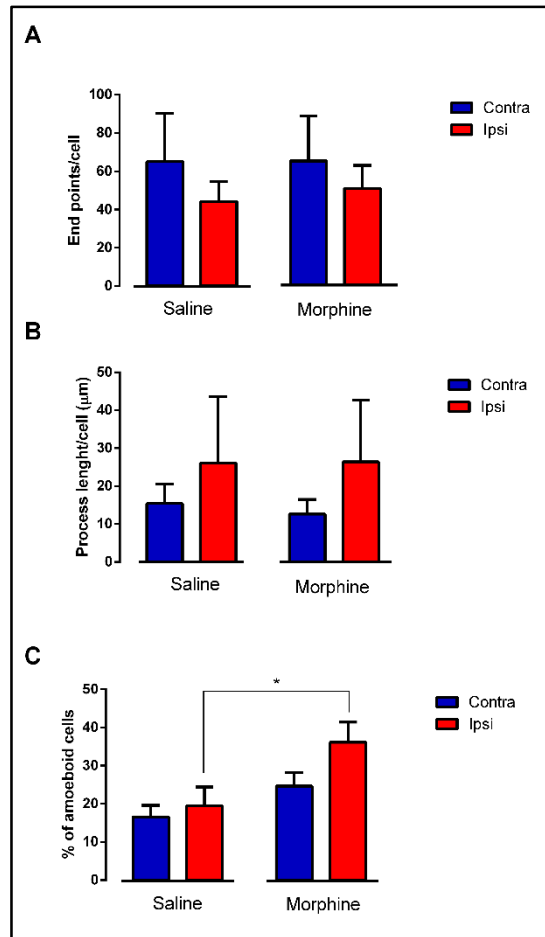


Figure 24: Effects of chronic administration of morphine on the microglia morphology at the DRt. Maximum projections of 0,720 µm z-stacks of Iba-1 expression in DRt were acquired and analysis of end point/cell, as an indicative of branch number per cell (A), process length per cell (B) and percentage of amoeboid cells (C) are shown. Data as means ± SEM (morphine n=3; saline n = 3). * - $p= 0,0300$

4.2 EFFECTS ON ASTROCYTES

The evaluation of GFAP expression in the DRt was performed using DRt sections from animals chronically administered with morphine or saline.

The GFAP expression quantified by densitometry was significantly higher in animals treated with morphine compared with saline at the left DRt (ipsilateral to SNI; $p=0,0444$; Figure 25 A, B, C). In morphine-treated animals, the ipsilateral DRt showed a significant increase of GFAP when compared to the contralateral DRt ($p=0,0418$; Figure 25 C).

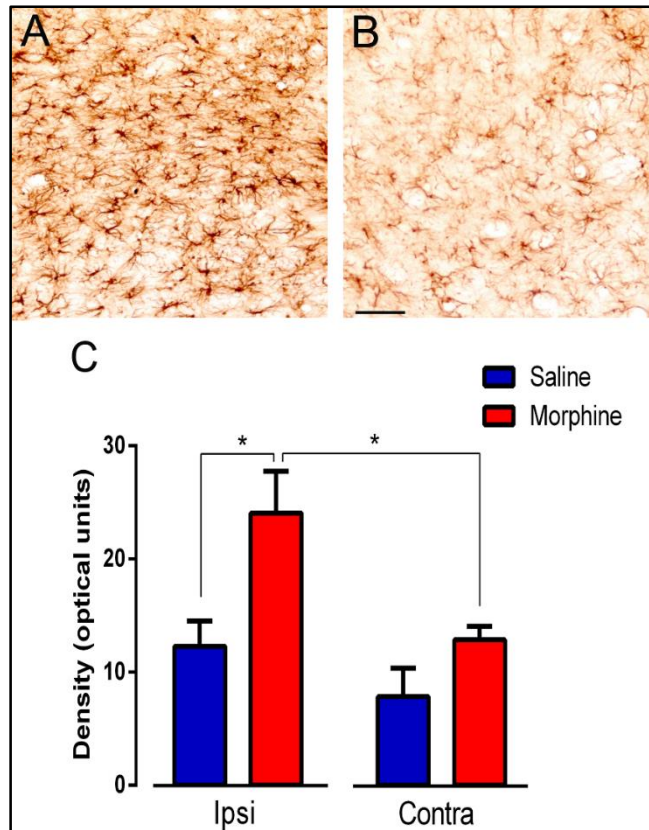


Figure 25: Effects of chronic administration of morphine on the expression of GFAP at the DRt. Representative photomicrographs of GFAP-IR labeling on the left DRt (ipsilateral to SNI) of animals chronically administered with morphine (A) and in animals chronically administered with saline (B). Data in C shows the expression of GFAP at the left (ipsilateral to SNI, Ipsi) and right (contralateral to SNI, Contra) DRt of saline and morphine treated animals. Data are presented as mean \pm SEM (saline n=4; morphine n=5) * p <0,05. Scale in B: 100 μ m (A is at the same magnification)

Discussion and Conclusions

The results gathered in the present thesis show that chronic administration of morphine in animals with chronic neuropathic pain induces an aggravation of pre-existing mechanical and thermal sensibility to pain. In the CPP test, the animals chronically treated with morphine failed to show preference for the morphine-paired chamber which indicates a loss of the analgesic effect of morphine. Lentiviral-mediated MOR knock-down at the DRt showed that the opioidergic system exerts an inhibitory effect at the DRt, in control animals, while the opposite occurs in animals chronically treated with morphine.

The morphological analysis of glial markers show that glia are likely to play an important role at the DRt during chronic morphine exposure, as suggested by the augmented expression of GFAP, an astrocytes marker, and Iba-1, a microglia marker, at the DRt of animals struggling with OIH.

1 EFFECTS OF CHRONIC ADMINISTRATION OF MORPHINE IN NEUROPATHIC PAIN

Chronic administration of morphine has already been shown to induce hyperalgesia in naïve animals during acute pain induction [75-77] and during inflammatory [78] and post-operative pain [43, 45, 79-83]. Our results show for the first time that chronic morphine administration also induces an aggravation of pain in a chronic neuropathic pain model. These results are clinically relevant since opioid drugs are used in patients struggling with moderate and severe chronic pain [17, 31, 32].

For the assessment of evoked pain, the pin-prick and the acetone test, respectively, showed that chronic administration of morphine led to an increase of mechanical hyperalgesia and cold allodynia. This increase of pain was not observed in the von-Frey test probably due to technical issues. Indeed, due to neuropathic pain, animals respond to the lowest microfilament available (0,008 g) at the baseline, therefore it was impossible to observe further decreases of the mechanical threshold. However, the mechanical effects on pain were still confirmed through the pin-prick test, as referred above.

With nonverbal animals, the assessment of spontaneous pain is a hurdle. In the past years, the CPP test, which was traditionally used to test the rewarding effects of drugs [84-86], has been used for the assessment of ongoing pain both in inflammatory [87, 88] and in neuropathic pain models [89-91]. Relief of pain is rewarding in humans [92] and rats [93] and so, if the ongoing pain is altered, rats will react in consonance, during the evaluation of their preference. Our results show that chronic administration of morphine reversed the preference of the animals for the morphine-paired chamber with even a trend towards a light aversion. Although these experiments were performed with a small number of animals, they are indicative of a loss of the analgesic effects of morphine in animals chronically treated with morphine. Increasing the number of animals should reinforce these results. These behavioural results combined with an increase of evoked-pain responses show that chronic administration of morphine results in loss of its analgesic effect leading to an increase of pro-nociception.

Activation of the c-Fos proto-oncogene in the SNI model was shown to require specific stimulus [94-96]. However, our results show that, even without stimulus, there were elevated levels of Fos-IR cells in animals treated with morphine as well as animals treated with saline and both at the spinal dorsal horn ipsilateral and contralateral to SNI surgery. A slight tendency for an increase of Fos-IR cells was observed in animals chronically treated with morphine, however, since control animals also display elevated values, these results probably result from unspecific staining of Fos-IR cells.

2 INVOLVEMENT OF THE DRT FACILITATION IN OIH

2.1 EFFECTS OF MOR KNOCK DOWN AT THE DRT DURING CHRONIC MORPHINE ADMINISTRATION

The animals injected with the lentiviral vectors recovered well from the surgery and regained weight progressively, in a manner similar to animals injected with neuronal tracers [24, 25] or subjected to cannula implantation [30] in the DRt, which indicates that the vectors did not affect the general animal well-being. The exploratory activity of the animals was normal which further indicates that lentiviral-mediated gene transfer did not interfere with these physiological functions.

EGFP expression from the control vector was confined to the DRt which indicates that lentiviral-mediated gene transfer was restricted to the DRt. The labelling of transduced cells showed a neuronal morphology indicating that gene transfer was targeted to

neurons. This is due to the use of the human synapsin promoter whose activity is restricted to neurons [97, 98]. Previous studies from our group (unpublished data) showed that the vector used for this study decreases MOR expression at the DRt. Several animal groups, during the evoked pain assessment, showed augmented withdrawal duration at day 2, however we hypothesize that the increase of pain observed at this time point results from the severe trauma caused by the stereotaxic injections.

By down-regulating MOR expression at the DRt, in animals treated with saline, we showed an increase of pain sensibility, which indicates that MOR plays an inhibitory role at the DRt. These results confirm previous reports on the inhibitory effects of the opioidergic modulation of the DRt [35, 61].

We also show that, knock-down of MOR expression at the DRt, during chronic morphine exposure, prevents opioid-induced hyperalgesia. In this sense, MOR presents opposing actions at the DRt, since in this case, MOR appears to modulate an increase in pain facilitation at the DRt. There are reported evidence of this switch of MOR signalling both *in vitro* [99-102] and at the locus coeruleus both *ex-vivo* [103] and *in vivo* [104] and at nucleus acumbens [104]. Usually MOR exerts a inhibitory action by inhibiting adenylate cyclase (AC) activity, causing activation of Kir3 K⁺ channels and inhibition of the voltage-dependent Ca²⁺ channels, leading to hyperpolarization of the cell [99, 104, 105]. However, the verified effect of MOR facilitation is consonant with the literature, where it is referred that prolonged exposure to opioids may change the normal signalling pathway. This alteration may be explained by a switch on the G-protein coupled with this receptor, from G_{i/o}-protein to G_s-protein, leading to an increase in AC, which causes augmented levels of cyclic adenosine monophosphate (cAMP), altering the hyperpolarized state of the neuron by changing the intracellular concentrations of Ca²⁺ and K⁺ [100, 101, 106, 107]. Ca²⁺ and/or cAMP will phosphorylate cAMP response element-binding protein (CREB) which is known to be critical for a variety of adaptive neuronal changes [103, 104, 108].

Results from an ongoing thesis in our group, show that lidocaine administered directly into the DRt, prevents OIH in naïve animals after chronic administration of morphine, which shows that the DRt is involved in descending facilitation during OIH. The results obtained in the present thesis demonstrate that the involvement of the DRt is mediated via the opioidergic system, namely MOR.

2.2 EFFECTS OF CHRONIC MORPHINE ADMINISTRATION ON GLIAL ACTIVATION AT THE DRt

Several studies report the fundamental role of glia in OIH in the spinal cord [109, 110], therefore we evaluated the role of these cells at the DRt. Our results showed an increase of the microglia marker Iba-1 and the astrocytes marker GFAP. Regarding microglia, we also performed a morphological analysis to evaluate morphological alterations, which complements the study of activation of these cells. However, the number of DRt sections analyzed must be augmented in order to seek for statistically relevant conclusions. Even though, we could already observe an increase in the rate of amoeboid cells, the most activated morphological state of microglia, in animals chronically treated with morphine. Activation of glia at the DRt is likely to be important in OIH and so it should be further studied. For instance, studies of co-localization of activation markers of these cells with p-p38 or pERKs should be performed to ascertain glial activation, downstream in the signalling pathway, and inhibition of glia at the DRt should be behaviourally evaluated.

3 CONCLUSIONS AND FUTURE PERSPECTIVES

Opioids are a common therapy for chronic pain and unravelling the molecular mechanisms involved in OIH is fundamental since, instead of relieving pain, these drugs may be responsible for hyperalgesia, in some patients. Unveiling the molecular mechanisms behind this condition is the key to overcome its side effects, namely the loss of their analgesic effect after chronic administration. In the future, it would be interesting to continue the studies of this thesis by using another analgesic drug combined with chronic administration of morphine could to confirm the loss of analgesic effect we observed during acute morphine administration at the CPP test.

Regarding the involvement of DRt in OIH, we show the effects of lentiviral injection at the DRt on evoked pain during chronic morphine treatment. The effects of the lentiviral injection at the DRt on spontaneous pain could also be assessed. To study how the facilitatory effects of opioids at the DRt are influenced by glia during chronic neuropathic pain and chronic morphine exposure, it would be interesting to inhibit glial cells at the DRt and assess evoked and spontaneous pain. Work from our group (data not published) has showed that DRt is involved in pain facilitation during OIH in an acute

pain model. The involvement of glia in this and in other pain models could also be interesting to further study.

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Appendix A: Composition of solutions

1. PHOSPHATE BUFFER SALINE (PBS) (1L)

Phosphate buffer (PB) 0,1M pH=7,2:

$\text{Na}_2\text{H}_2\text{PO}_4\text{H}_2\text{O}$ – 15,60g

K_2HPO_4 – 17,4g

H_2O up to 1L

PBS:

PB 250 ml

H_2O up to 1L

NaCl - 9g

2. PHOSPHATE BUFFER SALINE WITH TRITON X-100 (PBS-T)

PBS - 996ml

Triton X-100 - 4ml

3. TYRODE'S SOLUTION (1L)

NaCl – 6,8 g

KCl – 0,40g

$\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ – 0,32 g

$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ – 0,1 g

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ – 0,17 g

Glucose 1 g

NaHCO_3 – 2,2 g

H_2O up to 1L

4. CRYOPROTECTOR SOLUTION (1L)

PB 0,1M pH=7,2 - 125 ml

H_2O - 375 ml

Sucrose - 300g

Ethylene glycol - 300ml

PB 0,1M pH=7,2 up to 1L

5. THIONIN STAINING

1. Solutions

Acid acetone: acetone / acetic acid (4 vol / 1 vol)

0,1% thionin in 10% formalin

2. Protocol

Incubate the slides in acid acetone for 5 min;

Rinse with distilled water;

Stain in formol-thionin for 1 minute;

Rinse with distilled water;

Dry at 37 ° C;

Dehydrate in xylene for 5 min;

Mount with Eukitt.