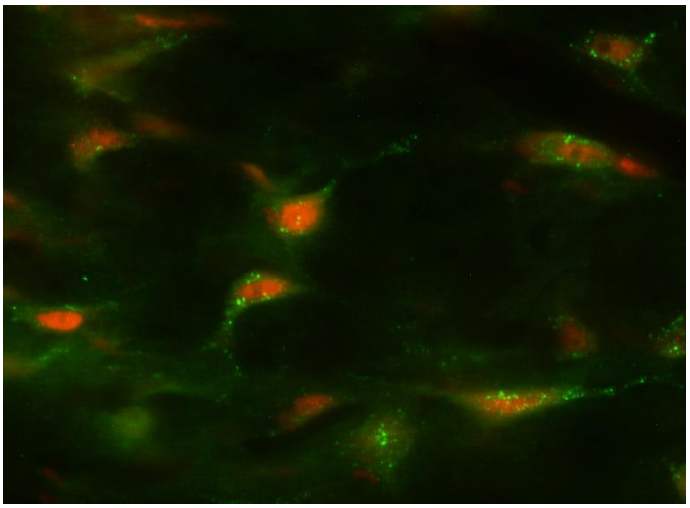

Unravelling the role of a pain facilitatory area of the brain during chronic opioid exposure

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Abstract

Opiates represent the most commonly used drugs for the treatment of moderate-to-severe postoperative and chronic pain. Chronic use of opioids can induce paradoxical hyperalgesia **(opioid-induced hyperalgesia; OIH)**. OIH is characterized by hypersensitivity to innocuous or noxious stimuli during sustained opiate administration, and is reported both in clinical and pre-clinical settings but its molecular mechanisms are not fully understood. One of the mechanisms that contribute to OIH is the activation of brain areas involved in pain facilitation. Here we studied the involvement of an area located in the medulla oblongata, the **dorsal reticular nucleus (DRt)**, which plays a unique and exclusive pain facilitatory role. The studies included in the present thesis aimed at ***i)*** determining whether chronic administration of morphine induces OIH in acute pain and the spared nerve injury (SNI) model of chronic neuropathic pain; ***ii)*** study the effects of chronic morphine on morphine reward and ***iii)*** evaluate the involvement of the DRt in the mediation of OIH and morphine reward.

To determine the effects of chronic morphine administration the animals were implanted with osmotic mini-pumps filled with morphine (45 $\mu g.\mu l^{-1}.h^{-1}$) or saline, which released their content continuously for 7 days. The effects of chronic morphine administration on pain behavior were tested before and at 2, 4 and 7 days after the mini-pump implantation. In naïve animals pain behavior was tested by the von-Frey and hotplate test which evaluate mechanical allodynia and thermal hyperalgesia respectively. Pain assessment in SNI animals was performed by the von-Frey test, the pin-prick test which evaluate changes in mechanical hyperalgesia and the acetone test to assess cold allodynia. The continuous infusion of morphine induced OIH in naïve animals and, for the first time, we show that chronic morphine administration induced OIH in an animal model of neuropathic pain.

To study the effects of chronic morphine on the reward behavior we used 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 reward effect of morphine.

To study the involvement of the DRt in the mediation of OIH, we first inactivated the DRt with lidocaine (0.5 µl; 4% w/v) in naïve animals. The injection of lidocaine was performed on day 7 after implantation of the osmotic pumps containing saline or morphine (45 $\mu g.\mu l^{-1}.h^{-1}$) and its effects were tested by behavioral tests mentioned above. The administration of lidocaine at the DRt fully reversed mechanical allodynia and thermal hyperalgesia in morphine-infused animals. Then we studied the

expression of the phosphorylated cAMP response element-binding protein (pCREB) and μ-opioid receptor (MOR), at the DRt, by immunohistochemistry. We show that chronic morphine treatment induces an increase of pCREB and MOR expression and that MOR immunoreactive cells co-localized with pCREB. Finally, we performed a lentiviral-mediated knock-down of the expression of MOR at the DRt. For that, the animals were stereotaxically injected with lentiviral vectors at the DRt and implanted with osmotic mini-pumps containing saline or morphine (45 $\mu g.\mu l^{-1}.h^{-1}$). The animals were tested before and 7 days after the lentiviral injections and mini-pump implantation by the behavioural tests mentioned above. The knock-down of MOR in control animals showed an increase of pain behaviours. In animals chronically treated with morphine, the knock-down of MOR prevented the development of OIH. We also evaluated the effects of MOR knock-down to assess the involvement of the DRt in morphine reward during chronic morphine exposure. Our preliminary results show that MOR knock-down results in a reversion of the loss of morphine reward.

Our results indicate that chronic morphine exposure induces OIH in naïve and neuropathic animals and, that the DRt is involved in the mediation of OIH, likely through MOR activation whose effects appear switch from inhibitors to facilitation upon chronic morphine. Our results also indicate that chronic morphine treatment induces a loss of morphine reward and that the DRt might also be involved in the mediation of such effects through MOR activation. Given the increase in the expression of pCREB at the DRt, it would be interesting to explore the involvement of this transcription factor in pain transmission from the DRt, during opioid-induced hyperalgesia. It would also be interesting to explore the interactions between the DRt and brain areas involved both in pain and reward on morphine reward.

Keywords: Opioid-induced hyperalgesia; Dorsal reticular nucleus**;** μ-opioid receptor; Reward

Resumo

Os opióides representam um dos tratamentos mais comuns tanto para o tratamento da dor pós-operativa como para o da dor crónica. O uso crónico de opióides promove um efeito paradoxal, a **hiperalgesia induzida por opióides (HIO)**. HIO é caracterizada por uma hipersensibilidade a estímulos inócuos ou nocivos durante a administração crónica de opióides, relatada tanto em ambientes clínicos como pré-clínicos mas os mecanismos moleculares subjacentes não estão ainda totalmente elucidades. Um dos mecanismos que contribui para a HIO é a activação de áreas supra-espinhais envolvidas na facilitação da dor. Neste trabalho estudou-se o envolvimento de uma área localizada no bolbo raquidiano, o **núcleo reticular dorsal (DRt)**, que desempenha um papel exclusivo na facilitação da dor. Os estudos incluídos na presente tese visam a ***i)*** determinar se a administração crónica de morfina induz HIO em modelos de dor aguda ou de lesão do nervo ciático, (o modelo "spared nerve injury"- SNI), um modelo de dor crónica neuropática; ***ii)*** estudar os efeitos da exposição crónica a morfina sobre os efeitos de recompensa da morfina e ***iii)*** avaliar o envolvimento do DRt na mediação da HIO e nos efeitos de recompensa da morfina.

Para determinar os efeitos da administração crónica de morfina, os animais foram implantados com mini-bombas osmóticas contendo morfina (45 $\mu g.\mu l^{-1}.h^{-1}$) ou soro, que permite a libertação contínua do seu conteúdo, durante 7 dias. Os efeitos da administração crónica de morfina no comportamento nociceptivo foram testados antes e aos 2, 4 e 7 dias após a implantação das mini-bombas. Em animais naïve o comportamento nociceptivo foi testado pelo teste de von-Frey e pelo teste de Hotplate que avaliam alodinia mecânica e hiperalgesia térmica, respectivamente. O comportamento nociceptivo em animais SNI foi realizado através do teste de von Frey, o teste de pin-prick que avalia hiperalgesia mecânica e pelo teste da acetona que avalia alodinia ao frio. A infusão contínua de morfina induziu HIO em animais naïve e, pela primeira vez, foi mostrado que a administração contínua de morfina induz HIO num modelo animal de dor neuropática.

Para estudar os efeitos do tratamento crónico com morfina no comportamento de recompensa foi utilizado o teste de "conditioning place preference" (CPP). Em animais cronicamente tratados com morfina, a administração aguda de morfina não induziu CPP, ao contrário dos animais controlo, o que indica uma perda do efeito de recompensa da morfina.

Para estudar o envolvimento do DRt na mediação da HIO, primeiro inactivou-se o DRt com lidocaína (0,5 mL; 4% m/v) em animais naïve. A injecção de lidocaína foi realizada no sétimo dia após a implantação das bombas osmóticas contendo morfina (45 $\mu g.\mu l^{-1}.h^{-1}$) ou soro e, os seus efeitos foram testados pelos testes comportamentais já mencionados. A administração de lidocaína na DRt reverteu totalmente a alodinia mecânica e hiperalgesia térmica nos animais tratados com morfina. Em seguida, estudou-se a expressão da proteína de ligação ao elemento de resposta ao AMP cíclico (pCREB) e do receptor μ-opióide (MOR), no DRt, por imuno-histoquímica. Mostrou-se que o tratamento crónico com morfina induziu um aumento da expressão de pCREB e MOR e as células marcadas com MOR apresentam uma percentagem elevada de co-localização com pCREB. Por fim, foi realizada uma diminuição da expressão, "knock-down", de MOR através de vetores lentivíricos no DRt. Para isso, os animais foram injetados com vectores no DRt e implantados com mini-bombas osmóticas contendo morfina (45 $\mu g.\mu l^{-1}.h^{-1}$) ou soro. Os animais foram testados antes e 7 dias após a injecção com os lentivírus e implantação de mini-bombas osmóticas, pelos testes comportamentais já mencionados. A diminuição de expressão de MOR em animais de controlo, induziu um aumento da sensibilidade à dor. Em animais tratados cronicamente com a morfina, o knock-down de MOR impediu o desenvolvimento de OIH. Também foram avaliados os efeitos do knock-down de MOR no DRt nos efeitos de recompensa da morfina durante a exposição crónica de morfina. Os resultados preliminares demonstram que a diminuição de expressão de MOR resulta numa pequena reversão da perda do efeito de recompensa da morfina.

Os resultados obtidos indicam que a exposição crónica à morfina induz HIO em animais naïve e neuropáticos e, que o DRt está envolvido na mediação de HIO, provavelmente através da ativação de MOR cujos efeitos parecem mudar de inibidores para facilitadores após a exposição prolongada de morfina. Os resultados obtidos também indicam que o tratamento crónico com morfina induz uma perda do efeito de recompensa da morfina e que o DRt também pode estar envolvido na mediação desses efeitos, através da ativação de MOR. Dado o aumento da expressão de pCREB no DRt, seria interessante no futuro explorar o envolvimento deste factor de transcrição na transmissão da dor a partir do DRt, durante a hiperalgesia induzida por opióides. Também seria interessante explorar as interações entre o DRt e as áreas cerebrais envolvidas tanto na dor como no efeito de recompensa da morfina.

Palavras-chave: Hiperalgesia induzida por opióides; Núcleo reticular dorsal; Recetor μ-opióide; Efeito de recompensa;

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Abbreviations

AC: Adenylate cyclase

ACC: Anterior cingulated cortex

AMY: Amygdala

AP: Anterior-posterior

Ca 2+-CaM: Calcium-calmodulin

CaMK: Ca^{2+}-calmodulin-dependent kinases

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

DOR: δ-opioid receptor

DRt: Dorsal reticular nucleus

DV: Dorso-ventral

EGFP: Enhanced green fluorescent protein

GAD: Glutamate decarboxylase

G-protein: Guanine-nucleotide binding protein

GDP: guanosine diphosphate

GTP: guanosine triphosphate

H: Height

HSV-1: Herpes-simplex vírus-1

hSYN-1p: Human synapsin promoter

Hyp: Hypothalamus

i.p: Intraperitoneal injection

IASP: Internacional Association for the Study of Pain

Ins: Insular cortex

IR: Immunoreactive

IRES: Internal ribosome entry site

KOR: κ-opioid receptor

L: length

LC: Locus coeruleus

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

NAc: Nucleus accumbens

NMDA: N-methyl-D-aspartate

NO: Nitric oxide

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

pCREB: phosphorylated cAMP response element binding protein

PFC: Prefrontal cortex

PI3K: Phosphotidylinositol-3-kinase

PKA: Protein kinase A

PKC: Protein kinase C

PNS: Peripheral nervous system

RNAi: RNA interference

RVM: Rostral ventromedial medula

s.c: Subcutaneous injection

Sol: Nucleus tractus solitaries

SNI: Spared nerve injury

Som: Somatosensory cortex

Sp5C: Spinal trigeminal nucleus, pars caudalis

TU: Transducing units

VLM: Caudal ventromedial medulla

VTA: Ventral tegmental area

W: Width

WAH: Withdrawl-associated hyperalgesia

WPRE: Woodchuck hepatitis virus post-transcriptional regulatory element

Introduction

1. Pain

1.1 Pain definition

According to the International Association for the Study of Pain (IASP), pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage [1]. Acute pain has a protective role. The capacity to experience pain alert us of imminent or actual tissue damage and leads to a behavioural response to minimize negative outcomes. On the other hand, persistent pain syndromes offer no biological advantage. Chronic pain persists beyond the expected normal time for healing, 3-6 months, and has no physiological purpose [2].

Pain can be considered a high plasticity process that leads to several changes in the neural structure and some of those changes are so drastic, specially chronic pain, that pain cannot be considered just a symptom but, instead, it should be considered as a pathological state [3].

Chronic pain is a major healthcare problem in Europe, it affects approximately 20% of the adult population, particularly women and elderly [4]. In Portugal it is estimated that 30 % of the population suffers from chronic pain [5]. Chronic pain may be inflammatory, neuropathic or functional and all forms share some common characteristics [3]. Inflammatory pain is caused by tissue damage occurring mainly after trauma, surgery or during chronic inflammatory diseases, having damaged and inflammatory cells recruited to the damaged tissue that release activators of peripheral nociceptors [3, 6]. Neuropathic pain is defined by IASP as a direct consequence of a lesion or disease affecting the somatosensory system [1], in other words, it is classified as an association of spontaneous pain and hypersensitivity with pathological changes in the peripheral nervous system (PNS) or in the central nervous system (CNS) [3]. Functional pain is a relatively new concept and is defined as pain sensitivity caused by an abnormal processing or function of the CNS in response to normal stimuli and may occur in fibromyalgia and irritable bowel syndrome [3].

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 [3, 7]. Primary afferent axons can be categorized by their peripheral targets, conduction velocity, response properties and neurochemical phenotype [8]. Aδ-fibers are characterized as medium cell bodies, thinly myelinated fibers and conduct at intermediate velocities; Aβ-fibers have larger cell bodies and are heavily myelinated; C-fibers have small cell bodies, unmyelinated fibers and conduct action potentials slowly [3, 5, 8, 9]. C- and Aδ-fibers are able to encode noxious chemical, thermal and mechanical stimuli and, for this reason, are considered the main nociceptive afferents signaling pain [9].

Primary afferent neurons convert noxious stimuli into electrical activity in peripheral terminals, causing depolarization of the neuronal membrane. If the stimuli are translated into a sufficiently intense electrical signal, voltage gated sodium channels will be activated generating the transmission of stimuli to central terminals of nociceptors in the spinal cord. The impulses generated in the dorsal horn travel through second order neurons, which constitute the ascending pathways to thalamus and brainstem where information is processed and pain is perceived, resulting in an appropriate response, transported by descending pathways to the spinal dorsal horn (Figure 1) [3, 10].

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, a region usually considered to process information relevant to the aversive properties of pain experience [11]. From these brainstem and thalamic loci, information reaches cortical structures [11, 12]. There is no single brain area essential for pain. Rather, pain results from activation of a group of structures, some of which are more related 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 well understood [9].

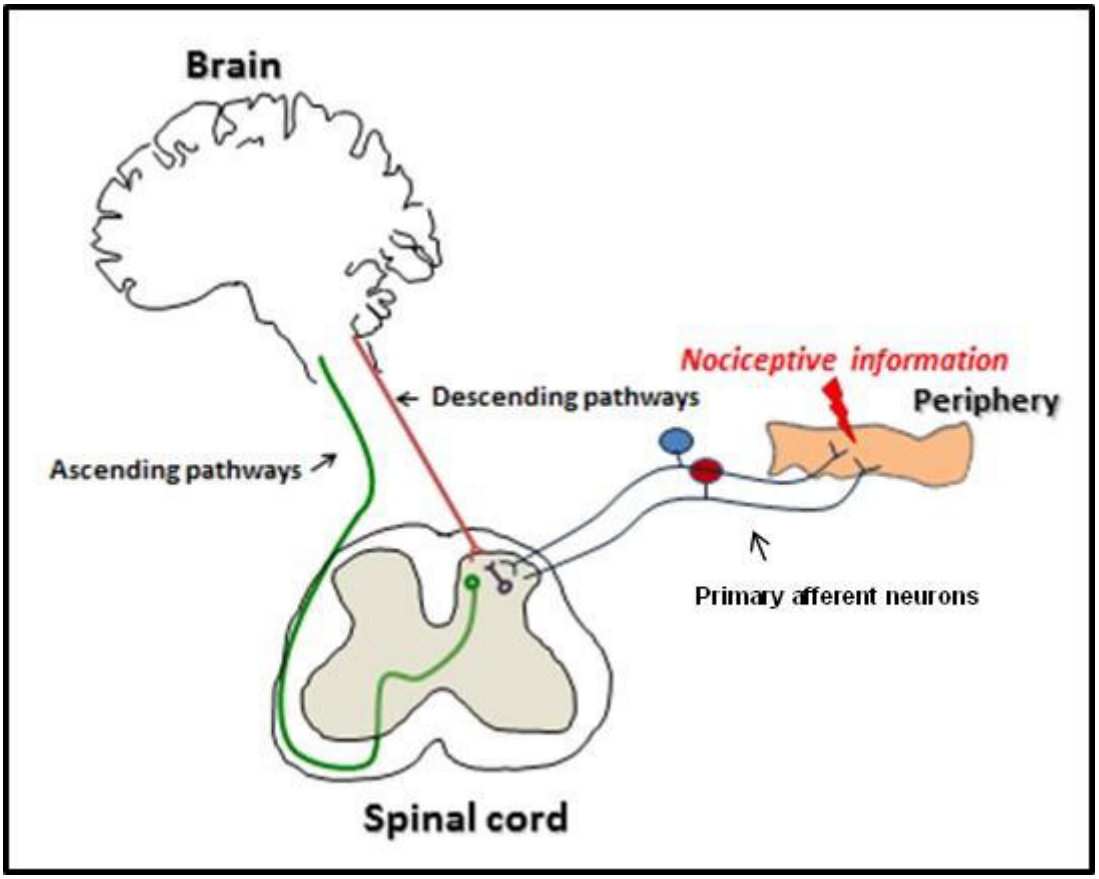


Figure 1: Transmission of nociceptive information. Nociceptive information is carried by primary afferent neurons from the periphery to the spinal cord and then reaches the brainstem through ascending pathways constituted by second order neurons. In the brain nociceptive information is evaluated and an appropriate response is generated and conveyed by descending pathways to the dorsal horn of spinal cord. *Adapted from Tavares & Martins [13].*

1.3 Descending pain modulation

1.3.1 The endogenous pain control system

The endogenous pain control system is a complex web of brain areas responsible for modulating pain transmission at the spinal cord. It is involved in pain inhibition and, more recently was discovered to be also involved in pain facilitation [14, 15].

Several supraspinal sites play an important role in pain modulation but, the most well characterized pain modulatory areas are the mesencephalic periaqueductal grey (PAG) and the rostral ventromedial medulla (RVM) (Figure 2) [16].

The PAG is connected with the hypothalamus and limbic forebrain structures including the amygdala, and also receives direct spinomesencephalic input. The PAG is also connected with the RVM, which in turn sends its output to the spinal dorsal horn [15]. The RVM is the final common relay in descending modulation of nociception from the PAG, since the PAG does not project directly to the spinal cord, and other supraspinal sites (Figure 2 and 4) [14, 15]. The RVM is constituted by the nucleus raphe magnus and adjacent reticular formation and projects to superficial layers of dorsal horn laminae and to deep dorsal horn [15]. In this area there are two distinct types of cells classified as ON- and OFF- cells, which exert facilitatory and inhibitory effects, respectively, on nociception. This two distinct populations of neurons 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 [17-19].

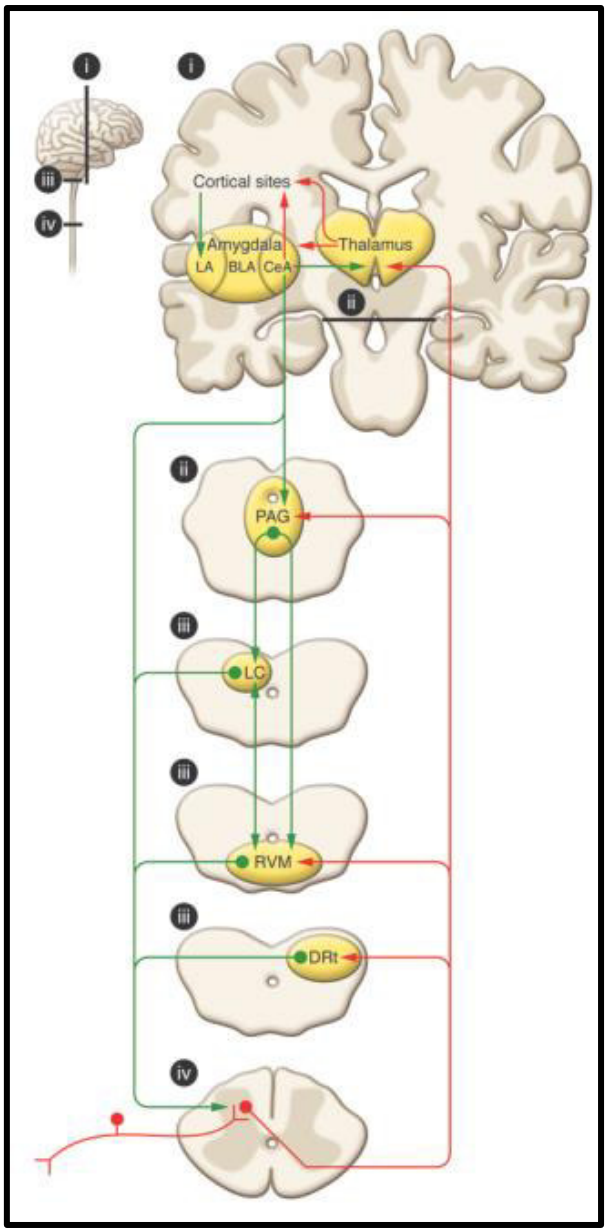


Figure 2: Schematic representation of the pain modularity circuitry. Primary afferent neurons convey nociceptive inputs to the spinal dorsal horn. From the dorsal horn there are ascending projections (labelled in red) targeting the thalamus, the DRt, the RVM and the PAG. The thalamus is connected to 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; RVM- rostral ventromedial medulla ; PAG- mesencephalic periaqueductal grey; LC- locus coeruleus. Adapted from Ossipov et al [14]

1.3.2 The dorsal reticular nucleus

The dorsal reticular nucleus (DRt) belongs to the endogenous pain control system and deserves special attention since this area will be 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].This area is located medially to the spinal trigeminal nucleus, pars caudalis (Sp5C), laterally to the nucleus tractus solitaries (Sol), ventral to the nucleus cuneate (Cu) and dorsal to the ventral reticular nucleus (VRt) (Figure 3)[20].

DRt neurons are exclusively activated by cutaneous or visceral noxious stimulation conveyed by Aδ- and C-fibers from the full body [20-23]. Glutamate administration in the DRt induces a long-lasting increase in the responsiveness of spinal nociceptive neurons [24], while lidocaine administration in the DRt results in the suppression of responsiveness [20]. At the behavioral level, the DRt was shown to be involved in pain facilitation both in acute and chronic pain models [25-27]. Recently, it was found that the facilitatory effects of the DRt, in inflammatory and neuropathic pain models, were mediated by noradrenaline release at the DRt [28, 29].

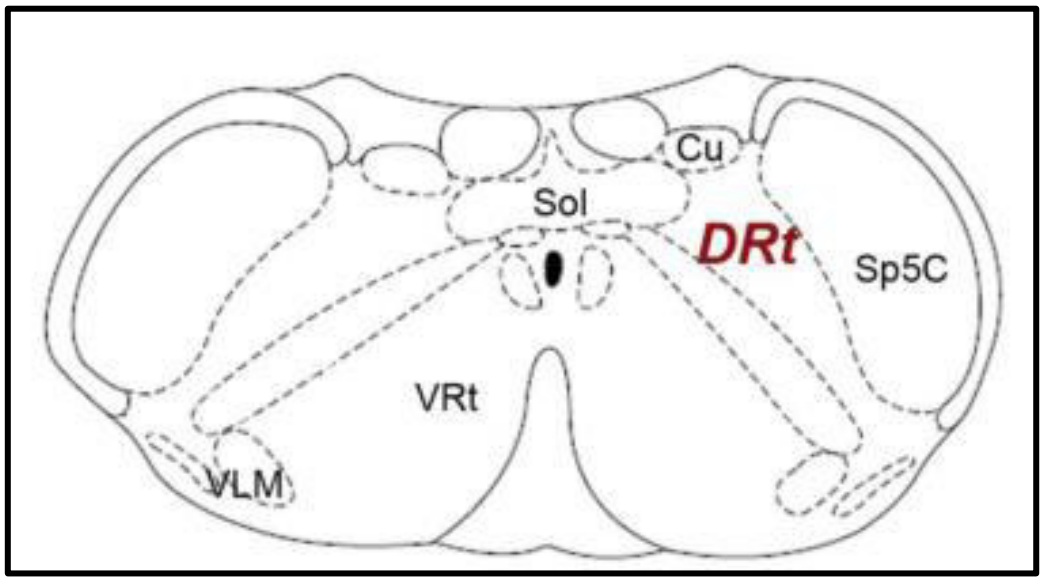


Figure 3: Diagram of a coronal section of the caudal medulla oblongata. Abbreviations: DRt-Dorsal reticular nucleus; Cu-Nucleus cuneate; Sol-Nucleus tractus solitaries; Sp5C-Spinal trigeminal nucleus, pars caudalis; VLM-Caudal ventromedial medulla; VRt-Ventral reticular nucleus. Adapted from Paxinos and Watson [30].

The DRt receives 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[20].

The DRt has connections with several brainstem areas such the ventrolateral medulla (VLM), PAG, RVM, locus coeruleus and the A5 and A7 noradrenergic cell groups. The DRt also projects to medial thalamus and the limbic system, which suggests an integration of DRt activity with emotional aspects of pain processing [16, 23]. Furthermore, the DRt is connected with the extrapyramidal and orofacial motor system, which suggests an involvement of DRt in motor reactions associated with pain [23].

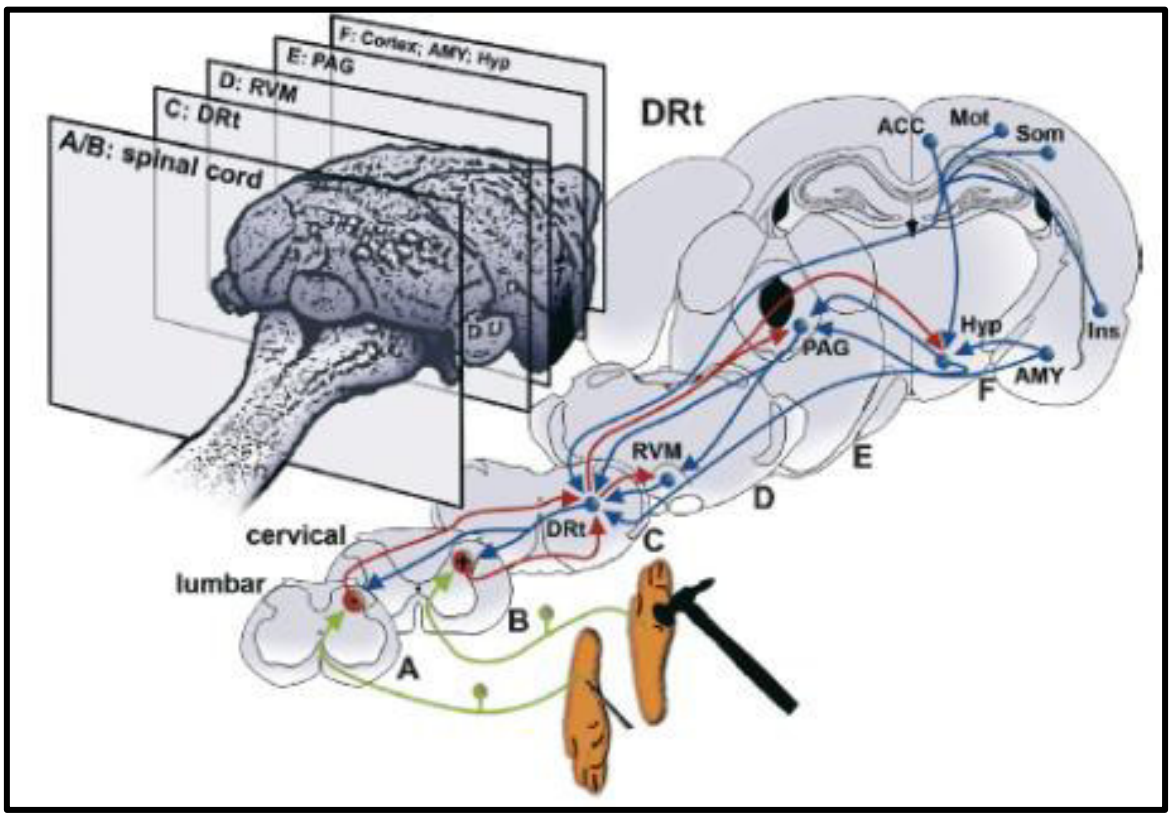


Figure 4: DRt involvement in pain modulating circuitries. Ascending connections are represented in red, descending projections are in blue and nociceptors are depicted in green. A–D are central nervous system sections and represent the spinal dorsal horn (A), the medulla oblongata and pons (B), the mesencephalon (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[23].

2. Opioids and pain

The Sumerians in Mesopotamia were among the earliest civilizations identified to have cultivated the poppy plant around 3400 BC [31]. Derived from opium poppy, opioids have been used for millennia for the treatment of moderate to severe pain [32, 33]. Opioids play a unique role in society. They are widely feared compounds, which are associated with addiction but they are also essential medications commonly used to treat postoperative, cancerous, and, more recently, chronic nonmalignant pain [19, 34] as consequence, opioids are among the most often prescribed drugs to treat pain [34].

2.1. Opioid receptors

Opioids activate peripheral, spinal and supraspinal opioid receptors. Currently there are four well-established groups of opioid receptors: μ (MOR), δ (DOR), κ (KOR) the and opioid receptor-like (ORL1) [18, 35, 36]. Subtypes of the receptors have been proposed. MOR is divided into two subtypes: MOR 1 mediates the analgesic and euphoric effects of opioids as well as the physical dependence and MOR 2 mediates the bradycardic and respiratory depressant effects. DOR, with two subtypes identified until now, DOR-1 and DOR-2, mediate spinal analgesic effects and have been associated to modulation of tolerance. The three KOR subtypes, KOR-1, KOR-2 and KOR-3, mediate spinal analgesia, miosis, sedation and diuresis [37].

The opioid receptors belong to the large family of seven-transmembrane G-protein-coupled receptors. The binding of opioids to the receptor results in a conformational change of the inhibitory Gi protein alternating from an inactive guanosine diphosphate (GDP) to an active guanosine triphosphate (GTP) which results in the activation of the α subunit of the G-protein. Once activated the α subunit dissociate from the β and γ subunits and binds to adenylate cyclase (AC) inhibiting it. As a consequence of the inhibition of AC the intracellular concentrations of cyclic adenosine monophosphate (cAMP) decrease, reducing phosphorylation and activation of multiple proteins resulting in decreased excitatory activity (Figure 5) [18, 35, 36].

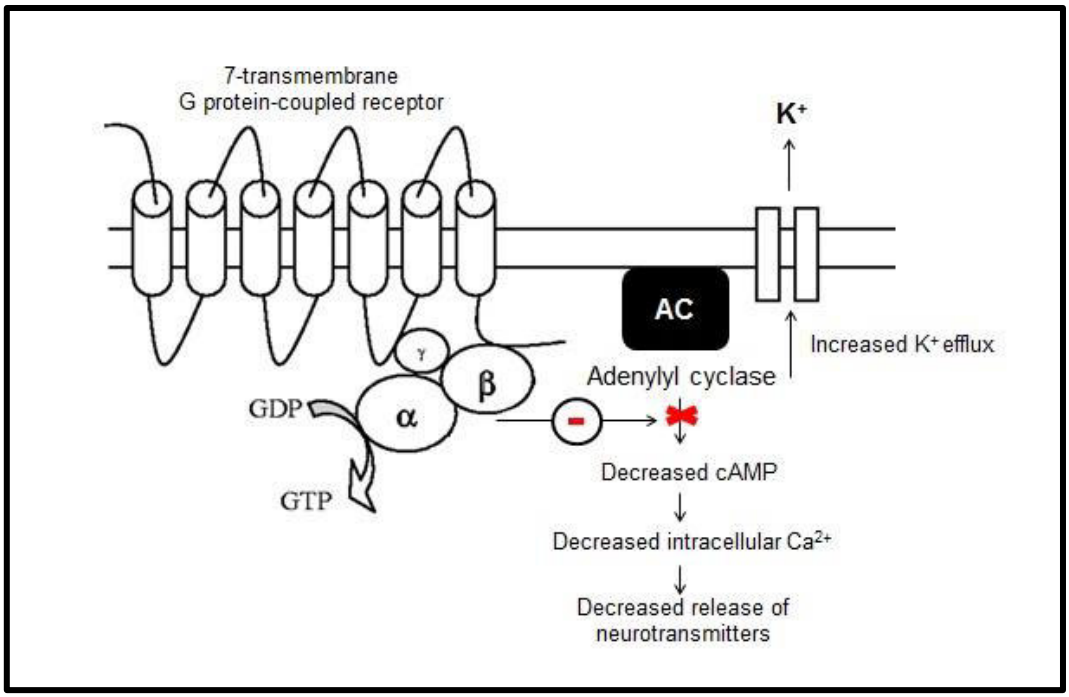


Figure 5: Seven transmembrane structure of opioid G-protein-coupled receptor. Receptor activation by opioid ligands leads to initiation of intracellular transduction pathways that include stimulation of potassium efflux, decrease of intracellular Ca^{2+} and inhibition of adenylyl cyclase resulting in decreased excitatory activity. Adapted from McDonald et al [38].

MOR presents the widest distribution in the brain and spinal cord, while DOR and KOR have a more restricted distribution. In peripheral tissues, opioid receptors are responsible for the modulation of several physiological functions including alterations during inflammation, analgesia and tolerance [36]. The ORL-1 has been detected in the amygdala septum, the hypothalamus, the thalamus, in the DRG and the spinal cord [39].

Investigations in pain have focused predominantly on MOR because its activation is essential for the action of the most powerful analgesics as morphine, oxycodone and hydrocodone [35]. The opioid receptors are expressed both on pre- and post-synaptic neurons in the CNS 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 [18, 35, 36]. Furthermore, MOR is expressed in the main brain areas associated to pain modulation, such as the insular cortex, amygdala, hypothalamus, PAG, RVM [35, 36], DRt [40] and are abundantly expressed in the limbic system which is associated the emotional perception of pain [36].

2.2. Effects of endogenous opioids in pain modulation

Opioid drugs act in peripheral, spinal, and supraspinal receptors which have endogenous ligands, known as endogenous opioid peptides [36].

Endogenous opioids, which are naturally produced in the organism are involved in the modulation of pain and also in other behavioural processes, such as reward, dependency, sedation and stress response [41]. There are three families of endogenous peptides that produce several active peptides: pro-opiomelanocortin that produce β-endorphin, proenkephalin that produce met- and leu-enkephalin peptides and prodynorphin that produced dynorphins and neo-endorphins (Table 1) [36].

Table 1: Endogenous opioid peptides and their receptors.

Precursor	Name	Receptor
Proenkephalin	Leu-enkephalin	δ and μ
Proenkephalin	Met-enkephalin	δ and μ
Pro-opiomelanocortin	β-Endorphin	μ and δ
Prodynorphin	Dynorphins	K
Unidentified	Endomorphin-1	μ
Unidentified	Endomorphin-2	μ
Pro-nociceptin/orphanin FQ	Nociceptin/orphanin FQ	ORL-1

Adapted from Ren and Dubner [42]

The opioid peptides β-endorphin, dynorphins and enkephalins are widely distributed throughout 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. Opioid peptides are also synthetized in nonneuronal cells, such as endocrine cells and cells of the immune system [41].

The enkephalins, activate mainly the DOR, while the dynorphins activate mainly the KOR (Table 1). The β–endorphin peptide can produce a response through all three receptors although this response is stronger when it acts through MOR and DOR (Table 1) [41]. Two additional peptides endomorphin-1 and -2, with no precursor for endogenous synthesis identified so far, bind with high affinity to MOR (Table 1) [36, 37, 41]. Also the endogenous opioid-like substance, nociceptin, is the product of a novel gene distinct from the gene families from which the classical endogenous opioids are derived (Table 1) [37]. In the CNS, opioids regulate nociceptive pathways both at spinal and supraspinal levels. At the spinal level, opioids inhibit nociceptive transmission conveyed by Aδ- and C-fibers [36].

Nonetheless, at the spinal cord, dynorphins have been associated with the development of hyperalgesia and allodynia since it increases the release of excitatory neurotransmitters, which contribute to intensify pain transmission [17, 36]. At the supraspinal level, opioid peptides inhibit ON-cells and disinhibit OFF-cells [35]. In the PAG, enkephalinergic neurons synapse with serotoninergic neurons in the RVM that project to the spinal cord inducing the release of enkephalins that produce inhibition of the activity of Aδ- and C- fibers entering the spinal cord [35, 36]. Noradrenergic cells from locus coeruleus projecting to the spinal dorsal horn are also regulated by the opioidergic system [35].

The DRt is under opioidergic modulation since it expresses MOR and DOR [40, 43]. As to the effects of opiods at this area the overexpression of proenkephalin at the DRt induced analgesia revealing thus that the effects of these opioid peptides inhibit DRt descending facilitacion of pain [44].

3. Opioid-induced hyperalgesia

3.1. Definition

It is well know that the use of opioids may be a double-edged sword [34].They provide straight analgesic and antihyperalgesic effects but, the knowledge that opioids might have pronociceptive effects might have been suspected as early as the American Civil War [45]. Opioids have several side effects such as the development of physical dependence, tolerance and addiction [46, 47]. Nowadays there is an increased number of evidences that opioids may cause another phenomenon often referred to as opioid-induced hyperalgesia (OIH) [45, 46]. This phenomenon is characterized by increased sensitivity to pain related to opioids exposure in the absence of disease progression or opioid withdrawal [34, 45-47].

OIH definition is often mistaken 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 [48]. Tolerance occurs when the patient seeks pain relief and increasing doses of opioids are necessary to maintain appropriate analgesia (Figure 6 B) [48, 49]. This definition could be confused with OIH, however, in opposition to tolerance, increasing doses of opioids will only worsen pain (Figure 6 A) [46].

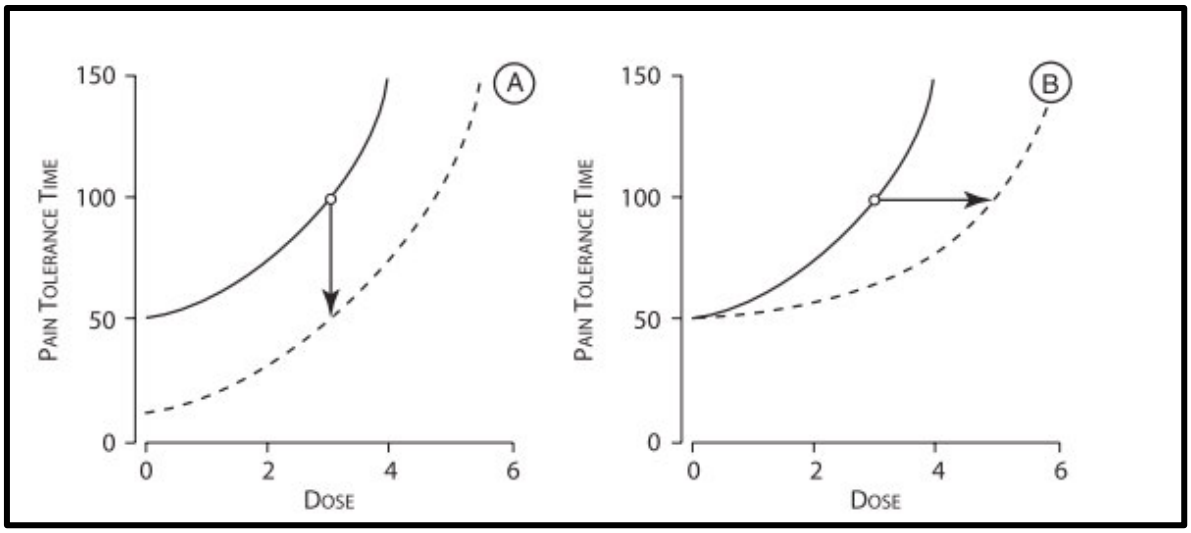


Figure 6: Alterations in opioid dose-response relationship with chronic opioid administration. It is a hypothetical experience, where an acute opioid infusion is used to detect changes in the analgesic dose-experimental pain response curve that occur as a result of chronic opioid exposure. The responses 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 [50].

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 [48].

3.2 Clinical and animal evidence

Several studies suggest that humans, as well as animals, treated with opioids can develop OIH. In humans the development of OIH already showed important clinical implications [34, 50]. Studies have been conducted using several distinct methodologies namely: former opioid addicts on methadone maintenance therapy; intraoperative exposure to opioids in patients undergoing surgery; healthy volunteers after acute opioid exposure; and prospective observational studies in opioid-naïve pain patients undergoing initiation of chronic opioid therapy [34, 50].

Diverse clinical studies have measured pain sensitivity in former opioid addicts, treated with methadone, and this set of patients are compatible with the hypothesis that OIH, when diagnosed, is caused by chronic opioid exposure [34, 51]. Evidences that patients exposure to higher doses of intraoperative opioids increased postoperative pain is also compatible with the view that OIH developed in these patients [34, 52]. There are also studies describing OIH in human volunteers after acute short-term

exposure to opioids and the results showed aggravation of induced hyperalgesic skin lesions, expansion of the area of mechanical hyperalgesia induced by transdermal electrical stimulation, aggravation of pressure-evoked pain or increased sensitivity to cold pressor pain in healthy human volunteers following precipitated opioid withdrawal after induction of acute physical opioid dependence [34, 50].

A few clinical studies also show evidences of the development of OIH. A small prospective study in which OIH was notable in 6 patients with chronic back pain, after one month of oral morphine treatment, when compared to baseline values [49, 53]. Another research, with a larger sample population, showed a significant negative correlation between experimental OIH and all clinical pain measures, in a dose dependent manner [53]. One additional prospective study, with indirect evidence of OIH, comes from 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 [53, 54].

The first time OIH was described in animals was 1971 [55] and now more than a hundred publications are available describing this phenomenon in an extensive diversity of animal models [50]. For more than three decades, it has been recognized that systemic exposure of opioids to rodents can lead to a hyperalgesic response after precipitating withdrawal with the administration of an opioid antagonist as well as during spontaneous withdrawal after cessation of opioid administration [34].

Chronic administration of opioids also was shown to cause a sustained pronociceptive response. In these experiments, OIH depended both on the dose of the opioid and on the experimental pain model (i.e. thermal, mechanical, electrical or chemical) [48]. 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 a transient hyperalgesic response which lasts for hours or for days in a dose dependent manner [34]. The second and most common pattern is observed after animals are exposed to opioids on a chronic time course for three to twelve days via repeated subcutaneous injections, implantation of subcutaneous opioid containing pellets or pumps, or intermittent administration or continuous infusions through indwelling intrathecal catheters. If animals were exposed to opioids by continuous techniques, antinoceptive 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 receives repeated systemic or

intrathecal boluses of opioids for several days, they progressively 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 [34].

3.3 Molecular mechanisms

The precise molecular mechanism of OIH are not yet well understood but, is thought to result from neuroplastic changes in the PNS and in the CNS resulting in the sensitization of pronociceptive pathways and it can been described based on the site of the plasticity [19], where the relevant mechanisms are probably unique [50]. Spinal cord plasticity underlying OIH has been demonstrated after both intraspinal and systemic administration of opioids [19, 34, 50]. The consequence of spinal sensitization is increased transmission of noxious inputs to supraspinal sites [17]. It is well accepted that repeated excitation of spinal cord neurons, along with persistent activation of the NMDA receptors, non-NMDA excitatory amino acid receptors, protein kinase C (PKC) [19, 34, 50], spinal dynorphin, spinal prostaglandins [34] and spinal cyclooxygenase (COX) [19] are involved in the sensitization of spinal neurons. 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 [19, 34, 50].

Regarding the molecular mechanisms underlying OIH, there are evidences suggesting that after morphine binding to MOR, on a post-signaling neuron, there is activation of G-protein mediated PKC translocation and the removal of the NMDA receptor Mg^{2+} plug (Figure 7 – item 1). Glutamate is released from pre-synaptic cells inducing the ionotropic NMDA receptor to allow Ca^{2+} influx, resulting in increased 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 7 – 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 [56]. There are also evidences suggesting that this process is not limited to neuronal cells and that glial cells also play an important part in OIH. Chronic opioid administration may act through MOR expressed 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 7 - 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 [56].

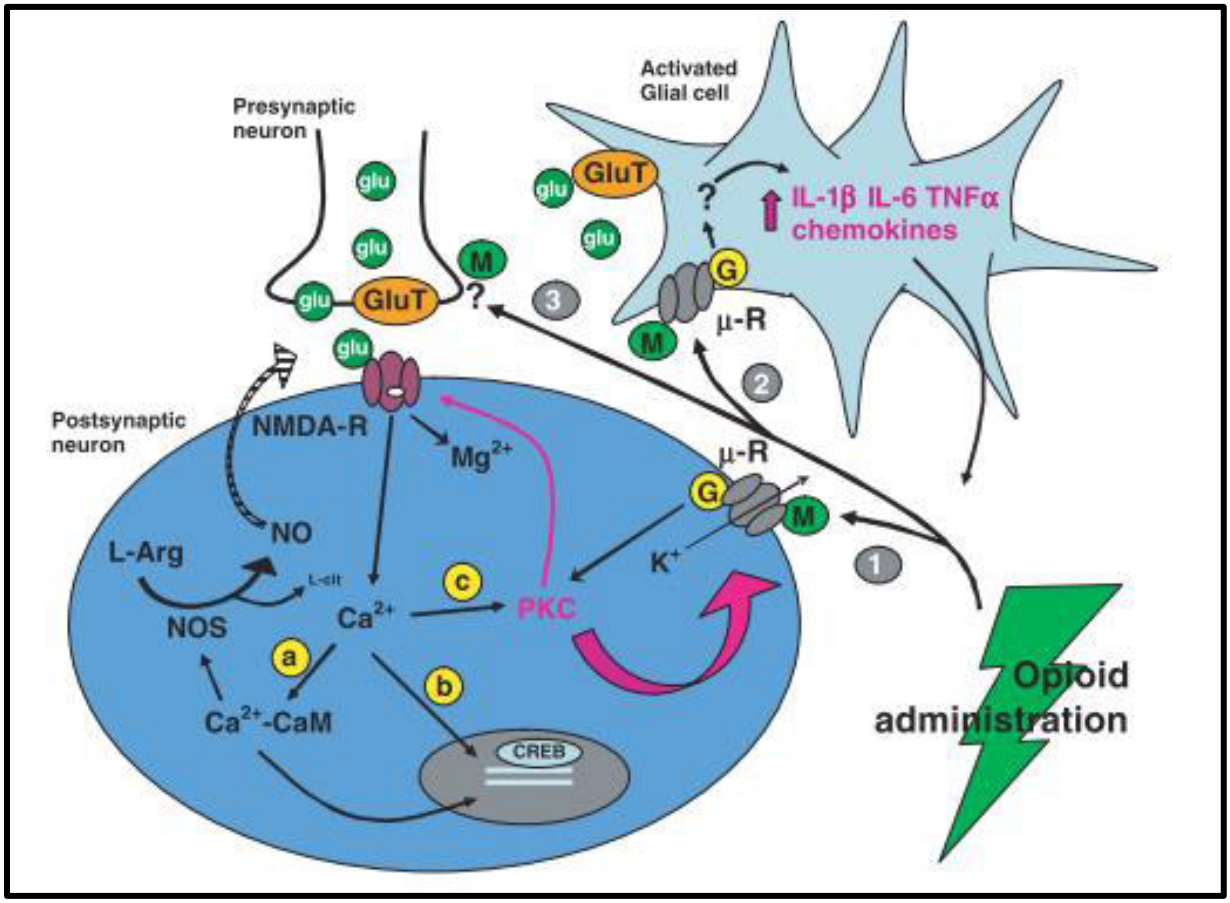


Figure 7: Cellular mechanisms of morphine hyperalgesia. Morphine (represented by M, as a representative opioid) may act on the post-synaptic neuron (1), on the glial cells (2) or on the pre-synaptic neuron (3). Regarding the post-synaptic neuron (1), it binds to the μ-receptor (μ-R) activates G-protein 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 [56].

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 [19, 34, 50].

3.4 cAMP response element binding protein (CREB) in OIH

CREB is a member of a superfamily of proteins that function as transcription factors and is expressed in all cells in the brain [57, 58]. Phosphorylation and successive activation of CREB is a site of convergence for several signal transduction cascades, including the cAMP pathway via protein kinase A (PKA), intracellular Ca^{2+} via Ca^{2+}-calmodulin-dependent kinases (CaMK), the Ras/ extracellular signal regulated kinase (ERK) protein kinase pathway, the phosphotidylinositol-3-kinase (PI3K)/ Akt kinase pathway, PKC pathways and stress-induced signaling cascades [58-60].

CREB is of particular interest since its activation is downstream of the cAMP signaling pathway, whose upregulation has been widely characterized as an adaptation to opioid chronic exposure [58]. Generally when morphine binds to MOR, adenylyl cyclase (AC) is inhibited and in consequence cAMP decrease and reduces the phosphorylation of CREB (pCREB) however, chronic opiate administration, increases levels of AC and pCREB implying a homeostatic or compensatory regulatory mechanism. This increased CREB activity appears to play an important role in physical opiate dependence and withdrawal [58, 61-63].

Several experimental evidences suggests that pCREB is involved in the opioid-induced effects in vitro and in vivo, beginning in cultured neuronal cell lines and extending to several brain areas [64]. In vitro it was shown that chronic administration of morphine increase pCREB in the coeruleus-like cell line [65]. In vivo it was shown that chronic administration of morphine increase pCREB in the locus coeruleus (LC) [58, 61, 66] or in the nucleus accumbens (NAc) [58, 67, 68]. Also mice containing targeted mutations on α and Δ isoforms of CREB gene showed attenuated physical symptoms of morphine withdrawal [58, 69]. The knockdown of CREB levels in the LC, using antisense oligonucleotides, blocks the capability of chronic opiates to upregulate some components of the cAMP pathway and consequently blocked some of the effects of morphine like physical dependence and withdrawal [62]. Similar results were observed when increasing or decreasing CREB levels in the LC using viral vectors [61].

A remaining mystery, however, is the exact mechanism which opiate exposure switches from acute inhibition of the cAMP pathway and CREB to chronic upregulation [61].

4. Opioids and reward

Reward is defined as a stimulus that brain interprets as intrinsically positive or as something to be approached [70]. Usually, rewards are conditionally learned based upon their positive influence on survival or reproduction [71, 72].

Pain and reward are opposite processes that can interact and influence each other. Some rewarding stimuli decrease pain sensitivity but on the other hand it has been proved that pain affect reward processing [73]. For example, chronic pain is associated with anhedonia, i.e. the incapacity to feel pleasure. Several brain systems are implicated in pain and reward processing such as the amydala, anterior and posterior insula, anterior cingulate cortex, dorsal and ventral striatum and the orbitofrontal cortex [73, 74].

Dopamine is a key neurotransmitter of reward [73, 75]. This catecholamine is released from the ventral tegmental area (VTA) that project mainly to the NAc and the prefrontal cortex (PFC) as a result of natural rewarding experiences, such as eating [71, 76] or in response to administration of drugs of abuse such as cocaine, amphetamine, opiates, nicotine and alcohol [71, 77, 78]. Although the dopaminergic system represents the cornerstone of the reward system other neurotransmitters, such as endogenous opioids, affect this mesolimbic dopaminergic system [72].

The opioid system represents an important substrate for the prejudicial effects of drugs of abuse. As a matter of fact activation of MOR reinforces the properties of countless abused drugs, which may be a potential molecular gateway to drug addiction [79, 80]. Van Ree et al [81] in 1980 was the first to demonstrate that rats self-administer an opioid receptor agonist into the VTA. Since then, several studies using intracerebral self-administration or conditioning place preference (CPP) in rats have confirmed the contribution of the VTA in opiate reinforcement [79, 82, 83]. More recently, genetic approaches using knockout animals have confirmed the role of opioid system in drug reinforcement and dependence [79]. Constitutive knockout mice for MOR, DOR and KOR have been used to study reward processes [70]. Several researches demonstrated an essential role of MOR in facilitating reward. Opiate reward studies in MOR knockout mice showed a loss of morphine reward on CPP as well as morphine self-administration test [70, 84, 85]. The DOR influence on reward is less evident. The analysis of DOR knockout animals showed a decreased morphine place preference but morphine self-administration was maintained, suggesting that this opioid receptor contribute to contextual learning rather than opioid reward [70, 79].

In addiction a large number of studies using KOR knockout mice confirm that this receptor negatively modulates reward, although this is not shown for all drugs of abuse, for example deletion of the KOR gene did not modify morphine CPP but in contrast reduced alcohol place preference [70, 79].

Chronic exposure to opioids, or another drugs of abuse enhance the activity of the cAMP–PKA pathway in NAc. Activation of PKA and subsequent phosphorylation of CREB within Nac reduces the rewarding effects of stimulant drugs, whereas PKA inhibition has the opposite effect [86]. Indeed, elevation of CREB levels within the rat NAc using viral vectors reduce the rewarding effects of morphine and cocaine and make low doses aversive, suggesting that CREB activity in this region can control reward qualities of drugs of abuse [57].

5. Genetic manipulation of the nociceptive system

Conventional drug treatment for pain has numerous limitations, such as drug dependence, tolerance, respiratory depression, and other systemic side effects [87]. The development of gene transfer has a possibility of using nonviral or viral vectors to transduce genes encoding antinociceptive substances to treat chronic pain and study the nociceptive system [87, 88].

An ideal delivery system would transduce cells with high efficiency, mediate high level and long-term expression, cause limited cytotoxicity, produce a small immune response in vivo and incorporate sufficient DNA so that transgenes of interest can be accommodated and enable regulated expression. These characteristics are difficult to achieve in a single vector system consequently, a variety of viral gene delivery systems have been developed, each with its own advantages and disadvantages [89]. Nonviral systems, like naked DNA or RNA, liposomes and nanoparticles, compared with viral vectors are less efficient since viral vectors are more capable of delivering exogenous genes to target cells and inducing long-term gene expression [87, 88].

In recent years, the development of selective genetic manipulation has largely enriched the understanding of molecular mechanisms of the descending pain modulatory system [13, 90]. 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 the nociceptive system [44, 91, 92]. All knowledge acquired on the mechanisms of pain, allowed to develop 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 [13]. Thereby, gene transfer allows the delivery or manipulation of genes with high specificity, avoiding side effects and off- target toxicity, mediating gene expression for a controlled and prolonged period of time [93]. 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 [13].
The vector is the carrier of the transcriptional cassette and its main function is to deliver its content to specific cell targets. 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 [13]. The most commonly used viral vectors for gene therapy for chronic pain are derived from the herpes-simplex virus (HSV-1), adeno-associated virus, adenovirus and lentivirus due to some characteristics, such as their low immunogenicity, natural integration ability and whether they can infect both dividing and nondividing cells [87, 91].

Lentiviral vectors belong to a subclass of retroviruses capable of inserting DNA into the host cell genome. They are interesting vectors due to their natural integration ability and tropism for non-dividing cells such as neurons. They have also been used for gene delivery in neural stem cells and progenitor cells [87].

The transgene is a coding sequence of a gene which can be fused with small unrelated sequences or even expressed under the same promoter with fluorescent proteins, so cells transfected with the transgene can be easily detected [93]. These coding sequences generally express antisense sequences or RNAi molecules in order to down-regulate gene expression [93], or neurotransmitters and receptors involved in pain transmission, neurotrophic factors and anti-inflammatory substances [13].

As for the promoter, cell-type specific promoters are preferred in order to restrict gene expression to a specific cell type. Synapsin I, calcium/calmodulin-dependent protein kinase II, tubulin alpha I and neuron-specific enolase are some examples of the promoters specifically targeting neurons [13].

Targeting brain circuits of pain is definitely challenging mainly because the access to brainstem areas is a great obstacle and the complex neuronal circuits are also difficult to manipulate. Gene transfer in the endogenous pain control system has been mainly achieved with HSV-1 vectors to express opioid peptides [92] glutamate decarboxylase (GAD) [94] and tyrosine hydroxylase [28] inducing analgesia in several pain models [13, 40, 44, 92].

Aims and methodology

The analgesic role of opioids for the treatment of chronic pain is 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 evidences 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.

The first goal of the present thesis was to determine the behavioural effects of chronic morphine administration in naïve animals and in a chronic pain model, the spared nerve injury (SNI), which is a model that presents substantial and prolonged changes in mechanical sensitivity and thermal responsiveness that mimic several features of clinical neuropathic pain [95]. First we assessed the effects of chronic administration of morphine on pain behaviors then on the reward effects of morphine. We used the von-Frey test by to evaluate mechanical allodynia and the hot plate test to verify changes in thermal hyperalgesia in naïve animals. Pain assessment in SNI animals was performed using the von-Frey test to assess mechanical allodynia, the pin-prick test to verify changes in mechanical hyperalgesia and the acetone test to study cold allodynia. To study reward behavior we used the conditioned place preference (CPP) test which is an established rodent paradigm of drug reward [96]. First, we performed the optimization of the different experimental conditions involved in the test. Then, we performed the CPP test to evaluate the effects of chronic morphine treatment in morphine reward.

The second aim of this thesis consisted on studying the involvement of the DRt in chronic morphine effects. For that, first we evaluated the effects of DRt inactivation by local injection of lidocaine on chronic morphine pain behavior, by the behavioural tests described above in naïve animals. Then we studied the effect of chronic morphine on the expression of the phosphorylated cAMP response element-binding protein (pCREB) and μ opioid receptor (MOR) by immunohistochemical detection, at the DRt. Additionally, we evaluated the effects of MOR knock-down at the DRt in naïve and SNI animals using a lentiviral vector. This vector was chosen since it does not undergo retrograde transport and , unlike other viral vectors, only transduce on local neurons [97]. We determined the effects of MOR knock-down on pain behaviour during chronic morphine administration. We also evaluated the effects of MOR knock-down to assess

the involvement of the DRt, in reward during chronic morphine administration. MOR knock-down was confirmed by immunohistochemical analysis.

Materials and Methods

1. Animals

Pathogen-free adult male Wistar rats (Charles River colony, France) were pair-housed in standard Plexiglas cages with free access to food and water. After stereotaxic injections, the animals were housed individually. The colony room was maintained at 22 ± 2°C on a standard 12/12h light/dark cycle. All experiments were conducted during the light phase. Upon arrival, rats were allowed five days of acclimation before any procedure. All procedures were performed in accordance with the European Community Council Directive (2010/63/EU) and the ethical guidelines for pain investigation (Zimmermann, 1983).

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-p), MOR cDNA in reverse orientation and the enhanced green fluorescent protein (EGFP) cDNA. The control vector (LV-Cont; Figure 8) only carries the EGFP transgene. The vectors further carry the encephalomyocarditis virus internal ribosome entry site (IRES) and transcriptional regulatory element (WPRE).

The lentiviral particles were produced by co-transfection of human embryonic kidney 293T cells with the lentiviral vectors, a packaging plasmid (pCMVΔR8.92), a plasmid encodingthe 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).

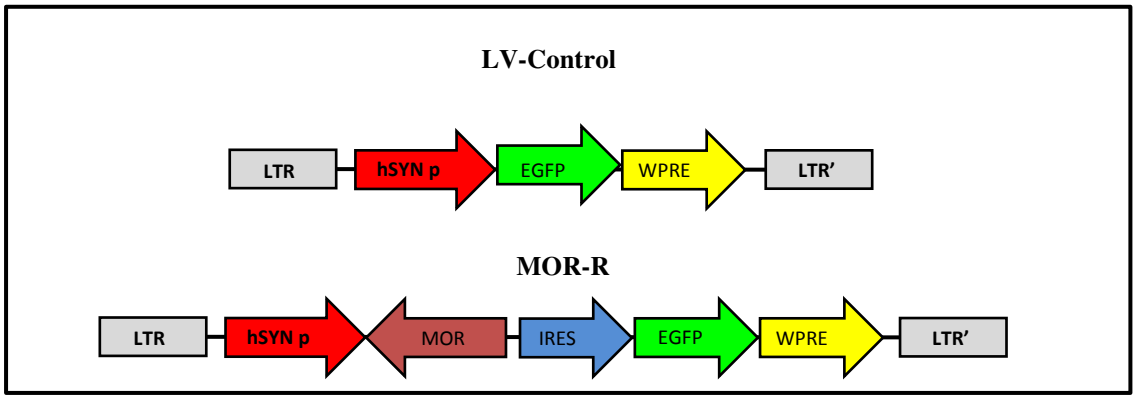


Figure 8: Schematic diagrams of the vectors. The lentiviral vectors contain the human synapsin promoter (hSYN-1p), the coding region of EFGP vector (LV-Cont) or MOR cDNA in reverse orientation (MOR-R vector). The vectors further carry the encephalomyocarditis virus internal ribosome entry site (IRES) and the transcriptional regulatory element (WPRE). Abreviations: LTR- long terminal repeat.

3. Surgical procedures

3.1. Osmotic mini-pump implantation

Mini-osmotic pumps (ALZET-model 2001- 200μL; figure 9) were filled with saline or morphine (45 μg^{-1}. $\mu L^{-1}.h^{-1}$), and immersed in a 0,9% saline solution at 37ºC for at least 4 hours before implantation, for stabilization purposes.

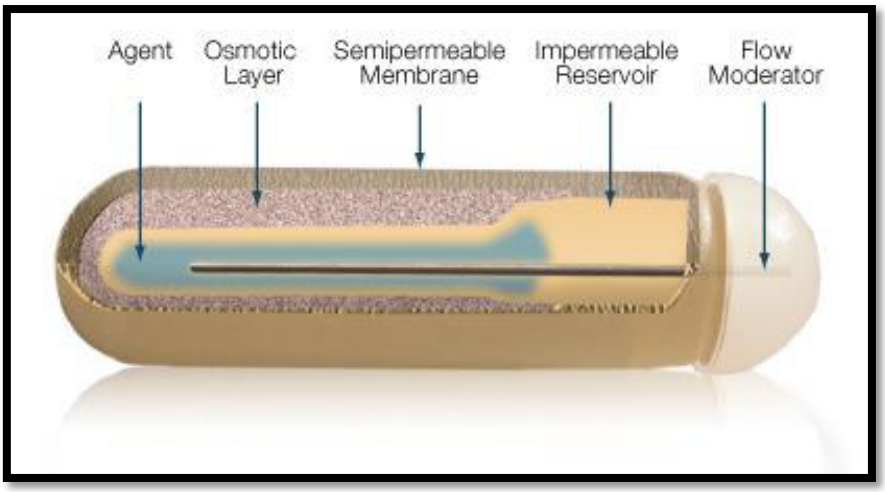


Figure 9: Schematic representation of a osmotic mini-pumps. These mini pumps act by osmotic pressure differences between their interior side (osmotic layer) and the tissue where the mini-pump is implanted. The higher concentration of the osmotic layer constituents triggers the entrance of water to the mini-pump through a semi-permeable membrane covering the surface of the mini-pumps. 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 the temperature (which should be around 37ºC) and by the osmolatily.

Animals were anesthetized with isofluorane (IsoFlo®) and their dorsum was shaved and cleaned with Betadine® solution. A midline incision was made in the skin and with a blunt-pointed scissors the skin was separated from the fascia and pumps were implanted subcutaneously (Figure 10). The incision was closed with surgery staples (Stoelting®, U.S.A.) and animals returned to their home cage.

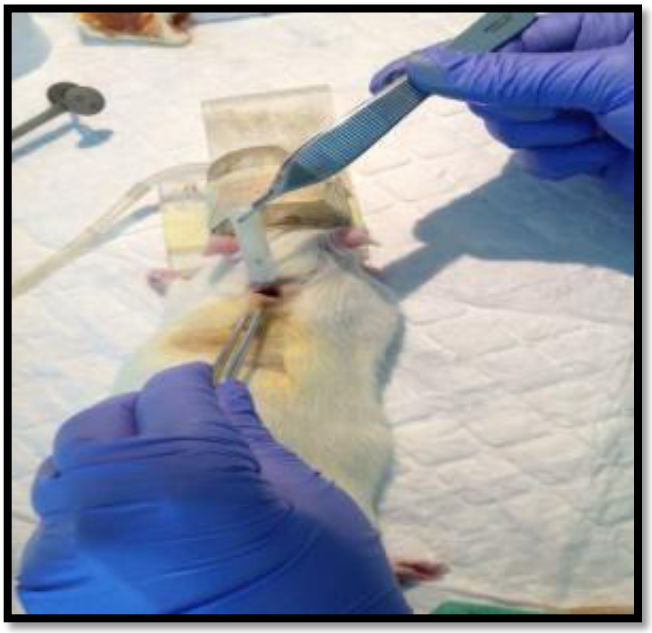


Figure 10: Implant of the mini-pumps in animals dorsum.

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, rhinorrhea, ptosis, irritability, lacrimation, escaping, penile erection and abnormal posture [98].

3.2. Neuropathic pain induction

The neuropathic pain model Spared Nerve Injury (SNI) was induced as described by Decosterd and Woolf [95]. 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, the left thigh of the animals was shaved and disinfected with 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 (Fig. 11, item 1), tibial (Fig. 11, item 2) and sural (Fig. 11, item 3) nerves.

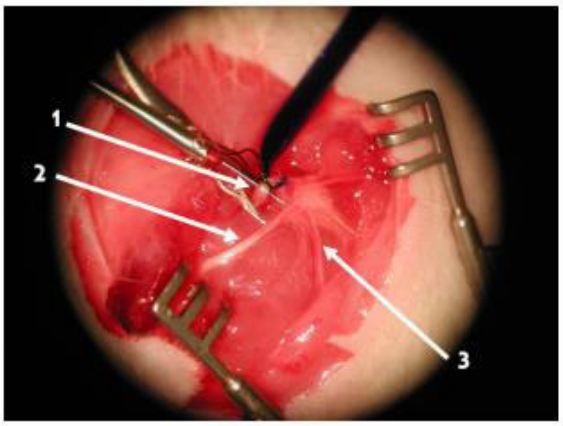


Figure 11: Exemplificative picture of the terminal branches of sciatic nerve. (1) common peroneal, (2) tibial (3) sural nerves.

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 4.0 silk and the skin was sutured with surgery 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 a s.c injection of atipamezole hydrochloride (Revertor®- 0,5 g/Kg). The staples suturing the skin were removed approximately 7 days after surgery.

Mini-pump implantation or mini-pump implantation in simultaneous with stereotaxic surgeries were performed between 2 to 3 weeks after SNI induction. This timing was chosen because after that period pain behaviours were shown to be robust and stable [28, 95].

3.3. Stereotaxic surgeries

Stereotaxic surgeries were performed for cannula implantation and lentiviral vectors injection into the left DRt of animals weighing 285-315g.

3.3.1 Cannula implantation

Naïve animals 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 animals were placed on a stereotaxic frame (David Kopf Instruments, U.S.A.) by positioning their head in the incisor bar (figure 12 B) and insertion of earbars into each ear canal(figure 12 C). 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. 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 four small burr holes were drilled, one over the left DRt and the other three in the surrounding area in order to implant screws to support the guide cannula (Plastics One® C315G Guide 266A 38172 11MM). The coordinates to target the left DRt, shown in Table 1, were determined according to the rat brain atlas relative to the interaural line. The interaural line was used as a reference to calculate the coordinates (Figure 13). The guide cannula was lowered (figure 12 A) until its tip was 3 mm above the DRt and was immobilized using a self-polymerizing acrylic (Vertex™ Self-Curing). A dummy Cannula (Plastics One®-Fit 11 MM C 315G W-O) was placed in the guide cannula to prevent its obstruction. At the completion of the stereotaxic procedure, the animals were implanted with osmotic mini-pumps filed with morphine ($45\mu g^{-1}.\mu L^{-1}.h^{-1}$) (n = 6) or saline (n= 7). The animals were rehydrated by subcutaneous (s.c.) administration of a saline solution 0,9% and the anesthesia was reverted with an s.c. injection of atipamezole hydrochloride (Revertor®-0,5 g/Kg). The animals were individually housed and monitored daily to evaluated body weight, and visible motor deficit and sedation. One week after cannula and mini-pumps implantation, a volume of 0.5µl of lidocaine at 4% was injected through the guide cannula and the behavioral effects of lidocaine were tested before and 30 min after injection (Scheme 2).

A guide cannula was also implanted into the left VLM (n = 3) or left Sol (n = 3) in order to verify that the effect of lidocaine was not the result of lidocaine spreading to surrounding areas of the DRt. The coordinates to target the left VLM and left Sol are shown in Table 2. At the completion of the stereotaxic procedure, the animals were

implanted with osmotic mini-pumps filed with morphine and 0.5µl of lidocaine at 4% was injected through the guide cannula as described above.

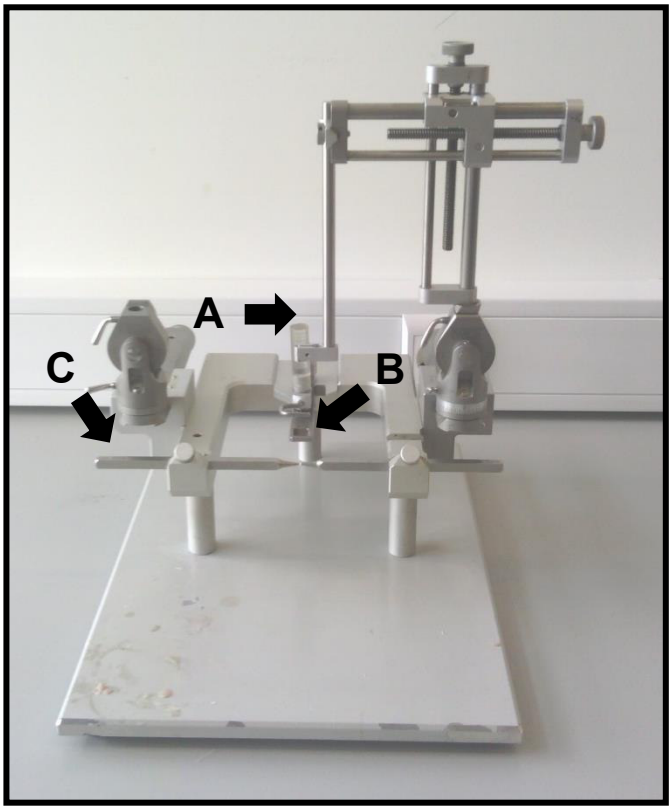


Figure 12: The stereotaxic frame. (A) cannula support, (B) Incisor bar and (C) earbars.

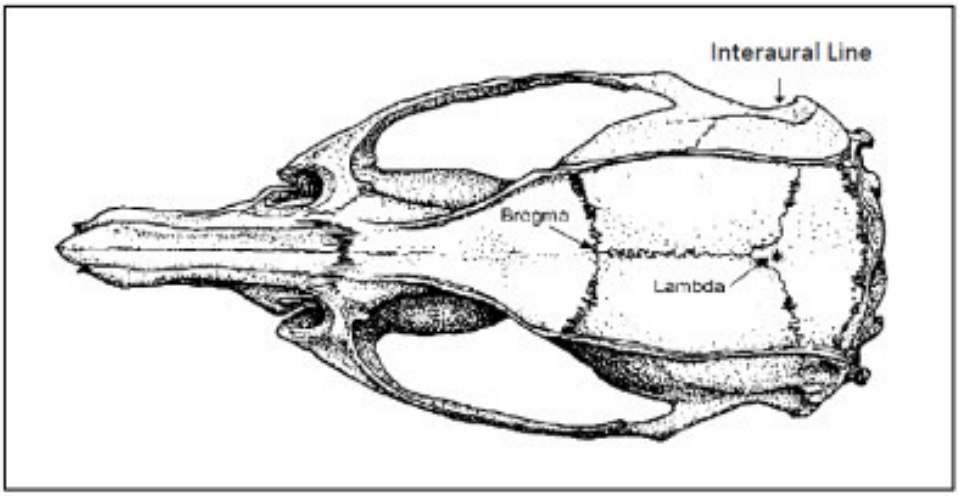


Figure 13: Dorsal view of the rat skull. Position of the interaural line used as a reference to calculate the coordinates to target the DRt. The positions of bregma and lambda points are also shown in the diagram. Bregma and lambda are used as references to calculate coordinates to target rostral brain regions. Adapted from Paxinos and Watson [30].

Table 2: Stereotaxic coordinates to target the left dorsal reticular nucleus (DRt), caudal ventromedial medulla (VLM) and Solitary nucleus (Sol) for implantation of a guide cannula.

Cordinates (mm) → Area ↓	AP (Anterior-posterior)	LM (Latero-medial)	DV (Dorso-ventral)
Left DRt	-6,0	+1,4	-1,5+3
Left VLM	−5.8	−2.2	−2.6+3
Left Sol	−6.0 mm	−0.8 mm	−1.2+3

3.3.2 Vectors injection

Stereotactic injections were performed to inject lentiviral vectors into the left DRt of naïve and neuropathic animals (2-3 weeks after SNI induction). Rats were deeply anesthetized by an i.p injection of a mixture of ketamine hydrochloride (0.06 g/Kg) and medetomidine (0.25 g/Kg) and placed on a stereotaxic frame as explained above. 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 a targeted area and, with a blunt needle, the dura was carefully pierced. Using a Hamilton syringe the rats received two injections of the control vector (LV-Cont; naïve animals n = 11; SNI animals n = 11) and MOR-R (naïve animals n = 11; SNI animals n = 12) at a volume of 0.6 μl each injection at 5.10^6 TU[1]/μl in two different rostrocaudal locations of the DRt following the stereotaxic coordinates shown in Table 3. The lentiviral suspensions were injected at the slow rate (0.2 μl/2 min) and, at the completion of each injection, the needle was left in place for 10 min to avoid reflow, before being slowly removed. At the completion of the stereotaxic procedure, the animals were implanted with osmotic mini-pumps filled with morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline. The animals were rehydrated by s.c. administration of a saline solution 0.9% and the anesthesia was reverted with a s.c. injection of atipamezole hydrochloride (Revertor®-0.5 g/Kg). The animals were then individually housed and monitored daily to evaluated body weight, and any visible motor deficit and sedation. The effects of the lentiviral vectors were behaviorally tested one week after stereotaxic surgery and mini-pump implantation (Schemes 3, 7).

[1] TU- Transducing units

Table 3: Stereotaxic coordinates to target the left DRt

Cordinates (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

4. Behavioural analysis

4.1. Nociceptive behavioural analysis

4.1.1. Pain assessment in naïve animals

Pain assessment in naïve animals was performed using the von Frey and Hot plate tests which allow to evaluate mechanical allodynia and thermal hyperalgesia, respectively. The tests were performed after a period of habituation of one week, during which the animals were handled by the experimenter in the test room for 30 min every day and placed in the testing apparatus for another 30 min (Figure 14 A). The criteria for adequate habituation were that animals did not freeze or defecate when placed in the testing apparatus.

The von Frey test was performed by the "up and down" method. The rats were placed on an elevated transparent cage with a mesh wire bottom allowing the stimulation of the plantar surface of left hind paw with a series of von Frey monofilaments (Stoeling, U.S.A.) ranged between 0.41 to 15 grams (Figure 14 B). A positive response was noted if the paw was sharply withdrawn upon application of the filament. Flinching or licking immediately upon removal of the filament were also considered a positive response [99].

The hot plate test was performed by placing the rat on a hotplate (BIO-CHP Cold Hot Plate Test) system, with a surface temperature of 52ºC. A rectangular Plexiglas chamber (35 cm high) with a removable top was used to confine the rat to a 16.5 cm × 16.5 cm hotplate surface during testing (Figure 14 C). Nociceptive threshold was quantified as the latency (in seconds) to licking, retraction of the hind paw or jump (all paws simultaneously leaving the plate surface) after placement of the rat on the hotplate. All rats was removed from the hotplate after a 30-s cutoff to avoid tissue injury.

These behavioral tests were used to evaluate:

i) the time course effects of chronic morphine administration. For this, the animals were implanted with a mini-pump filled with morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline (n=6 each group; Scheme 1). Von Frey and Hot plate tests were performed before and at 2, 4 and 7 days after mini-pump implantation (Scheme 1);

ii) the effects of DRt inactivation by lidocaine on chronic morphine administration. For this, two subsets of animals were used, as described above. One subset of animals was implanted with a guide cannula into DRt and with osmotic mini-pumps for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) (n=7) or saline (n=6). The second subset of animals was implanted with off target cannulas and with osmotic mini-pumps for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$; n=6). The behavioural tests were performed 7 days after guide cannula and mini-pump implantation before and 30 min after lidocaine injection through the guide cannula (Scheme 2);

iii) the effects of MOR knock down at the DRt on chronic morphine administration. For this, the animals were injected with lentiviral vectors at the DRt, as explained above, and implanted with mini-pumps for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$;LV-Cont n = 6; MOR-R n= 5) or saline (LV-Cont n = 5; MOR-R n= 6) as described above. The behavioural tests were performed before and at 2, 4 and 7 days after lentiviral vectors injection and mini-pump implantation (Schema 3).

The time course effects of chronic morphine administration was analysed by two-way repeated measures of ANOVA followed by Tukey post-hoc test for multiple comparisons. The effects of lidocaine or lentiviral vectors injection into the DRt were analysed by two-way ANOVA followed by Tukey post-hoc test for multiple comparisons. The effects of lidocaine injection into the off-target sites were analysed by paired t tests.

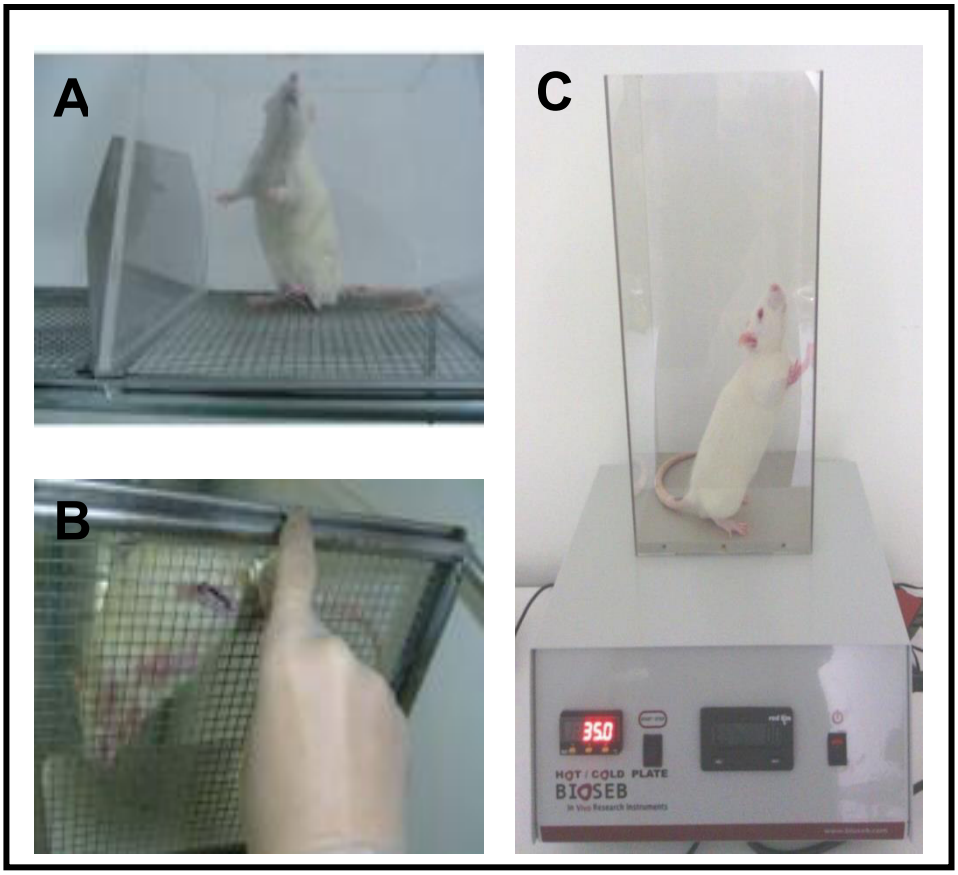
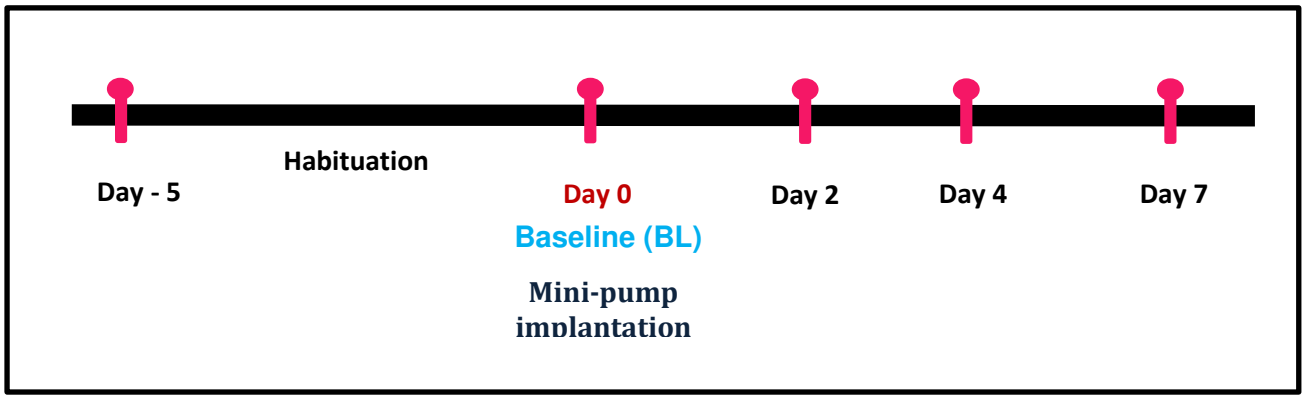
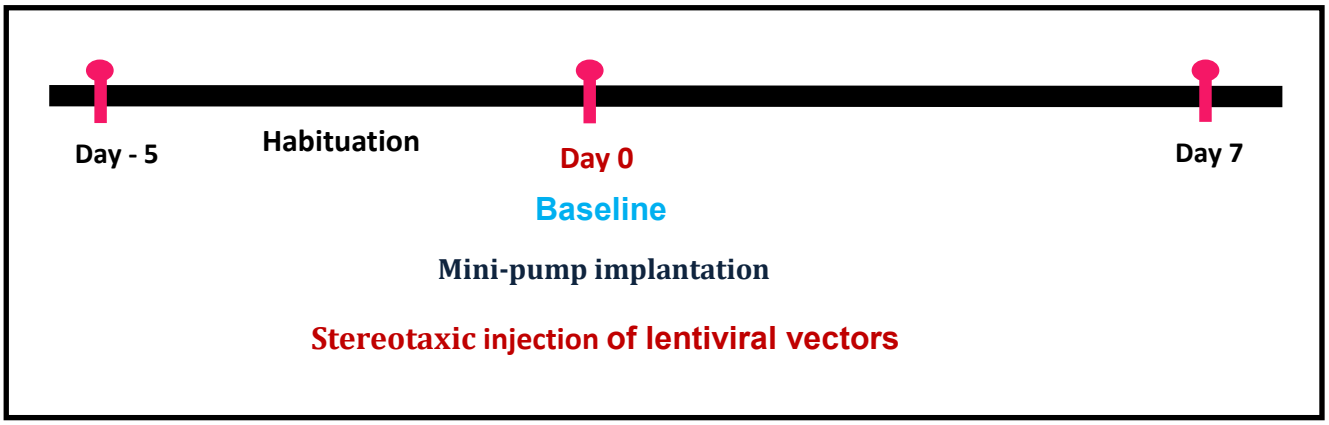


Figure 14: Behavioural tests apparatus. (A) Animal placed in the individual Plexiglas container in the wire grid; (B) von-Frey test; (C) Hotplate test.



Scheme 1: Time course effects of chronic morphine administration in naïve animals. Mini-pump implantation for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline was performed at Day 0. The animals were tested at Day 0 (Baseline; BL), before mini-pump implantation and at Days 2, 4 and 7 after mini-pump implantation.

Scheme 2: Effects of DRt inactivation by lidocaine on chronic morphine admnistration in naïve animals. Stereotaxic surgery for the implantation of a guide cannula and osmotic mini-pumps implantation for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline were performed at Day 0. The animals were tested at Day 7 before and after lidocaine injection through the guide cannula.



Scheme 3: Effects of MOR knock down at the DRt on chronic morphine admnistration in naïve animals. The stereotaxic injection of the lentiviral vectors LV-Control and MOR-R and the implantation of osmotic mini-pumps for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline was performed at Day 0. The animals were tested before the surgical procedures at Day 0 and at Day7.

4.1.2. Pain assessment in neuropathic animals

Pain assessment in neuropathic animals was performed by the von Frey, pin prick and acetone tests which allow to evaluate mechanical allodynia, mechanical hyperalgesia and cold allodynia, respectively. The tests were performed after a period of habituation of one week, during which the animals were handled by the experimenter in the test room for 30 min every day and placed in the testing apparatus for another 30

min. The criteria for adequate habituation were that animals did not freeze or defecate when placed in the test apparatus.

To perform the tests, the rats were placed on an elevated transparent cage with a mesh wire bottom allowing the stimulation of the lateral surface of the left hind paw (Figure 15). The von-Frey test was performed by applying von Frey monofilaments (Stoeling, U.S.A.) in a sequence of increasing stiffness for 5 seconds [100]. The threshold was taken as the lowest force that evoked a brisk withdrawal response to one of five repetitive applications (Figure 15 A). The pin prick test was performed by the application of a brief stimulation with a safety pin (Figure 15 B) at an intensity sufficient to produce a reflex withdrawal response but not penetrate the skin and the duration of paw withdrawal was clocked [101]. The acetone test was performed by application of 40 µL of acetone using a micropipette tip connected to a micropipette without touching the skin (Figure 15 C) and the duration of the withdrawal was timed [102].

These behavioral tests were used to evaluate:

i) the time course effects of chronic morphine administration. For this, 2-3 weeks after SNI induction, the animals were implanted with a mini-pump filled with morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline (n=6 each group; Scheme 4). Von-Frey, pin-prick and acetone tests were performed before and at 2, 4 and 7 days after mini-pump implantation (Scheme 4);

ii) the effects of MOR knock down at the DRt on chronic morphine administration. For this, 2-3 weeks after SNI induction, the animals were injected with lentiviral vectors at the DRt and implanted with mini-pumps for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$;LV-Cont n = 6; MOR-R n= 6) or saline (LV-Cont n = 5; MOR-R n= 6) as described above. The behavioural tests were performed before and at 2, 4 and 7 days after lentiviral vectors injection and mini-pump implantation (Scheme 5).

The time course effects of chronic morphine administration and the effects of lentiviral vectors injection into the DRt were analysed by two-way ANOVA followed by Tukey post-hoc test for multiple comparisons.

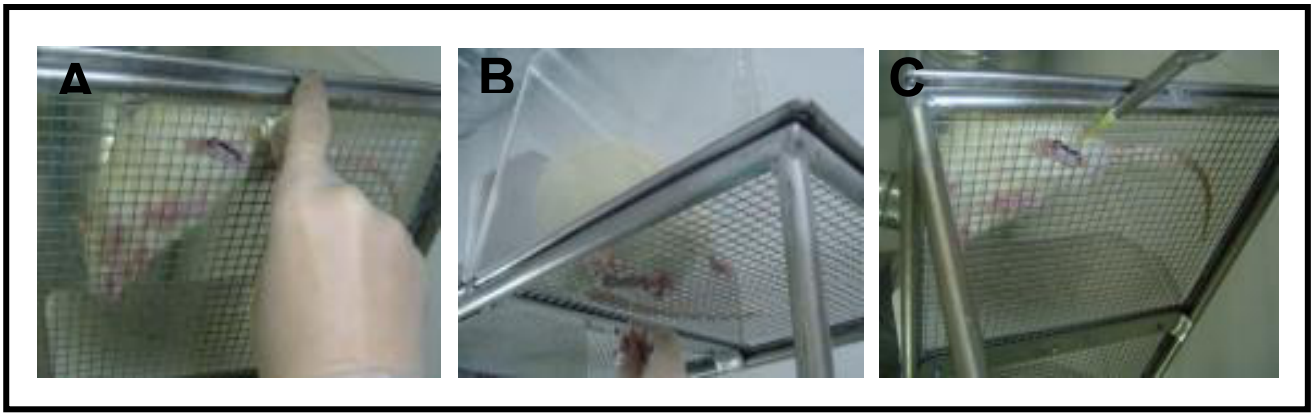
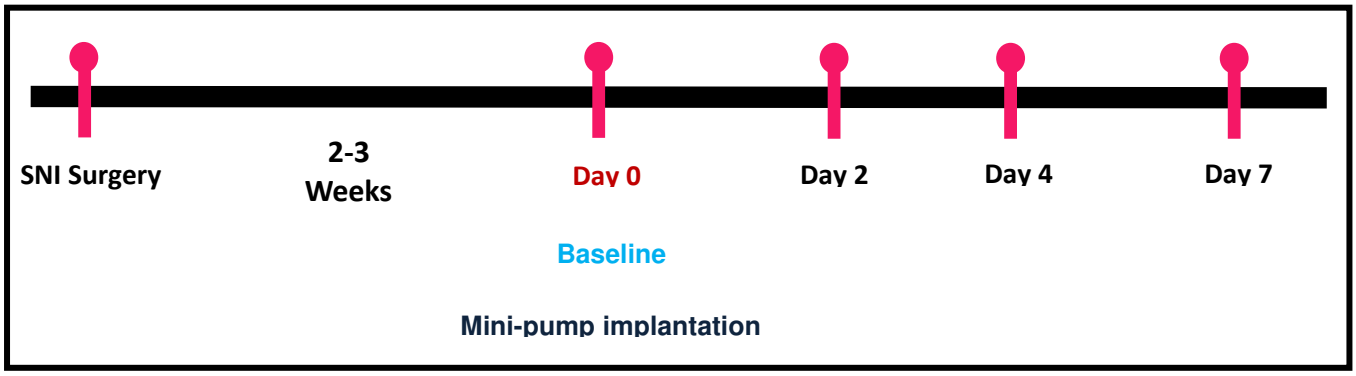
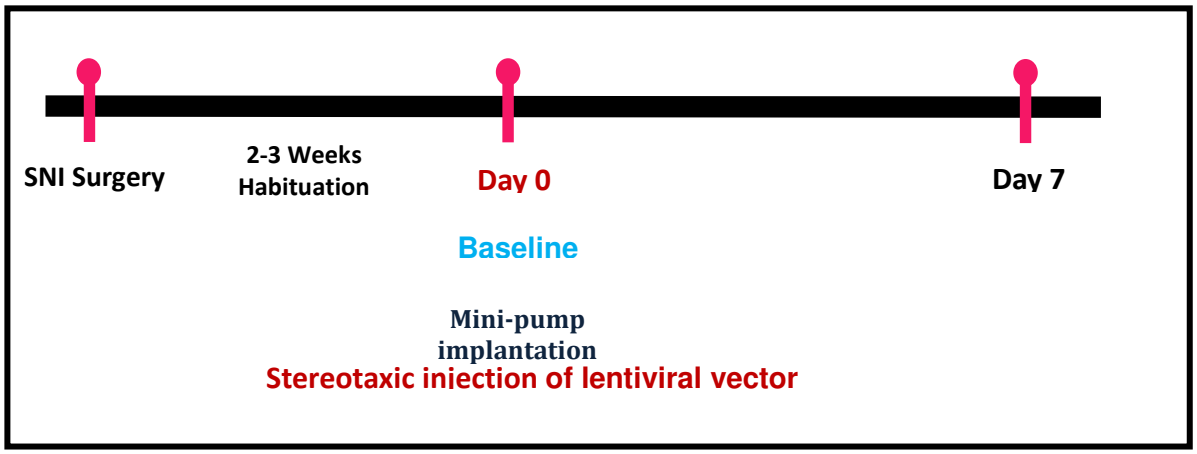


Figure 15: Behavioural tests apparatus. (A) von-Frey test; B) Pin-prick test; (C) Acetone test.



Scheme 4: Time course effects of chronic morphine administration in neuropathic (SNI) animals. Mini-pump implantation for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline was performed at Day 0, i.e. 2-3 weeks after SNI surgery. The animals were tested at Day 0 (Baseline; BL), before mini-pump implantation and at Days 2, 4 and 7 after mini-pump implantation.



Scheme 5: Effects of MOR knock down at the DRt on chronic morphine administration in neuropathic (SNI) animals. The stereotaxic injection of the lentiviral vectors LV-Control and MOR-R and the implantation of osmotic mini-pumps for the s.c. delivery of morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline was performed Day 0, i.e. 2-3 weeks after SNI surgery. The animals were tested before the surgical procedures at Day 0 and at Day7.

4.2. Morphine reward behavioural analysis

The analysis of the reward behaviour was performed using the Conditioned Place Preference test (CPP). The CPP paradigm is a standard behavioural test used to study the rewarding and aversive effects of drugs. The test involves the association of a particular environment, through visual and tactile cues, with a drug treatment. This is known as the **conditioning phase** of the test. The conditioning phase is followed by a **post-conditioning phase**, during which the animals associate a different environment with a drug but in the absence of the drug [103]. A common variation of this design consists of a **three-compartment chambers** with **two different conditioning chambers/contexts**, distinguished by visual and sensory cues, to which the animal is exposed once or several times, that are connected by **a neutral chamber**, with no special characteristics and is not paired with a drug. Before the conditioning phase (i.e. during the **pre-conditioning phase**), the animal should not present a previous preference for any chamber/context [103, 104].

The CPP test was used for the first time in the laboratory therefore we first optimized the test. We tested different visual and tactile cues/contexts in order to obtain contexts towards which the animals present no preference before the conditioning sessions (i.e. during the pre-conditioning phase). We also tested the number of conditioning sessions, which is the number of conditioning trials/sessions the animal is

exposed to one of the different chambers/contexts during the conditioning phase. All experiments were performed using neuropathic animals 2 to 3 weeks after SNI induction.

4.2.1. Optimization of visual cues

The optimization of the visual cues was performed in a Plexiglas apparatus measuring 100 [length (L)] X 40 [width (W)] X 40 [height (H)] cm and comprising two distinct conditioning chambers separated by a neutral chamber. The neutral chamber measured 20 X 40 X 40 cm with black Plexiglas walls. Each conditioning chamber measured 40 (L) X 40 (W) X 40 (H) cm and contained different visual clues. One chamber had walls with alternating 3 cm wide black and white horizontal stripes. The second chamber had alternating 3 cm wide black and white vertical stripes. The tactile cues were the same in the three chambers, which is all chambers, had black Plexiglas floors.

Rats (n = 5 were placed in the neutral chamber with full access to all chambers for 15 min for 2 consecutive days. Day 1 was used for habituation purposes. On day 2 (pre-conditioning phase), rats behaviour was recorded for 15 minutes using a video camera. The time spend in each chamber was analysed by Ethlog 2.2.

4.2.2. Optimization of tactile cues

The optimization of tactile cues was performed in a Plexiglas apparatus with the characteristics described above. The visual clues in the conditioning chambers were the ones tested above. The following tactile textures in the floor of each conditioning chamber were tested:

1- Mesh wire floor in one chamber / Black Plexiglas floor in the other chamber;
2- Mesh wire floor in one chamber / Corn cob in the floor of the other chamber;
3- Mesh wire floor in one chamber / Metallic floor with 0.5 cm diameter circles spaced 1 cm in the other chamber;
4- Mesh wire floor in one chamber / 0.5 cm diameter metal rods spaced 2 cm in the other chamber.

Rats (n = 15) were placed in the neutral chamber with full access to all chambers for 15 min for 2 consecutive days. Day 1 was used for habituation purposes. On day 2 (pre-conditioning phase), rats behaviour was recorded for 15 minutes using a video camera. The time spend in each chamber was analysed by Ethlog 2.2.

4.2.3. Optimization of the number of conditioning trials/sessions.

The optimization of the number of conditioning sessions was performed in a Plexiglas apparatus with the dimensions described above and with visual and tactile cues that showed unbiased pre-conditioning results. One chamber of the CPP apparatus had a floor with 0.5 cm diameter metal rods spaced 2 cm and walls with alternating 3 cm wide black and white horizontal stripes. The floor of the second chamber was a mesh wire and the walls had with alternating wide black and white vertical stripes.

We tested the effect of morphine (10 mg/Kg) on a single and a multi-trial conditioning protocol.

Rats were placed in the neutral chamber with full access to all chambers for 15 min for 2 consecutive days, Day 1 was used for habituation purposes and on day 2 (pre-conditioning phase), rats behaviour was recorded for 15 minutes, as explained above. 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 chamber/environment in a counterbalanced fashion.

During the conditioning phase, Plexiglas partitions matching their respective environments were inserted to restrict the rats to a specific designated chamber/environment. In the single-trial conditioning protocol (n = 3), on Day 3 the animals, were subcutaneously injected with saline and randomly placed in a conditioning chamber for 1 hour. Four hours later, the animals were subcutaneously injected with morphine (10 mg/Kg) and placed in the other chamber for 1 hour. In the multi-trial conditioning protocol (n = 3), the animals were conditioned on Days 3-8. The animals were subcutaneously injected with saline or morphine (10 mg/Kg) once a day on alternate days. The animals were placed into the designated conditioning chamber for 1 hour immediately after the injection.

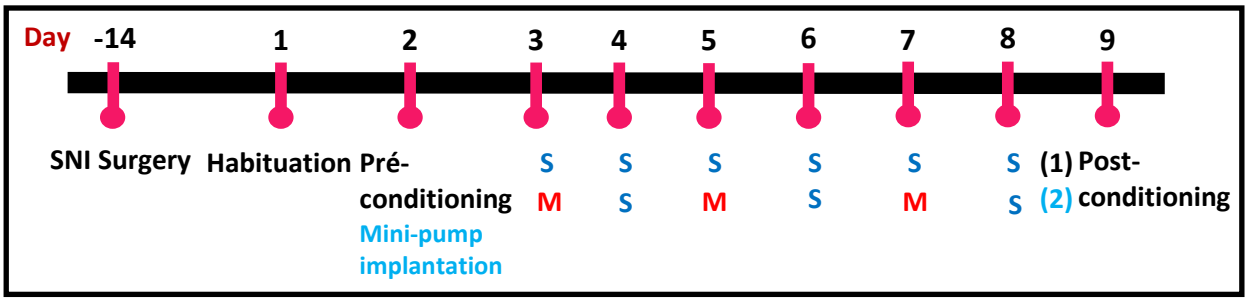
On post-conditioning day (Day 4 in the single single-trial conditioning protocol; Day 9 in the multi-trial conditioning protocol), rats were placed in the neutral chamber in a drug-free state, with access to all chambers, their behaviour was recorded for 15 min. The time spend in each chamber was analysed by Ethlog 2.2., as above, and the difference between post- and pre-conditioning time spent in each chamber was calculated to determine the preference score for each chamber.

4.2.4. Reward effects of morphine after chronic morphine administration

The evaluation of the reward effects of morphine after chronic morphine treatment was performed in a Plexiglas apparatus with the dimensions described above. The visual and tactile cues in the conditioning chambers, optimized above, were as follows: one chamber with a floor constituted by 0.5 cm diameter metal rods spaced 2 cm and walls with alternating 3 cm wide black and white horizontal stripes; the second chamber with a mesh wire floor and walls with alternating wide black and white vertical stripes. The multi-trial conditioning protocol was chosen to perform the CPP test.

Rats were placed in the neutral chamber with full access to all chambers for 15 min for 2 consecutive days, Day 1 was used for habituation purposes and on day 2 (pre-conditioning phase; Scheme 6), rats behaviour was recorded for 15 minutes, as explained above. 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 chamber/environment in a counterbalanced fashion. Immediately after recording their behavior, the rats were implanted with osmotic mini-pumps filled with morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline for the chronic treatment (Scheme 6). On days 3-8 (conditioning phase; Scheme 6) one group of animals was subcutaneously injected with saline or morphine (10 mg/Kg) once a day on alternate days (saline mini-pumps group n = 7; morphine mini-pumps group n = 8). The animals were placed into the designated conditioning chamber for 1 hour. immediately after injection (Scheme 6 – item 2). The animals from the control group (saline mini-pumps group n = 4; morphine mini-pumps group n = 3) were subcutaneously injected with saline every day and placed into the designated conditioning chamber for 1 hour immediately after injection (Scheme 6 – item 1). On day 9 (post- conditioning phase; Scheme 6) rats were placed in the neutral chamber in a drug-free state, with access to all chambers, their behaviour was recorded for 15 min and the videos were analyzed by Ethlog 2.2 to determine the difference between post- and pre-conditioning time spent in each chamber. The number of chamber crossings was also analysed as it is considered a good indicator of locomotor activity [105, 106].

The statistical analysis was performed by two-way ANOVA followed by Tukey post-hoc test for multiple comparisons.



Scheme 6: Evaluation of the reward effects of morphine after chronic morphine administration by the CPP test. Animals were allowed a 15 min period of habituation at Day 1 and were recorded on Day 2 (pre-conditioning phase) to ascertain preferences. Mini-pumps filled with morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) or saline for chronic treatment were implanted on Day 2 immediately after behavioural recording. Days 3-8 (conditioning phase): one group (1) received saline (S) s.c every day and the other group (2) received morphine (M, 10 mg/Kg) or saline on alternate days. Immediately after injection the animals were placed in the assigned chamber/environment for 1 hour each day. On day 9 (post-conditioning phase) animals were allowed to explore the apparatus in a drug free state for 15 min and were recorded to ascertain preferences.

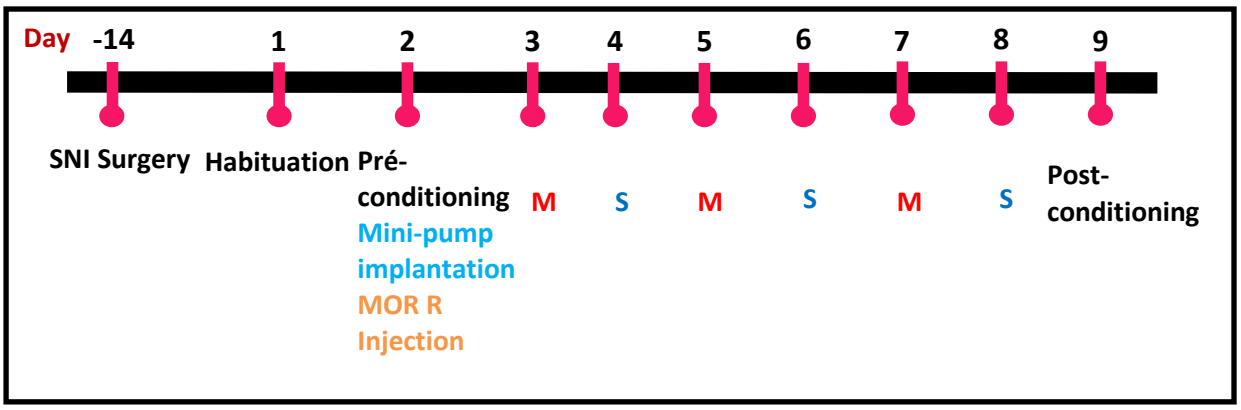
4.2.5. Effects of MOR knock-down at the DRt on morphine reward after chronic morphine administration

The evaluation of the effects of MOR knock-down at the DRt on morphine reward was performed in a Plexiglas apparatus with the dimensions and characteristics described above.

Rats were placed in the neutral chamber with full access to all chambers for 15 min for 2 consecutive days, Day 1 was used for habituation purposes and on day 2 (pre-conditioning phase; Scheme 6), rats behaviour was recorded for 15 minutes, as explained above. 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 chamber/environment in a counterbalanced fashion. Immediately after recording their behavior, the rats were stereotaxically injected MOR-R into the left DRt (n = 3), as decribed above, and implanted with osmotic mini-pumps filled with morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) for the chronic treatment (Scheme 7). On days 3-8 (conditioning phase; Scheme 7) the animals were subcutaneously injected with saline or morphine (10 mg/Kg) once a day on alternate days. The animals were placed into the designated conditioning chamber for 1 hour immediately after injection (Scheme 7). On day 9 (post- conditioning phase; Scheme 7) rats were placed in the neutral chamber in a drug-free state, with access to all chambers, their behaviour was recorded for 15 min and the videos were analyzed by

Ethlog 2.2 to determine the difference between post- and pre-conditioning time spent in each chamber. The number of chamber crossings was also analysed.

The statistical analysis was performed by was one-way ANOVA followed by Tukey post-hoc test for multiple comparisons.



Scheme 7: Effects of MOR knock down at the DRt on morphine reward. Animals were allowed a 15 min period of habituation at Day 1 and were recorded on Day 2 (pre-conditioning phase) to ascertain preferences. MOR-R was injected at the DRt and mini-pumps filled with morphine (45 µg-1.µL-1.h-1) for chronic treatment were implanted on Day 2 immediately after behavioural recording. Days 3-8 (conditioning phase): the animals received morphine (M, 10 mg/Kg) or saline on alternate days. Immediately after injection the animals were placed in the assigned chamber/environment for 1 hour each day. On day 9 (post-conditioning phase) animals were allowed to explore the apparatus in a drug free state for 15 min and were recorded to ascertain preferences.

5. Material processing for histological and immunohistochemical analysis

After the last behavioral evaluation, the animals that received lidocaine injections were deeply anaesthetized by an overdose of sodium pentobarbital (65 mg/Kg i.p.), injected with 0.5 µl of 0.6% Chicago sky blue dye (Sigma, St. Louis, USA) through the guide cannula and sacrificed by decapitation. After decapitation, the brain was removed and immersed in a fixative solution containing 4% paraformaldehyde in 0.1 M PB, pH 7.2 (Appendix A) for 4h followed by 30% sucrose in 0.1M PB, pH 7.2 overnight at 4ºC.

At the completion of the experiments performed with naïve animals used to study the time course effects of chronic morphine administration and all animals injected with the lentiviral vectors, the animals were anaesthetized by an overdose of

sodium pentobarbital (65 mg/Kg i.p.). The animals were then 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 brains were removed, immersed in fixative for 4 hours followed by 30% sucrose in 0.1M phosphate-buffered saline (PBS) overnight, at 4ºC.

The brainstems of all animals were serially cut in a freezing microtome at 40 µm, collected in 4 sets and stored in a cryoprotector solution (Appendix A) at -20ºC. Brainstem sections from naïve animals were used for the immunohistochemical analysis of MOR and pCREB. Brainstem sections from all animals injected with the lentiviral vectors were used for the immunohistochemical analysis of MOR.

6. Histological verification of injection sites

The location of the injection site of animals injected with lidocaine was determined by the location of the Chicago sky blue dye after counterstaining medullary sections encompassing the DRt with thionin. Only animals with cannula correctly targeting the DRt were included in data analysis.

To analyze 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 medullary sections encompassing the DRt with thionin (Appendix A) [107]. Only animals with vector injections centred in the DRt were included in data analysis.

7. Immunohistochemical analysis.

7.1. pCREB immunodetection.

One in every fourth brainstem section, after washing with PBS, was incubated with a 30% Hydrogen peroxide solution to block endogenous peroxidase activity, followed by an incubation with a blocking solution (Glycine + 10% PBS-T) to prevent the nonspecific binding of the antibodies. After blocking, the sections were incubated with a rabbit polyclonal anti-pCREB antibody (Neuromics; U.S.A.) in PBS-T at 1:10000 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 mounted on gelatin-coated slides, cleared in xylol and coverslipped with Eukitt. Five sections encompassing the rostro-caudal extent of the DRt were taken from each animal and photomicrographs of the DRt were taken using a Zeiss® light microscope with a high-resolution digital camera. The number of pCREB positive nucleus/particles was calculated using an automated cell counting in the ImageJ® software. Briefly, all images were transformed into a 8-bit greyscale followed by an adjustment of the threshold, to highlight the structures to be counted. Then the images were converted to a binary watershed image to separate overlapped particles. The statistical analysis was performed using an unpaired t-test.

7.2. MOR immunodetection

One in every fourth brainstem section, after washing with PBS, was incubated with a 30% Hydrogen peroxide solution to block endogenous peroxidase activity, followed by an incubation with a blocking solution (Glycine + 10% PBS-T) to prevent the nonspecific binding of the antibodies. After blocking, the sections were incubated with rabbit polyclonal anti-MOR antibody (Neuromics; 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 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 mounted on gelatin-coated slides, cleared in xylol and coverslipped with Eukitt. Five sections encompassing the rostro-caudal extent of the DRt were taken from each animal and the numbers of MOR

neurons into the DRt were counted using an Olympos® light microscope with a high-resolution digital camera. The statistical analysis was performed by was two-way ANOVA followed by Tukey post-hoc test for multiple comparisons.

7.3. Double immunodectetion of MOR and pCREB

One in every fourth brainstem section, after washing with PBS, was incubated with a 1% sodium borohydride solution to reduce the tissue autofluorescence followed by an incubation with a blocking solution (Glycine + 10% PBS-T) to prevent the nonspecific binding of the antibodies. After blocking, the sections were incubated with a guinea-pig polyclonal anti-MOR antibody (Neuromics; U.S.A.) in PBS-T at 1:500 for 72 hours at 4°C. After washing with PBS-T the sections were incubated for 1h with a goat biotinylated anti-guinea-pig serum (Dako, Denmark) diluted in PBS-T containing 2% normal goat serum. Sections were washed again with PBS-t and were incubated with streptavidin Alexa 488 (Molecular Probes®) at 1:500 for 1h. After that, sections were again incubated with a blocking solution as above, followed by the incubation with rabbit polyclonal anti-pCREB antibody (Neuromics; 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 donkey anti-rabbit Alexa 594 (Molecular Probes®). The sections were mounted on gelatin-coated slides, coverslipped with glycerol–phosphate buffer (3:1, vol/vol) and stored at 4°C. Five sections encompassing the rostro-caudal extent of the DRt were taken from each animal and the total number of MOR-IR neurons and double-IR for MOR and pCREB were counted using ApoTome Slider (Zeiss®) fluorescence microscope. The statistical analysis was performed using the unpaired t-test.

Results

1. Effects of chronic morphine administration on pain behaviors

1.1. Effects on naïve animals

The effects of chronic morphine administration on naïve animals were tested before and at 2, 4 and 7 days after mini-pumps implantation (Scheme 1) by the von Frey and hot plate tests. In the von-Frey test, animals chronically administered with morphine showed a significant decrease of withdrawal thresholds at day 4 ($p=0.0128$; Figure 16 A) and day 7 ($p=0.0001$; Figure 16 A) compared to baseline. At day 7, the withdrawal threshold of the morphine group was also significantly lower than in the saline group ($p=0.0012$; Figure 16 A). No significant differences were observed in the saline group compared to baseline.

In the hot plate test, animals treated with morphine showed a significant decrease of the withdrawal latency at day 4 ($p= 0.0094$; Figure 16 B) and day 7 ($p=0.0001$; Figure 16 B) compared to baseline. At day 7, the withdrawal latency of the morphine group was also significantly lower than in the saline group ($p=0.0129$; Figure 16 B). No significant differences were observed in the saline group compared to baseline.

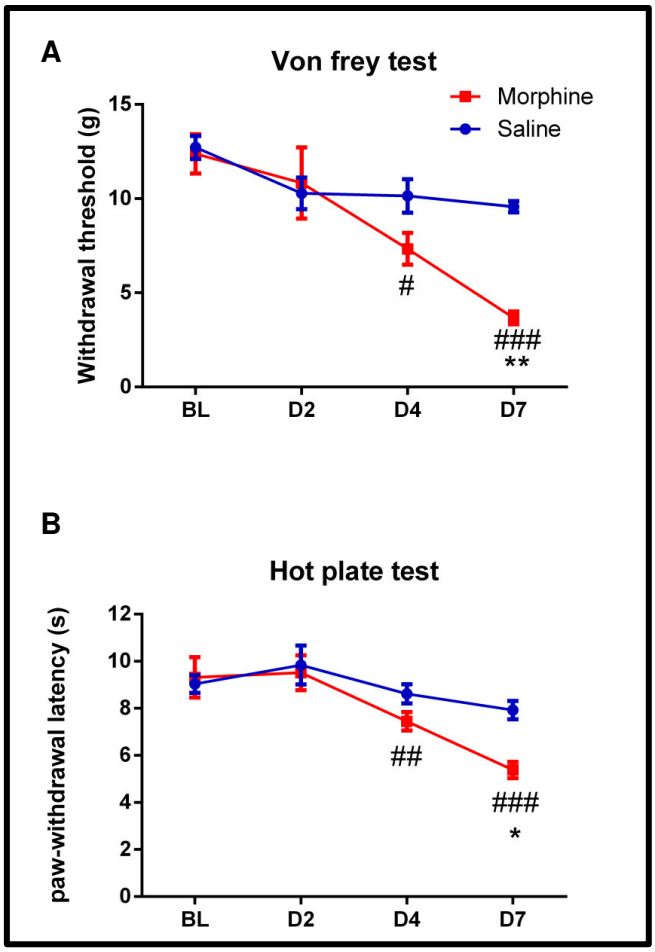


Figure 16: Time course effects of morphine administration on mechanical allodynia tested by the von Frey test (A) and on thermal hyperalgesia tested by the hot plate test (B). Data are presented as mean ± SEM. (saline – blue line n=6; morphine – red line n=6 # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ vs. BL; * $p < 0.05$; ** $p < 0.01$ saline vs treated group.)

1.2. Effects on neuropathic animals

The effects of the chronic morphine administration were tested before and at 2, 4 and 7 days after mini-pumps implantation (Scheme 4) by the von Frey, pin prick and acetone tests. In the von-Frey test, no significant differences were observed within each group and between both groups (Figure 17 A).In the pin-prick test, the animals chronically administered with morphine showed a significant increase of the withdrawal duration at day 4 ($p=0.0027$; Figure 17 B) and day 7 ($p=0.0001$; Figure 17 B) compared to baseline. At day 7, the withdrawal duration in the morphine group was also significantly higher than in the saline group ($p=0.0159$; Figure 17 B). No significant differences were observed in the saline group compared baseline.

In the acetone test, animals treated with morphine showed a significant increase of the withdrawal duration at day 7 compared both to BL (p=0.0287; Figure 17 C) and the saline group (p=0.0289; Figure 17 C). No significant differences were observed in the saline group compared to baseline.

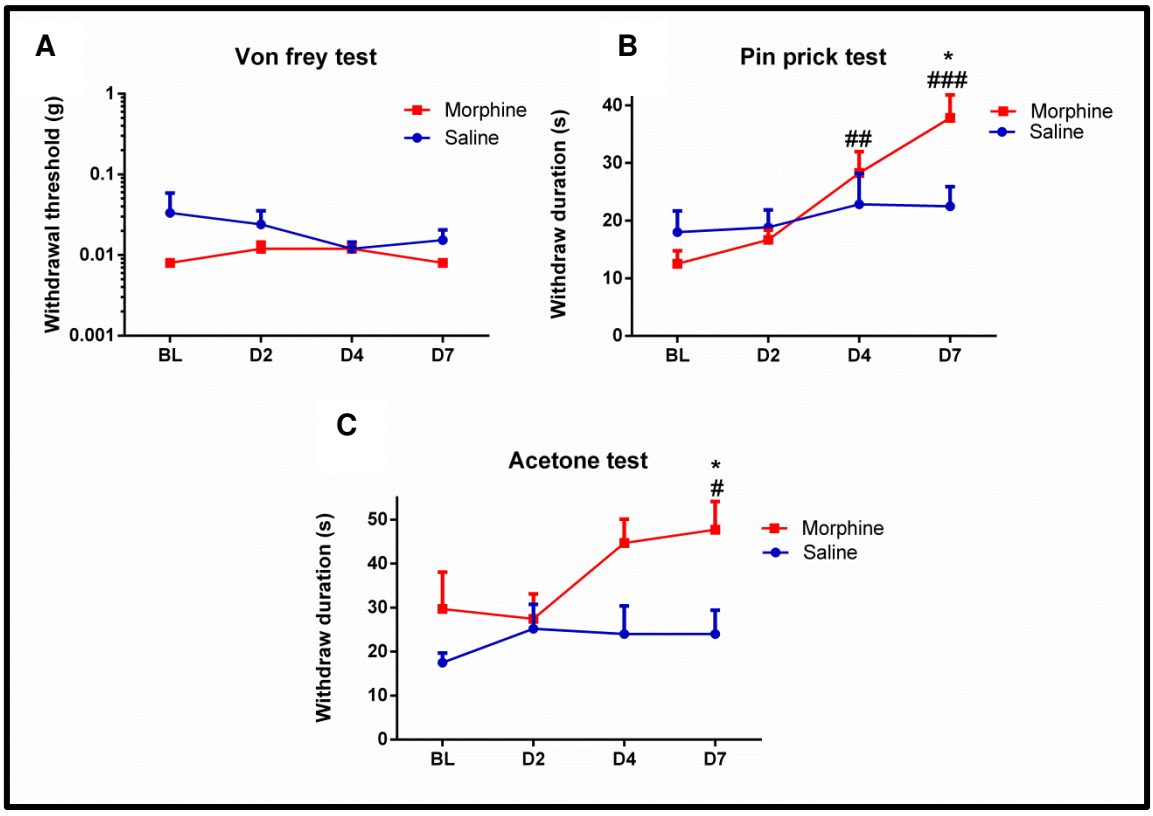


Figure 17: Time course effects of morphine administration on mechanical allodynia tested by the von Frey test (A), mechanical hyperalgesia tested by the pin prick test (B) and cold allodynia tested by the acetone test (C). Data are presented as mean ± SEM. (saline – blue line n=6; morphine – red line (n=6) $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ *vs.* BL; * $p < 0.05$ saline *vs* treated group).

2. Effects of chronic morphine administration on the reward behavior

2.1. Optimization of the conditioned place preference test

2.1.1 Visual cues

Two different visual contexts were tested in the CPP apparatus. One conditioning chamber had walls with alternating 3 cm wide black and white horizontal stripes. The second conditioning chamber had alternating 3 cm wide black and white vertical stripes. The chambers were separated by a neutral chamber with black Plexiglas walls.

The animals did not show a preference for any context (Figure 18). Given the unbiased results obtained, these two visual contexts were elected to carry on the CPP test.

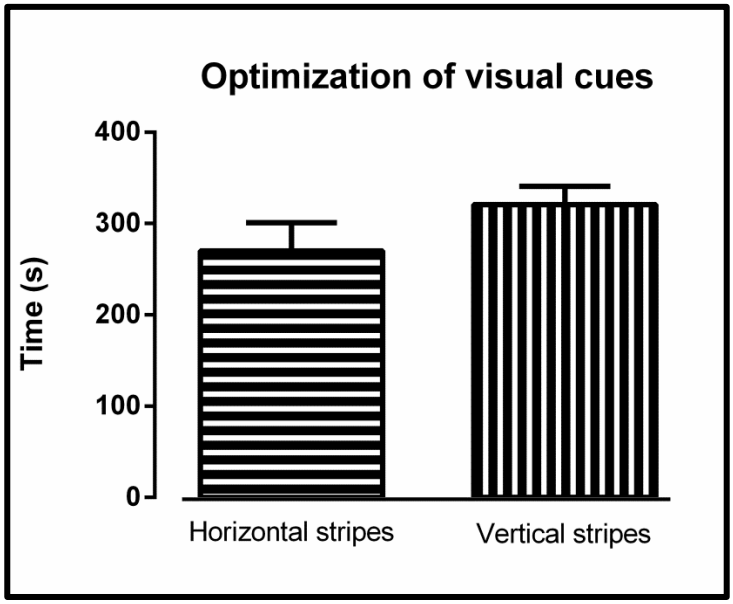


Figure 18: Optimization of the visual cues of the CPP test. The CPP apparatus contained one chamber with walls containing alternating 3 cm wide black and white horizontal stripes and a second chamber containing alternating 3 cm wide black and white vertical stripes. The chambers were separated by a neutral chamber with black Plexiglas walls. Data are presented as mean ± SEM (n=5).

2.1.2. Tactile cues

To optimize the tactile cues, four different combinations of floors in the conditioning chambers were tested: *i)* "Mesh wire floor vs black Plexiglas floor"; *ii)* "Mesh wire floor vs Corn cob floor"; *iii)* "Mesh wire floor vs metallic floor with circles" and *iv)* "Mesh wire floor vs metal bars floor".

The animals presented a clear preference for the compartment presenting the mesh wire floor when it was combined with the black Plexiglas (Fig. 19 A), corn cob (Fig. 19 B) and metallic floor with circles (Fig. 19 C). When the mesh wire floor was combined with the metal bars floor, the animals no longer presented any preference (Figure 19 D). Given the unbiased results obtained with the "Mesh wire floor" and the "Metal bars floor" combination, these two tactile contexts were elected to carry on the CPP test.

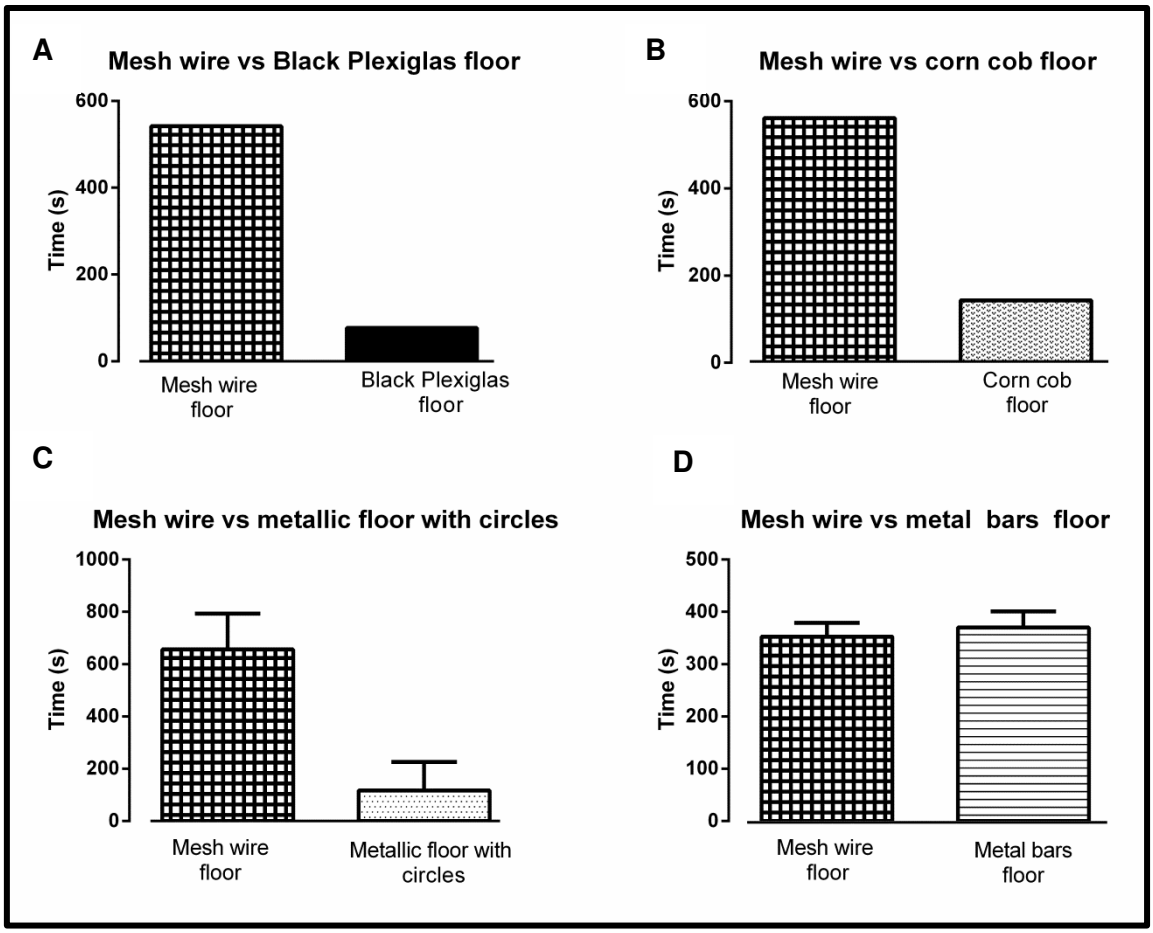


Figure 19: Optimization of the tactile cues of the CPP test. Four different environments were tested: Mesh wire floor vs black Plexiglas floor (A; n=1); Mesh wire floor vs Corn cob floor (B; n=1); Mesh wire floor vs metallic floor with circles (C; n=4) and Mesh wire floor vs metal bars floor (D; n=9). Data are presented as mean ± SEM.

2.1.3. The number of conditioning trials

To optimize the number of conditioning trials, the animals first underwent the pre-conditioning phase during which they were placed in the neutral chamber with full access to all chambers and their behaviour was recorded for 15 minutes. The pre-conditioning phase was followed by a conditioning phase during which the animals were administered saline or morphine (10 mg/Kg; s.c.) in a specific compartment. We tested a single-trial conditioning phase protocol during which the animals, were subcutaneously injected with saline and randomly placed in a conditioning chamber for 1 hour. Four hours later, the animals were subcutaneously injected with morphine (10 mg/Kg) and placed in the other chamber for 1 hour. Then we tested a multi-trial conditioning phase protocol during which the animals were subcutaneously injected

with saline or morphine (10 mg/Kg) once a day on alternate days for 6 days. The animals were placed into the designated conditioning chamber for 1 hour immediately after the injection. The conditioning phase was then followed by the post-conditioning phase during which the rats were placed in the neutral chamber in a drug-free state, with access to all chambers, their behaviour was recorded for 15 min and the difference between pre- and post-conditioning time spent in the morphine-paired chamber was determined.

The animals that underwent the multi-trial conditioning protocol, showed higher preference scores in the morphine-paired chamber compared to the animals that underwent the single-trial conditioning protocol (Figure 20). Given the higher scores of the multi-trial conditioning protocol, this conditioning protocol was elected to carry on the CPP test.

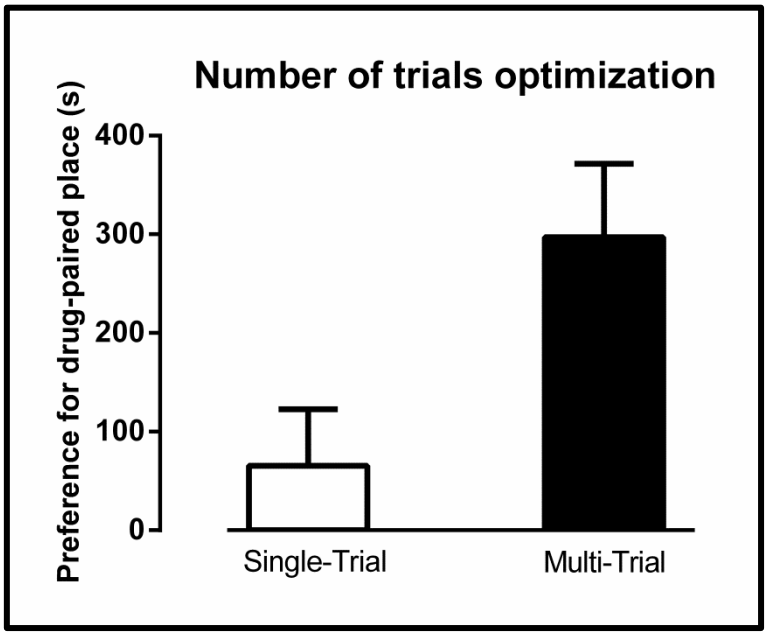


Figure 20: Optimization of the number of the conditioning trials. During the conditioning phase, the animals were administered saline or morphine (10 mg/kg; s.c.) and then placed on a specific compartment. In the single-trial protocol (n = 3) the animals, were subcutaneously injected with saline and randomly placed on a conditioning chamber, 4 hours later, they were subcutaneously injected with morphine (10 mg/Kg) and placed in the other chamber. In the multi-trial conditioning protocol (n = 3) the animals were subcutaneously injected with saline or morphine (10 mg/Kg) once a day on alternate days for 6 days. On the post-conditioning day, rats behavior was recorded for 15 min and the videos were analyzed to determine the difference between pre - and post -conditioning time spent in the morphine-paired chamber. Data are presented as mean ± SEM.

2.2. Effects of chronic morphine administration on morphine reward

The CPP test was performed on animals chronically administered morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$; n=11) or saline (n=11). Animals chronically treated with saline and conditioned with morphine (10 mg/kg; s.c.) on alternate days (n=7) spent longer time in the morphine-paired chamber compared to control animals conditioned with saline (n=4) every day (p= 0.0087;Figure 21). Animals chronically treated with morphine and conditioned with morphine in alternate days (n=8) spent significantly less time in the morphine paired chamber compared to animals chronically treated with saline and conditioned with morphine in alternate days (p=0.0438; Figure 21).

We also analyzed the effect of chronic morphine treatment in the locomotion by analyzing the number of compartment crossings (Table 4). The statistical analysis reveals that in general chronic morphine treatment induces a higher number of crossings compared to chronic saline treatment ($F_{1,19}$=5.26; p = 0.03). Within the chronic morphine treated group the number of compartment crossing was statistically higher when conditioning was performed with saline (p = 0.0002). Nonetheless, when conditioning was performed with morphine, the number of crossings was not statistically different between animals chronically treated with saline and morphine (p = 0.98). Within the chronic saline treated group no differences were observed between conditioning with saline and morphine (p = 0.25).

Table 4: Effects of chronic morphine administration on locomotor activity. Locomotion was evaluated by the number of compartment crossings. Data are presented as mean ± SEM. *** p < 0. 001 *vs*. CPP-Saline/Saline of the morphine treated group.

Saline mini-pumps		Morphine mini-pumps	
Saline/Saline	Morphine/Saline	Saline/Saline	Morphine/Saline
73.25± 14.1	53.5± 5.72	111±12.2	50.875± 2.01***

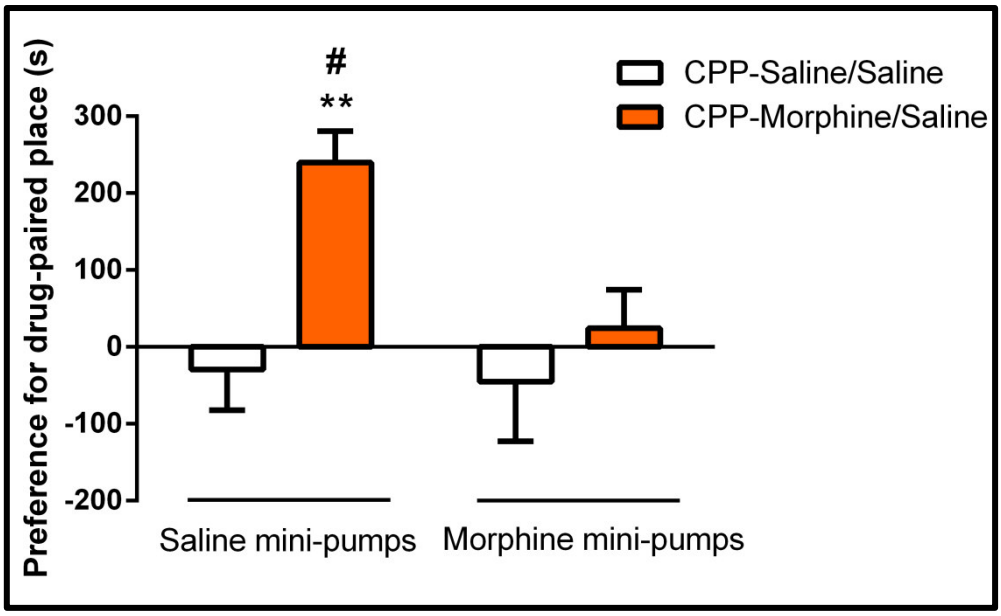


Figure 21: Effects of chronic morphine administration on morphine reward. During conditioning morphine (orange bars - 10 mg/kg, s.c.) was injected in alternate days in animals chronically administered with saline (n=7) or morphine (n=8). Control animals were injected saline (white bars) every day in animals chronically administered with morphine (n=3) or saline (n=4). Data are presented as mean ± SEM. (*p < 0. 01 *vs.* CPP-Saline/Saline; # p < 0. 05 *vs.* CPP-Morphine/Saline of the morphine treated group).

3. Involvement of the DRt in chronic morphine effects

3.1. Effects of DRt inactivation on nociceptive behavior

DRt inactivation was achieved by local injection of lidocaine. The effects of lidocaine on nociceptive behavior were 7 days after mini-pump implantation before (Figure 22; -Lido) and 30 min after (Figure 22; +Lido) lidocaine injection (Scheme 2).

In the von-Frey test, before lidocaine injection (i.e. at day 7 after mini-pump implantation) animals chronically administered with morphine showed a significant decrease of the withdrawal threshold compared with animals chronically administered with saline (p=0.0001;Figure 22 A). Lidocaine injection completely reversed the effects of chronic morphine (p=0.0001; Figure 22 A). No significant differences were observed after lidocaine injection on animals chronically administered with saline (Figure 22 A).

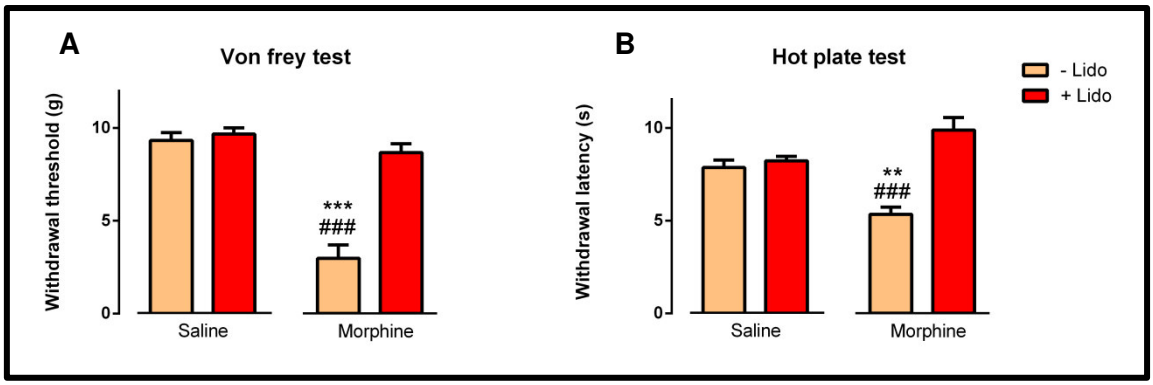


Figure 22: Effects of the DRt inactivation on mechanical allodynia tested by the von Frey test (A) and on thermal hyperalgesia tested by the hot plate test (B). Data are presented as mean ± SEM. (Saline group n=6; morphine group n=6 ; $^{###}$ $p < 0.001$ *vs.* + Lido ** $p < 0.01$; *** $p < 0.001$ *vs.* - Lido of the saline treated group).

In Hot plate test, before lidocaine injection animals chronically administered with morphine showed a significant decrease of the withdrawal latency compared with animals chronically administered with saline ($p=0.0044$;Figure 22 B). Lidocaine injection completely reversed the effects of chronic morphine ($p= 0.0001$; Figure 22 B). No significant differences were observed after lidocaine injection on animals chronically administered with saline.

Lidocaine injection in neighboring areas of the DRt like the VLM and Sol did not revert the effects of chronic morphine both in the von Frey (Fig. 23 A and C) and hot plate test (Fig. 23 B and D).

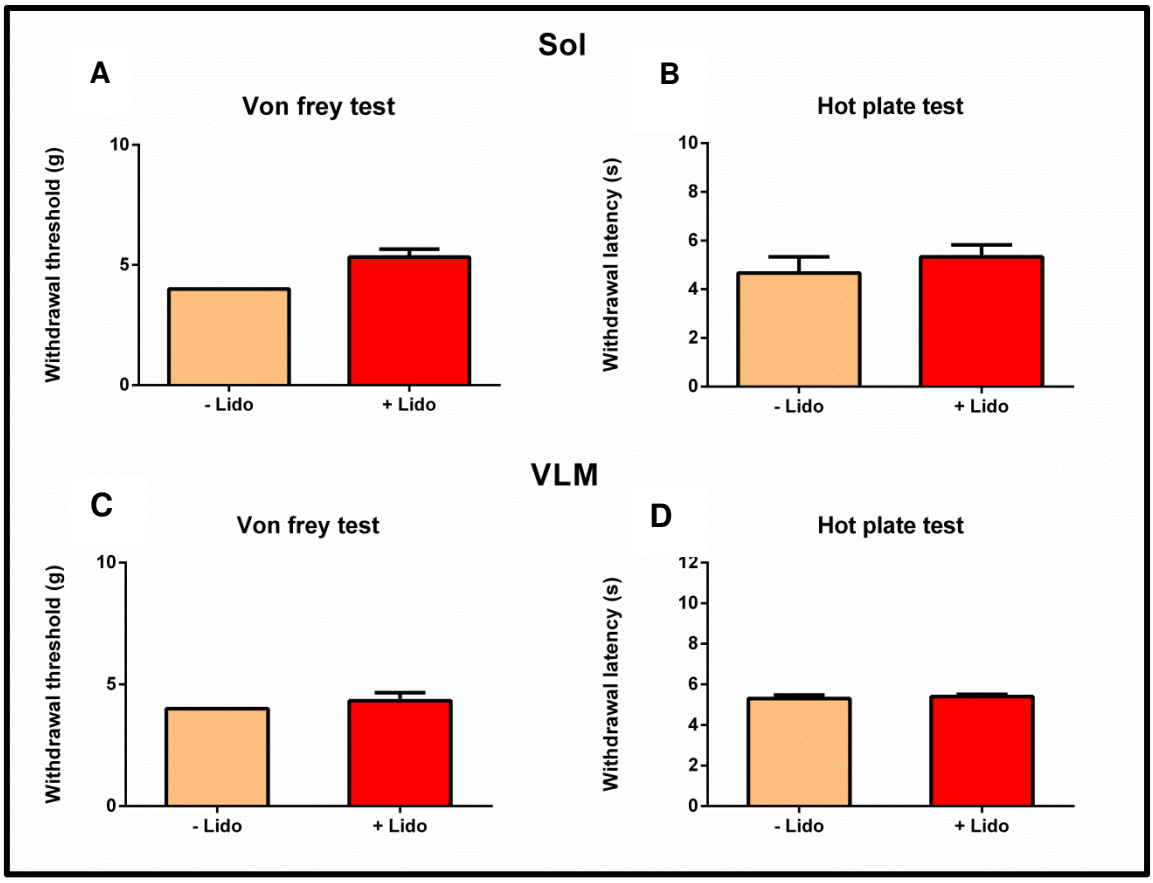


Figure 23: Effects of the VLM and Sol inactivation on mechanical allodynia tested by the von Frey test (A and C) and on thermal hyperalgesia tested by the hot plate test (B and D). Data are presented as mean ± SEM (n=3).

3.2. Effects of chronic morphine on pCREB and MOR expression at the DRt

Chronic morphine administration in naïve animals induced a significant increase of pCREB at the DRt compared with animals chronically treated with saline (p= 0.0304; Figure 24).

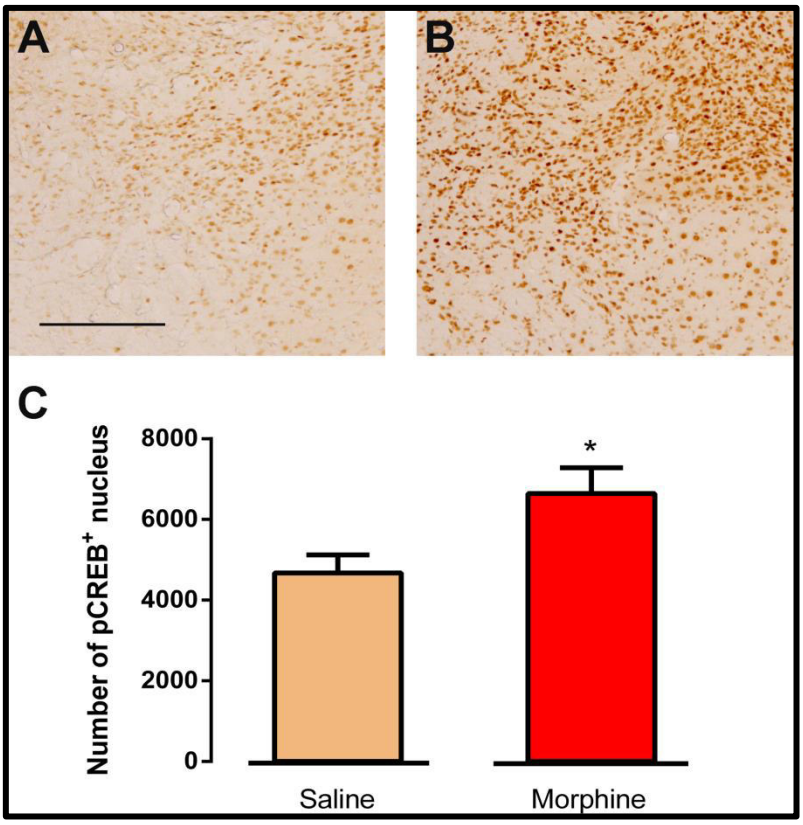


Figure 24: Effects of chronic administration of morphine on the expression of pCREB at the DRt. Representative photomicrographs of pCREB labeling at the DRt of animals chronically administered with morphine (A) and saline (B). Scale bar in A: 100μm (B is at the same magnification). Data in C are presented as mean ± SEM (n=6/group *p<0,05).

Chronic morphine administration in naïve animals induced a significant increase in the number of MOR cells at the DRt compared with animals chronically treated with saline (p= 0.0022; Figure 25 A). The percentage of double labelled cells for MOR and pCREB (Figure 25 C) was high both in saline and morphine treated animals and did not differ berween the groups (p= 0.2098 ; Figure 25 B); Saline treated animals showed an average of 92.6 ± 1.73% of co-localization (201.0 ± 11.0 double labelled cells; Figure 25 A and B). Morphine treated animals showed an average of 95.4 ± 0,85% co-localization (Figure 25 B).

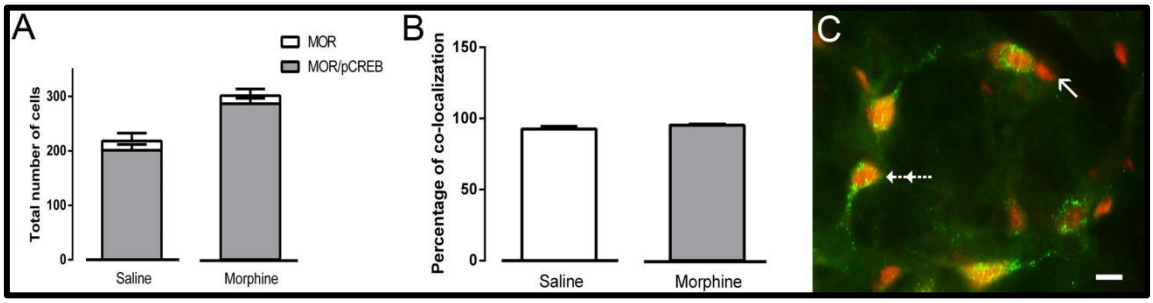


Figure 25: Effects of chronic administration of morphine on the expression of MOR and pCREB . Total number of neurons single labelled for MOR and double labelled for MOR and pCREB (A); Percentage of neurons that co-localize with CREB (B) in animals chronically treated with saline and morphine. Representative photomicrograph of double-labeled neurons for MOR and pCREB (yellowish; double arrows) and pCREB labeling (sigle arrow) at the DRt (C). Data in A and B presented as mean ± SEM (saline n=6; morphine n=5)

3.3. Effects of MOR Knock-down at the DRt

3.3.1. Pattern of lentiviral transduction

The pattern of lentiviral transduction was analyzed in animals injected with LV-Control and with the injection site centered at the DRt (Figure 26 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 26 B) around this central region. 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 26 C). Only animals with the injection site placed at the DRt were included in data analysis.

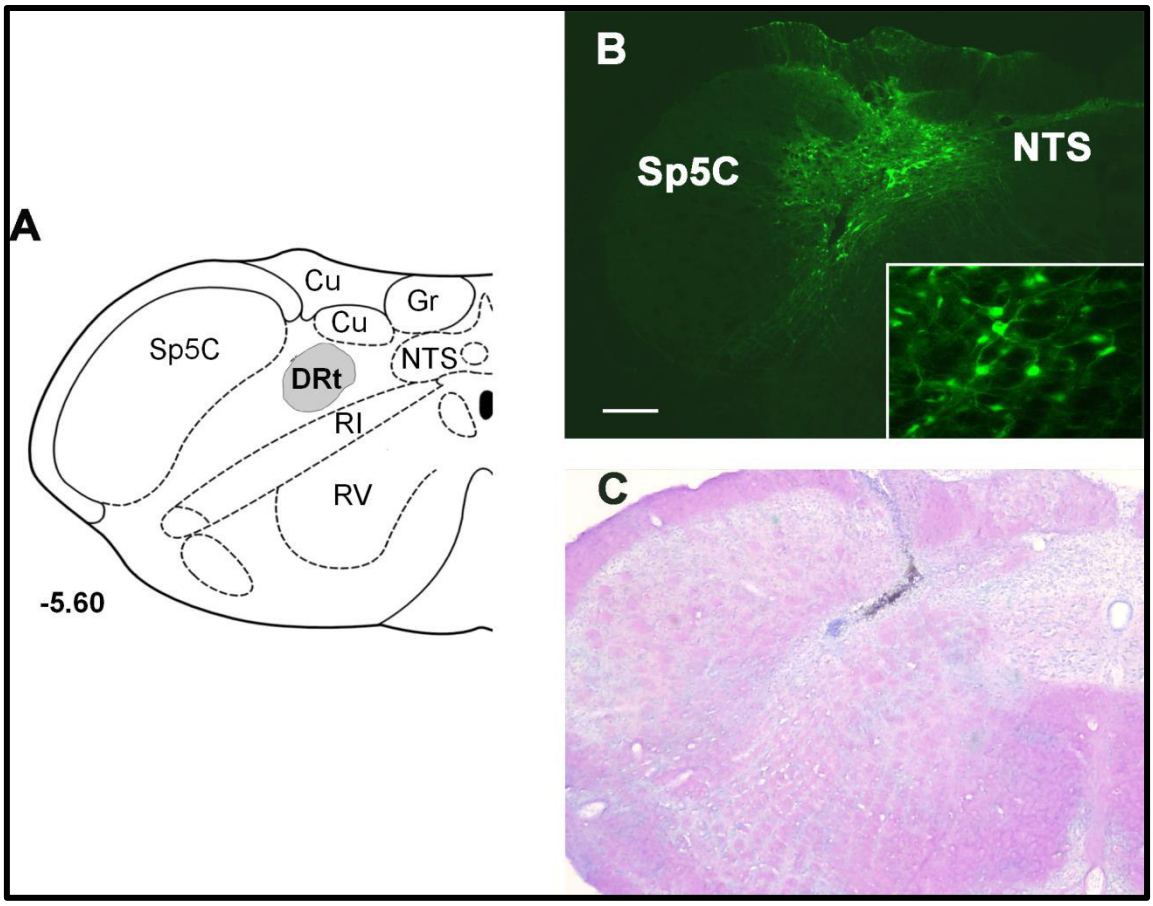


Figure 26: Localization of the injection site in the DRt. (A) Diagram depicting the location of the DRt, at 5.60 mm caudal to the interaural line adapted from the Paxinos and Watson (2007 Paxinos) (B) Fluorescence photomicrograph of the injection site at the DRt showing EGFP neurons better depicted in the insert. (C) Representative photomicrograph of a thionin-stained section illustrating a correct vector injection at the DRt. Scale bar in B: 200 µm (C is at the same magnification**).**

3.3.2. Effects of lentiviral transduction on MOR expression

MOR expression at the DRt was evaluated in naïve and SNI animals chronically treated with saline or morphine and injected with LV-cont or MOR-R. LV-cont-injected animals treated with morphine showed increased numbers of MOR-IR cells compared to LV-cont-injected animals treated with saline both in naïve (p=0.0001; Figure 27 C) and SNI animals (p=0.0001; Figure 27 C). In naïve rats, MOR-IR-injected animals showed a significant reduction of MOR-IR cells compared LV-cont-injected rats both after chronic treatment with saline (p=0.0001; Figure 27 C) and morphine (p=0.0001; Figure 27 C). In SNI animals MOR-IR also induced a significant reduction of MOR-IR cells compared to LV-cont-injected rats both after chronic treatment with saline (p=0.0040 Figure 27 C) and morphine (p=0.0001; Figure 27 C).

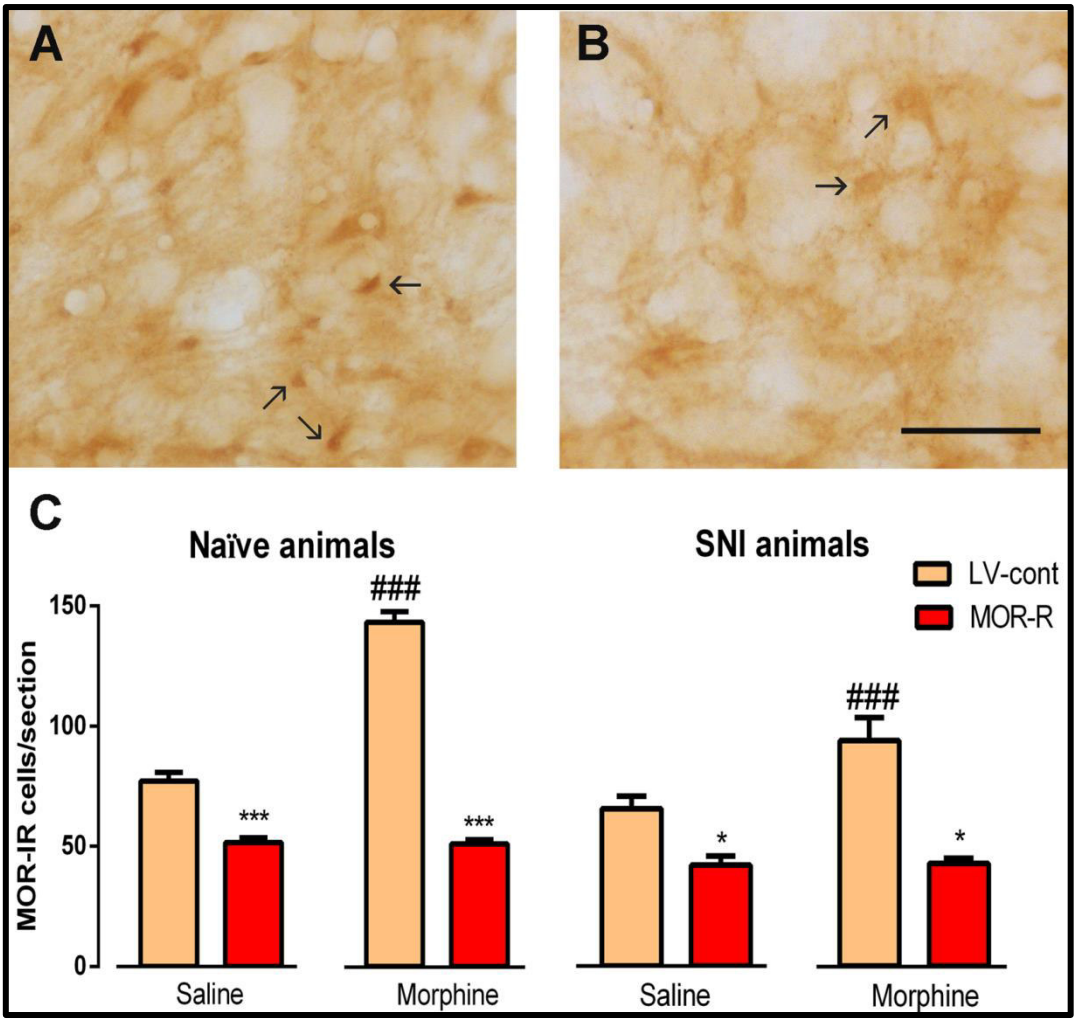


Figure 27: Evaluation of MOR expression by immunohistochemistry after injection of a lentiviral vector that knocks down MOR (MOR-R vector) and a control vector (LV-cont) at the DRt. Representative photomicrographs of MOR-IR cells in the DRt of naïve rats chronically treated with morphine and injected with LV-cont (A) and MOR-R (B). Typical MOR immunolabeling is marked by arrows. Scale bar in B: 50μm (A is at the same magnification). Data in C are represented as mean ±SEM (naïve animals treated with saline LV-Cont n = 7; MOR-R n = 6; or morphine: LV-Cont n= 6; MOR-R n=6 and SNI animals treated with saline: LV-Cont n = 6; MOR-R n = 5; or morphine: LV-Cont n= 5; MOR-R n=6; ###p < 0. 001 vs. LV-Cont of saline group; *p < 0. 05; *** p < 0. 001 vs. Lv-Cont).

3.3.3. Effects of lentiviral transduction on nociceptive behavior

3.3.3.1. Effects on naïve animals

The effect of the MOR knock-down at the DRt on naïve animals during chronic treatment with morphine was evaluated before and at 7 days after the mini-pumps implantation and stereotaxic injections of the lentiviral vectors (Scheme 3).

In von-Frey test, animals chronically treated with morphine and injected with the control vector showed a significant decrease of the withdrawal threshold compared to baseline ($p=0.0001$; Figure 28 A). Animals injected with MOR-R and chronically exposed to morphine also showed a significant decrease of the withdrawal threshold compared to baseline ($p=0.0001$; Figure 28 A). Nonetheless, this decrease was less important than that observed in animals injected with the control vector, indeed the withdrawal thresholds of MOR-R-animals were significantly higher than in the LV-Control group ($p= 0.0149$; Figure 28 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 decrease of the withdrawal threshold compared to the baseline ($p= 0.0001$; Figure 28 A) and the LV-Control group ($p= 0.0105$; Figure 28 A).

In the Hot plate test, animals chronically treated with morphine and injected with LV-Control showed a significant decrease in the withdrawal latency compared to baseline ($p= 0.0001$; Figure 28 B). Animals injected with MOR-R showed higher withdrawals latencies compared to the LV-Control group ($p= 0.0032$; Figure 28 B) with values similar to the baseline (Figure 28 B). In animals chronically treated with saline, the injection of LV-Control did not induce significant alterations compared to baseline (Figure 28 B). MOR-R induced a significant decrease of the withdraw latency compared to baseline ($p = 0.0129$; Figure 28 B).

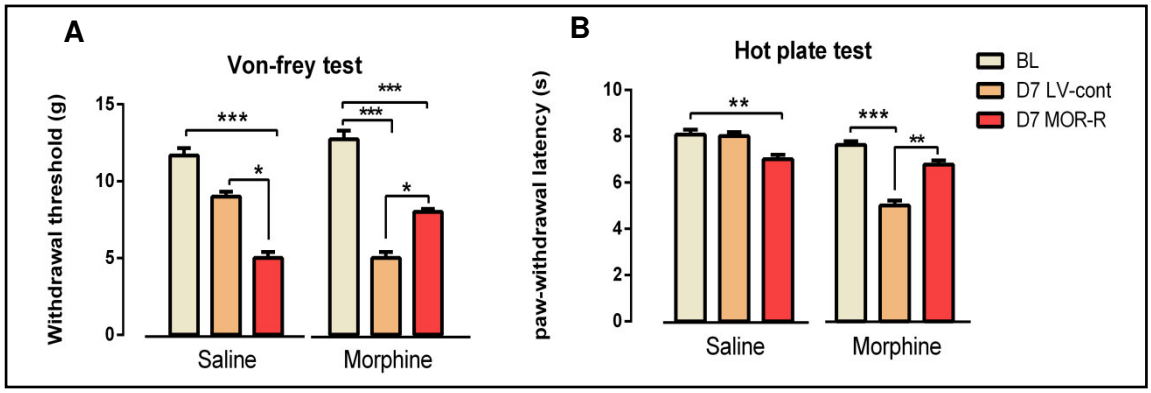


Figure 28: Effects of MOR knock-down at the DRt on mechanical allodynia tested by the von Frey test (A) and on thermal hyperalgesia tested by the hot plate test (B) during chronic saline or morphine administration. Data are presented as mean ± SEM. (chronic morphine treatment: LV-Control-injected n=6, MOR-R-injected n=5; chronic saline treatment: LV-Control-injected n=5; MOR-R-injected n=6; * p < 0. 05; **p< 0. 01; *** p < 0. 001).

3.3.3.2. Effects on SNI animals

The effect of MOR knock-down at the DRt on SNI animals during chronic treatment with morphine was evaluated before and 7 days after the mini-pumps implantation and stereotaxic injections (i.e 3 to 4 weeks after SNI induction; Sheme 5).

In von-Frey test no significant differences were observed (Figure 29 A).

In the pin-prick test, animals chronically treated with morphine and injected with the control vector showed a significant augmented withdrawal duration compared to baseline (p=0.0045; Figure 29 B). Animals injected with MOR-R showed a significantly lower withdrawal duration compared to the LV-Control group (p= 0.0014: Figure 29 B) with values similar to the baseline (Figure 29 B). In animals chronically treated with saline, the injection of LV-Control did not induce significant alterations compared to baseline (Figure 29 B). MOR-R induced a significant increase of the withdrawal duration compared both to the LV-Control group (p= 0.0017; Figure 29 B) and baseline (p= 0.0001; Figure 29 B).

In the acetone test, animals chronically treated with morphine and injected with LV-Control showed a significant increase of the withdrawal duration compared to baseline (p= 0.0041; Figure 29 C). Animals injected with MOR-R showed a significantly lower withdrawal duration compared to the LV-Control group (p= 0.0001; Figure 29 C) with values similar to the baseline (Figure 29 C). In animals chronically administered with saline the injection of LV-Control did not induce significant alterations compared to baseline (Figure 29 C). MOR-R induced a significant increase of the withdraw duration

compared both to the LV-Control group (p= 0.0001; Figure 28 C) and baseline (p= 0.0001; Figure 29 C).

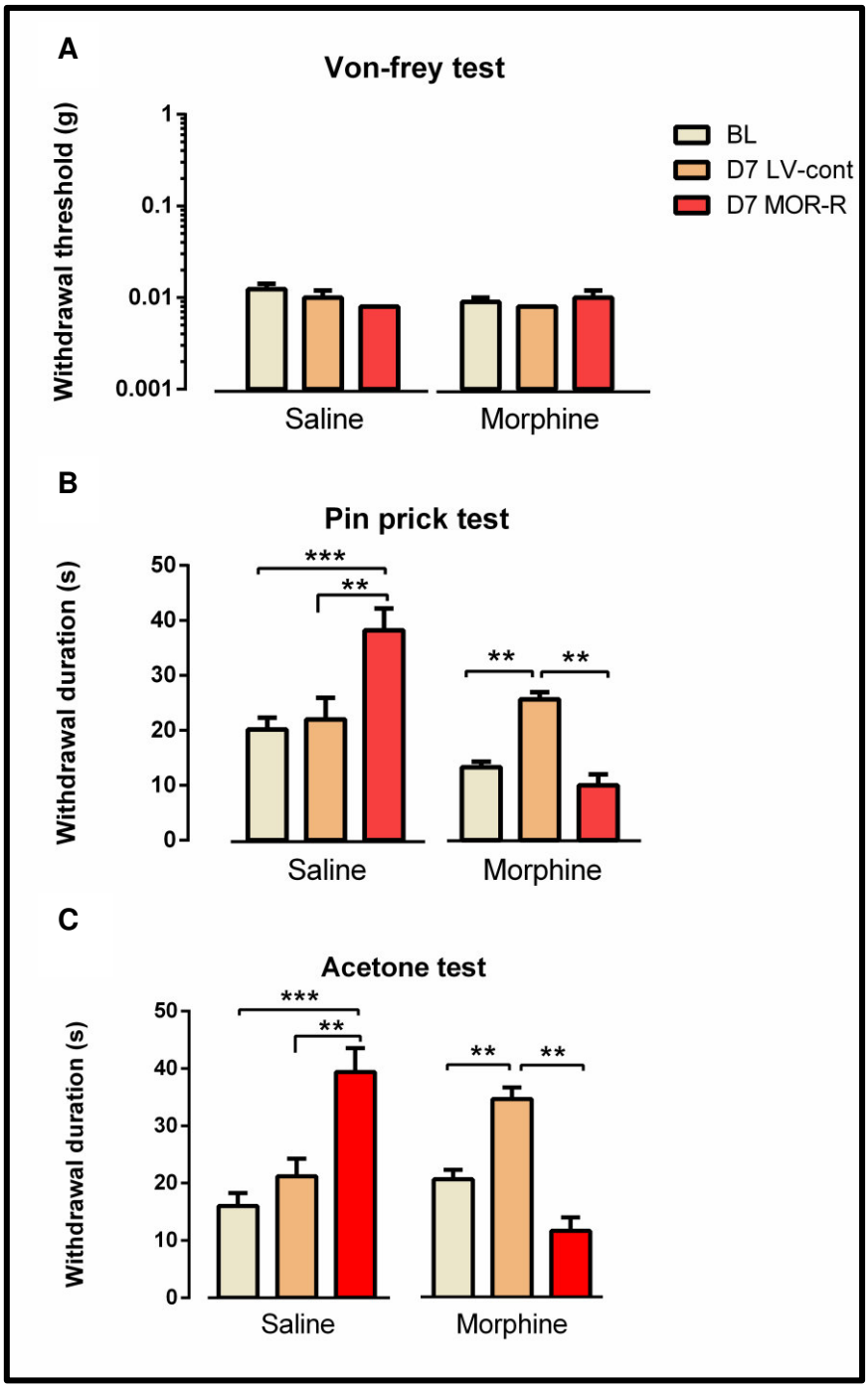


Figure 29: Effects of MOR knock-down at the DRt on mechanical allodynia tested by the von Frey test (A), mechanical hyperalgesia tested by the pin prick test (B) and cold allodynia tested by the acetone test (C) during chronic saline or morphine administration. Data are presented as mean ± SEM. (chronic morphine treatment: LV-Control-injected n=6, MOR-R-injected n=6; chronic saline treatment: LV-Control-injected n=5; MOR-R-injected n=6 ** p < 0. 01; *** p < 0. 001)

3.3.4. Effects of lentiviral transduction on reward behavior

The CPP test was performed on animals chronically administered morphine (45 $\mu g^{-1}.\mu L^{-1}.h^{-1}$) and injected with MOR-R at the DRt. Animals injected with MOR-R and chronically treated with morphine and conditioned with morphine (10 mg/Kg; s.c.) (n=3) spent more time in the morphine-paired chamber compared to animals only chronically treated with morphine and conditioned with morphine (n=8) although this comparison did not reach statistical significance (Figure 30).

The statistical analysis showed no differences in the number of crossings between the groups (Table 5).

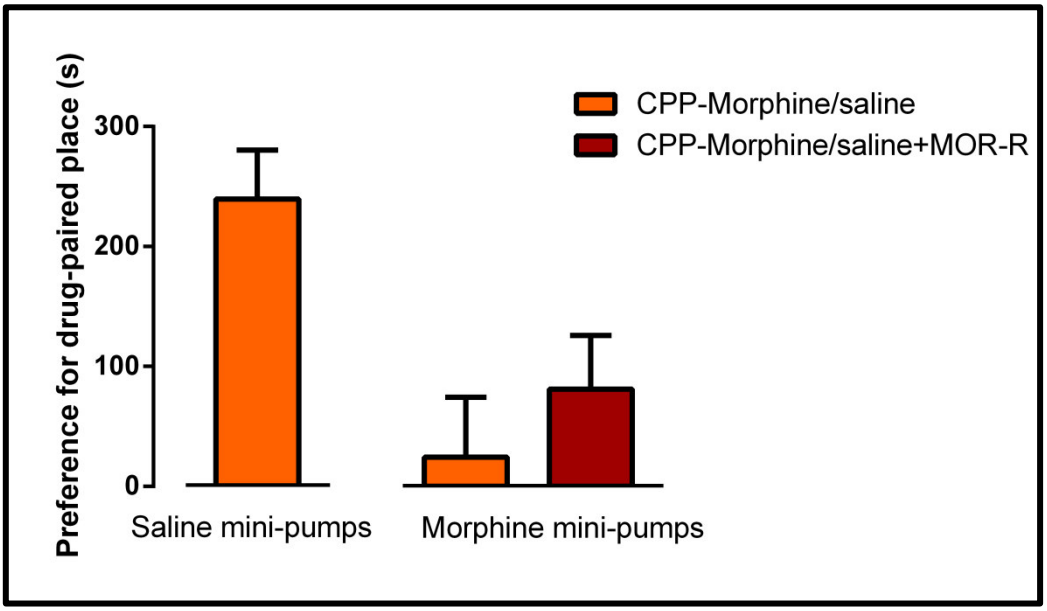


Figure 30: Effects of MOR knock-down at the DRt on morphine reward. During conditioning morphine (10 mg/kg, s.c. - orange bars) was injected in alternate days in animals chronically administered with saline (n=7) or morphine (n=8). In MOR-R-injected animals morphine (red bar) was injected in alternate days in animals chronically administered with morphine (n=3). Data are presented as mean ± SEM.

Table 5: Effects of chronic morphine administration on locomotor activity. Locomotion was shown as the number of compartment crossings. Data are means ± SEM.

Saline mini-pumps	Morphine mini-pumps	
Morphine/Saline	Morphine/Saline	Morphine/Saline+ MOR-R
53.5± 5.72	50.875± 2.01	63.75± 3.42

Discussion and Conclusions

The results gathered in the present thesis show that chronic administration of morphine induces mechanical and thermal sensibility in naïve animals and, an aggravation of pre-existing pain in animals with chronic neuropathic pain. In the CPP test, neuropathic animals chronically treated with morphine failed to show preference for the morphine-paired chamber which indicates a loss of the reward effect of morphine. Inactivation of the DRt with lidocaine fully reversed mechanical allodynia and thermal hyperalgesia of morphine-infused animals. Chronic morphine treatment induces an increase of pCREB and MOR expression at the DRt. Lentiviral-mediated MOR knock-down at the DRt prevented the development of opioid-induced hyperalgesia and in the CPP test, the preliminary results obtained indicate a small reversion of the loss of the reward effect of morphine.

1. Effects of chronic administration of morphine

Our results show that chronic morphine administration in naïve animals induce enhanced pain responses during acute pain induction, which is in agreement with previous reports [108-110]. Moreover, we show for the first time that chronic morphine administration 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, 34, 50].

Unlike naïve animals, neuropathic animals did not show an aggravation of mechanical thresholds upon chronic morphine administration. This is probably due to technical issues. Indeed, due to neuropathic pain, animals respond to the lowest microfilament available (0.008 g), therefore it was impossible to observe further decreases of the mechanical threshold. However, since in naïve animals chronic morphine induced a decrease of mechanical thresholds it is likely that in SNI animals this pain modality might be affected.

2. Involvement of the DRt in OIH

The injection of lidocaine directly into the DRt fully reversed mechanical allodynia and thermal hyperalgesia after chronic morphine exposure. These results show that descending facilitation from the DRt is involved in OIH. Furthermore, our results indicate that MOR is likely involved in increased DRt facilitation during chronic administration of morphine. Usually MOR exerts a inhibitory action by inhibiting AC activity, causing activation of Kir3 K^+ channels and inhibition of the voltage-dependent Ca^{2+} channels, leading to hyperpolarization of the cell [111, 112]. This inhibitory effect would decrease DRt facilitation and MOR knock-down at the DRt would then lead to disinhibition of the DRt and thus hyperalgesia. The hyperalgesic effects observed in naïve animals or the aggravation of pain behaviors in SNI animals upon saline treatment are consistent with the inhibitory effects of MOR. Previous reports also confirm the inhibitory effects of opioids at the DRt in acute pain models [40, 44]. The opposite effects of MOR knock-down during chronic morphine, i.e. MOR knock-down prevented the development of OIH both in naïve and SNI animals, are more consistent with MOR mediating facilitatory effects. This switch of MOR signalling have been also reported during chronic morphine in vitro [111, 113-115], and at the locus coeruleus [112, 116] and at the nucleus acumbens [112].

Accelerated desensitization, increased constitutive activity or endocytosis of the receptor are suggested as cellular adaptations to chronic morphine exposure [117]. The most likely explanation might lie on a switch on the G-protein coupled to MOR, from $G_{i/o}$ to a G_s-protein, leading to an increase in AC, which causes augmented levels of cAMP, altering the hyperpolarized state of the neuron by changing the intracellular concentrations of Ca^{2+} and K^+ [118, 119]. Futher corroborating this, chronic morphine exposure in naïve animals also increased pCREB levels at the DRt and MOR-IR cells colocalized with pCREB. The increased expression of pCREB during chronic morphine administration has been previously described in other areas like the nucleus of the solitary tract [59] and locus coeruleus [62]. The evaluation of the co-localization of MOR and pCREB in neurophatic animals was not performed but will be held in the future.

It is worth noting that chronic morphine increased MOR expression more significantly in naïve animals than in neuropathic animals. This may be the due to the neuropathy. Indeed, several studies reveal that chronic pain induces down-regulation of MOR signaling and expression [120, 121]. This decrease of the MOR expression likely contributes to increased descending facilitation during chronic pain [11, 122]. In

spite of this chronic treatment with morphine induces an increase of MOR expression, leading to an increase of pain sensibility characteristic of the OIH.

3. Reward effects of morphine

Pain and reward are opponent and interacting processes with a large overlap of the brain systems that control them. Opioids are particularly important for processing the aspects of reward as well as the processes of pain modulation [73]. Relief from an unpleasant state is usually associated with rewarding qualities. Pain relief is an important type of reward, in particular in chronic pain [73, 74], and has been proven in humans [123] and rats [124]. In fact, in the past years, the CPP test, has also been used for the assessment of ongoing pain both in inflammatory [125, 126] and in neuropathic pain models [127, 128].

Our results show that chronic morphine exposure reversed the preference of the animals for the morphine-paired chamber. This loss morphine reward in neuropathic animals is not associated with sedative effects of morphine since the number of the compartments crossing is similar to the animals chronically treated with saline. On the contrary, the chronic administration of morphine in animals increased locomotor activity [105, 129] which was also demonstrated in our work in animals chronically administrated with morphine and conditioned with saline.

In the reward system, morphine binds to MOR expressed on GABAergic neurons which normally inhibit dopaminergic neurons in the VTA. MOR activation on GABAergic neurons leads to disinhibition of dopaminergic neurons witch induces an increase of dopamine release in the NAc [120]. Therefore, the loss of morphine reward should be the result of decreased inhibition of GABAergic neurons, also consistent with the switch of MOR activity, resulting thus in a reduction of dopamine release in areas involved in the reward system.

Given the overlap of these two systems, to assess the influence of pain modulatory areas in the reward system, we studied the effect of the MOR knock-down at the DRt on the effect of morphine reward. Our hypothesis is that during chronic pain, increased descending facilitation [11, 122] along with the aggravation of DRt descending facilitation, due to chronic morphine exposure shown by our results, likely lead to a less pain relief/reward (Figure 31). Therefore, if we reduce MOR expression

at the DRt, this should lead to more pain relief and consequently more morphine reward.

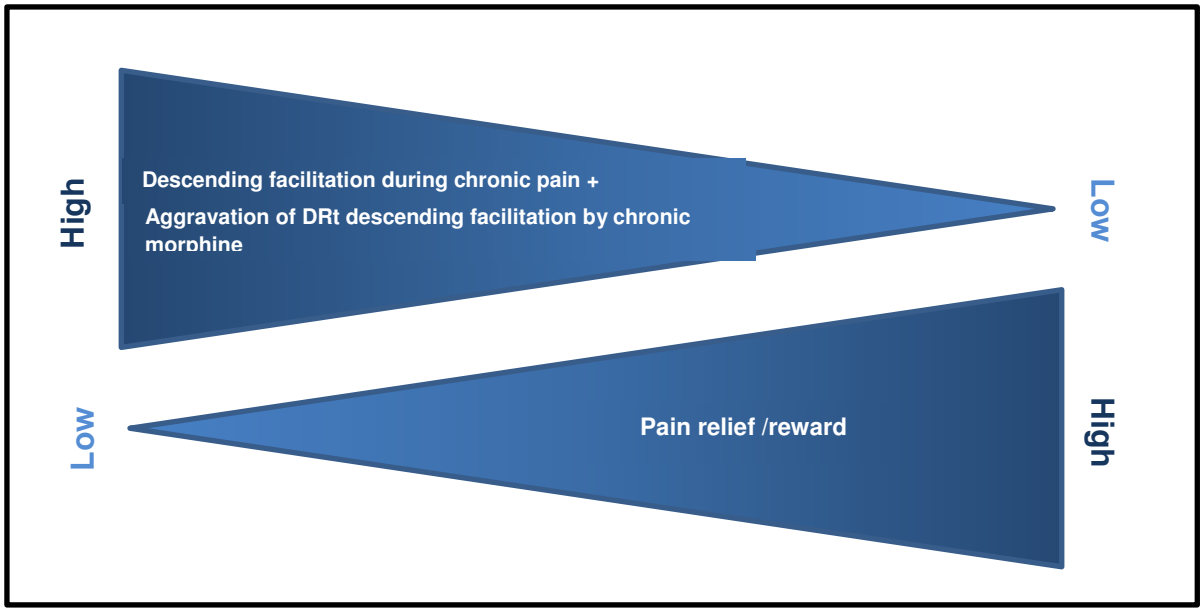


Figure 31: Schematic representation of the relationship between descending facilitation and pain relief/reward.

Although these experiments were performed with a small number of animals, they are indicative of a reversion of the loss of the reward effect of morphine. Increasing the number of animals should reinforce these results. Our results suggest that the DRt is connected to the reward system. These connections are probably relayed by other areas connected to the DRt [130] that are involved both in pain and reward such as the amygdala, the dorsal and ventral striatum and insular cortex. In future studies it would be interesting to evaluate the effect of these connections on the effects of pain relief and reward mediated by morphine.

4. Conclusions and future perspectives

Opioids are a common therapy to treat moderate and severe pain. Unravelling the molecular mechanisms involved in OIH is fundamental since, instead of relieving pain, these drugs may be responsible for hyperalgesia, in some patients.

We show in this thesis that prolonged exposure to opioids likely induces plastic changes in opioidergic circuits causing an exacerbation of DRt descending facilitation that contributes to OIH. In continuity of this thesis it would be interesting to further

extend the study of pCREB and MOR in neuropathic animals mainly by inhibiting some kinases responsible for the activation of CREB at the DRt.

Unveiling the molecular mechanisms behind OIH is the key to overcome its side effects, namely the loss of the pain relief observed after morphine chronic administration at the CPP test. In the future, it would be interesting to continue the studies of this thesis by evaluating the connections between the DRt and areas involved in pain and reward on the effects of pain relief mediated by morphine.

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Appendix A: Composition of solutions

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

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

Na_2 $H_2PO_4H_2O$ – 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

$MgCl_2$ 6 H_2O – 0,32 g

$MgSO_4$ 7 H_2O – 0,1 g

NaH_2PO_4 H_2O – 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.