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Opioidergic modulation of descending pain facilitation:

Studies in models of neuropathic pain and opioid induced hyperalgesia

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TESE DE DOUTORAMENTO APRESENTADA

À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO EM

NEUROCIÊNCIAS, RAMO NEUROCIÊNCIAS EXPERIMENTAIS

Dissertação apresentada à Faculdade de Medicina da Universidade do Porto para candidatura ao grau de Doutor no âmbito do Programa Doutoral em Neurociências.

A candidata realizou o presente trabalho com o apoio de uma bolsa de doutoramento de quatro anos, concedida pelo Programa Operacional Regional do Norte, NORTE2020, e referenciada NORTE-08-5369-FSE-000026

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Walter Friedrich Alfred Osswald

À Professora Doutora Isabel Martins

À Professora Doutora Isaura Tavares

Ao meu pai

Ao meu irmão

Agradecimentos

Todo o trabalho desenvolvido na presente dissertação não teria sido possível sem o apoio de inúmeras pessoas. Um doutoramento é uma longa viagem, cheia de desafios e inúmeros percalços, que se torna mais fácil quando temos a sorte de encontrar pessoas nos ajudam a encontrar o melhor rumo em cada momento da caminhada. É com enorme prazer que aqui expresso toda minha gratidão para com todas elas.

O mestrado em Bioquímica trouxe-me até à Unidade de Biologia Experimental onde conheci a minha orientadora Professora Isabel Martins. À Professora Doutora Isabel Martins, agradeço a dedicação, paciência e orientação no decorrer desta jornada. A ela devo tudo o que aprendi e todas as palavras do mundo nunca serão suficientes para lhe agradecer. Agradeço, de igual forma, à Professora Doutora Isaura Tavares, por me ter aceitado no seu grupo de investigação, pelos conhecimentos transmitidos e pela amizade durante todos estes anos. À Professora Deolinda Lima, anterior diretora do programa doutoral, agradeço a oportunidade de seguir com a minha formação e, apesar de todas as responsabilidades que tinha a seu cargo, por estar sempre disponível para batalhar pelos interesses dos alunos.

É claro que este percurso não teria sido o mesmo sem os amigos que fiz. Ao Zé Tiago, companheiro desta caminhada, agradeço não só toda a ajuda que me deu mas também por me fazer rir todos os dias. Agradeço à Marília, a sua sempre inteira disponibilidade, as boas energias e todos os momentos de boa disposição. Aos restantes, atuais e antigos, elementos do grupo, agradeço a amizade e partilha de experiências. Foi um privilégio trabalhar com todos vocês. À Raquel e à Joana, agradeço os bons momentos que passamos. O caminho foi difícil mas a nossa amizade e cumplicidade fez com que conseguíssemos ultrapassar os desafios que surgiram. Obrigada Sílvia, Alonso, Bárbara, Marta, Lena e Raquel Silva pela vossa companhia. Vocês tornaram tudo mais divertido. Aos restantes elementos do Departamento de Biomedicina um enorme obrigada por estarem sempre dispostos a ajudar e tornarem esta jornada mais completa e especial.

Por terem estado sempre do meu lado, não posso deixar de agradecer ao meu pai e ao meu irmão. Obrigada pela força que me transmitiram e pelo conforto que sempre encontrei nas vossas palavras. A toda a minha família e aos amigos de longa data, obrigada por estarem sempre presentes e pelo apoio constante.

Por fim, ao Rafael, por ser a pessoa que mais acredita em mim e me encoraja.

Em obediência ao disposto no Decreto-Lei 388/70, Artigo 8º, parágrafo 2, declaro que efetuei o planeamento e execução do trabalho experimental, observação do material e análise dos resultados e redigi as publicações que fazem parte integrante desta dissertação.

Publication I. Costa, A. R., Carvalho, P., Flik, G., Wilson, S. P., Reguenga, C., Martins, I., & Tavares, I. (2019). Neuropathic Pain Induced Alterations in the Opioidergic Modulation of a Descending Pain Facilitatory Area of the Brain. Frontiers in cellular neuroscience, 13, 287.

Publication II. Costa, A. R., Sousa, M., Wilson, S. P., Reguenga, C., Teixeira-Pinto, A., Tavares, I., Martins, I. (2020) Shift of μ-opioid receptor signaling in the dorsal reticular nucleus is implicated in morphine-induced hyperalgesia in male rats. Anesthesiology.

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List of abbreviations

5-HT	5-hydroxytryptamine or serotonin
5-HT3	5-hydroxytryptamine receptor 3
AC	adenylate cyclase
ACC	anterior cingulate cortex
AMPA	a-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid
Amy	amygdala
BDNF	brain-derived neurotrophic factor
Ca^{2+}-CaM	calcium-calmodulin
CALCRL	calcitonin receptor-like receptor
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
Cu	nucleus cuneate
DLF	dorsolateral funiculus
DNIC	diffuse noxious inhibitory control
DOR	δ-opioid receptor
DRt	dorsal reticular nucleous
ERK	extracellular-signal-regulated kinase
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GLuT	glutamate transporter
GPCR	G protein-coupled receptors
GTP	guanosine triphosphate
Hyp	hypothalamus
IASP	international association for the study of pain
IL-1β	interleukin 1 beta;
IL-6	interleukin 6
Ins	insular cortex
KOR	κ-opioid receptor
L-Arg	L-arginine
LC	locus coeruleus

mGluR1	metabotropic glutamate receptor 1
mMRF	medial medullary reticular formation
MOR	μ-opioid receptor
Mot	motor cortex
NK-1	neurokinin-1
NMDA	N-methyl-D-aspartic acid
NO	nitric oxide
NOP	nociceptin opioid receptor
NOS	nitric oxide synthase
NTS	nucleus tractus solitaries
OIH	opioid induced hyperalgesia
PAG	periaqueductal gray matter
pCREB	phosphorylated cAMP response element binding protei
pERK	phosphorylated extracellular-signal-regulated kinase
PKA	protein kinase A
PKC	protein kinase C
pMOR	phosphorylated μ-opioid receptor
RVM	rostral ventromedial medulla
SNI	spared nerve injury
Som	somatosensory cortex
SP	substance P
Sp5C	spinal trigeminal nucleus, pars caudalis
TLR-4	toll-like receptor 4
1TM	one transmembrane domain
6TM	six transmembrane domain
7TM	seven transmembrane domain
TNFα	tumor necrosis factor alpha
VLM	ventrolateral medulla
VRt	ventral reticular nucleus
WAH	withdrawal-associated hyperalgesia

Abstract

The opioidergic system plays a major role in the control of descending pain modulatory areas of the brain. However, our knowledge is less comprehensive regarding the alterations of the system during pathological conditions, namely in the chronic neuropathic pain and in the paradoxical opioid induced hyperalgesia (OIH) phenomena. This is particularly relevant at supraspinal areas devoted to descending pain facilitation, namely because the maintenance of neuropathic pain and OIH may rely on increased descending facilitation. The studies included in the present PhD thesis aimed at studying the opioidergic modulation during neuropathic pain and OIH at the dorsal reticular nucleus (DRt), a medullary area that stands out for its descending facilitatory role. We focused our studies on the μ-opioid receptor (MOR), since MOR agonists are among the most powerful analgesic drugs available to treat pain, and alterations in MOR signaling appear to be involved in the pathophysiology of neuropathic pain and OIH.

To study the opioidergic modulation of the DRt during neuropathic pain we used the spared nerve injury (SNI) model of neuropathic pain. First, we performed a series of behavioral experiments in naïve animals which consisted on the evaluation of the effects of the pharmacological activation and lentiviral mediated knock-down of MOR at the DRt. We showed that MOR-knockdown at the DRt increased the sensitivity to thermal and mechanical stimuli while the MOR agonist DAMGO induced the opposite effect. We also evaluated in naïve animals the effects of genetic or pharmacological blockade of MOR at the DRt on the analgesic effects of systemic morphine. We showed that MOR-knockdown or pharmacological blockade of MOR with the antagonist CTAP decreased or inhibited, respectively, the analgesic effects of systemic morphine. Then, we evaluated the extracellular levels of the methionine- and leucine- enkephalin peptides at the DRt of

sham- and SNI- animals using *in vivo* microdialysis. Our results show increased levels of extracellular enkephalinergic peptides at the DRt of SNI-animals compared to sham-animals. We also evaluated in the DRt MOR mRNA levels by quantitative real-time PCR and the expression of MOR and phosphorylated MOR by immunohistochemistry. Compared to sham, SNI-animals showed no alterations in MOR mRNA levels, lower numbers of MOR-labeled cells and increased numbers of phosphorylated MOR-labeled cells. Finally, we performed a series of behavioral studies in SNI animals to determine the potency of systemic morphine and the effects of the genetic and pharmacological manipulation of MOR at the DRt. We showed a reduced antinociceptive potency of systemic morphine in SNI-animals compared to sham animals. Lentiviral-mediated MOR-overexpression at the DRt of SNI-animals produced no effects on mechanical sensitivity. DAMGO induced antinociceptive effects only after MOR-overexpression. Together, these results indicate that neuropathic pain induce MOR down-regulation, desensitization and phosphorylation at the DRt, impairing the opioidergic inhibition of DRt pain facilitatory actions.

To study the opioidergic modulation of the DRt during OIH, we used a validated model by performing a subcutaneous implantation of osmotic mini-pumps containing morphine. We performed a time-course evaluation of the effects of morphine administration on pain-like behaviors before and 5 hours, 2, 4 and 7 days after mini-pumps implantation. The continuous infusion of morphine initially produced antinociception, followed by the development of a marked hypersensitivity to mechanical and thermal stimuli. Then, to study the involvement of the DRt in the mediation of OIH, we used lidocaine to inactivate pharmacologically the DRt. Lidocaine administration at the DRt fully reversed mechanical and thermal hypersensitivity in morphine-infused animals. Afterwards, we evaluated the role of MOR at the DRt in OIH. Morphine-infusion

increased the number of MOR-labeled cells, without altering MOR mRNA levels. MOR knockdown in morphine-infused animals attenuated the development of mechanical and thermal hypersensitivity, while in saline-infused animals it increased pain behaviors. Finally, we evaluated the effects of chronic morphine infusion on MOR function and signaling pathways at the DRt. For that, we used immunohistochemistry to evaluate the expression of the phosphorylated cAMP response element-binding (pCREB), a downstream marker of the excitatory signaling transduction pathway of MOR. Morphine-infusion increased the number of pCREB-labeled cells at the DRt. Then we determined the effects of DAMGO, an ultra-low dose of naloxone, which prevents MOR coupling to the Gs-excitatory protein, and the PKA inhibitor H-89, on mechanical and thermal sensitivity. DAMGO induced antinociceptive effects in saline-infused animals and increased mechanical hypersensitivity in morphine-infused animals. Naloxone restored the antinociceptive effect of DAMGO in morphine-infused animals, and decreased pCREB levels. The PKA inhibitor H-89, produced no effect. These results indicate that chronic morphine infusion switches MOR coupling to an excitatory Gs-protein at the DRt, altering MOR signaling from inhibitory to excitatory, likely enhancing DRt pain descending facilitation.

Collectively, the data gathered in this dissertation reveal that neuropathic pain and OIH induce different types of adaptations of MOR at the DRt which lead, in both situations, to an impairment of the MOR inhibitory function at the DRt. Taking into account the important role of opioids in the inhibition of pain facilitatory actions from the DRt, those alterations probably contribute to enhancing descending facilitation from that medullary area. MOR adaptations at supraspinal pain facilitatory areas likely underly the involvement of descending pain facilitation during neuropathic pain and OIH. New

avenues directed to the opioidergic brain system may be considered in the future for pain treatment.

Resumo

O sistema opioidérgico desempenha um papel fundamental no controlo das áreas encefálicas envolvidas na modulação descendente da dor. No entanto, o nosso conhecimento é menos abrangente relativamente às alterações que o sistema sofre em situações patológicas, como no caso da dor neuropática e na hiperalgesia induzida por opioides (HIO). Isto é particularmente relevante em áreas supra-espinhais envolvidas na facilitação descendente da dor, uma vez que a manutenção da dor neuropática e da HIO pode depender do aumento da facilitação descendente. Os estudos incluídos na presente tese visaram estudar a modulação opiódergica do núcleo reticular dorsal (DRt), uma área localizada no bolbo raquidiano que se destaca pelo seu papel na facilitação da dor, durante a dor neuropática e a HIO. Os estudos focaram-se no recetor μ-opioide (MOR), uma vez que os agonistas deste recetor estão entre os fármacos mais eficazes para o tratamento da dor, e alterações nas suas vias de sinalização aparentam estar envolvidas na fisiopatologia da dor neuropática e da HIO.

Para estudar a modulação opioidérgica do DRt durante a dor neuropática, foi utilizado o modelo de lesão do nervo ciático (modelo "SNI" do inglês "spared nerve injury"). Começamos por realizar uma série de experiências comportamentais em animais naïve que permitiram avaliar os efeitos da ativação farmacológica ou da diminuição da expressão (*knockdown*), mediada por um vetor lentivírico, de MOR no DRt. O *knockdown* de MOR resultou no aumento da sensibilidade a estímulos térmicos e mecânicos, enquanto a ativação farmacológica do recetor, com o agonista DAMGO, produziu o efeito oposto. Também foi avaliada a consequência do *knockdown* ou do bloqueio farmacológico de MOR no DRt no efeito analgésico da morfina administrada de forma sistémica. O *knockdown* de MOR diminuiu o efeito analgésico da morfina, enquanto o

bloqueio farmacológico do recetor, com o antagonista CTAP, inibiu os efeitos da morfina. De seguida, avaliámos por microdiálise os níveis extracelulares de metionina-encefalina e de leucina-encefalina no DRt de animais *sham* e SNI. Animais SNI apresentaram níveis mais elevados de encefalinas no DRt em comparação com animais sham. Foram também avaliados os níveis de mRNA do gene que codifica MOR por PCR quantitativo em tempo real e a expressão do recetor e da sua forma fosforilada por imuno-histoquímica. Nos animais SNI, comparados com os animais *sham*, não se encontrou alterações nos níveis de mRNA, mas os números de células a expressar MOR eram mais baixos e o número de células a expressar a forma fosforilada do recetor estava aumentado. Por fim, foram realizadas uma série de experiências comportamentais em animais SNI para avaliar a potência analgésica da morfina administrada de forma sistémica e os efeitos da manipulação genética e farmacológica de MOR no DRt. Nos animais SNI, por comparação com animais *sham*, verificou-se uma diminuição da potência analgésica da morfina. A sobre-expressão de MOR no DRt de animais SNI, mediada por lentivírus, não produziu nenhum efeito na sensibilidade mecânica. A administração de DAMGO no DRt apenas teve efeito antinociceptivo após a sobre-expressão de MOR. Estes resultados sugerem que a dor neuropática induz diminuição da expressão, dessensibilização e fosforilação de MOR, comprometendo a inibição opioidérgica sob as ações facilitatórias do DRt.

Para estudar a modulação opioidérgica do DRt durante a HIO, usou-se um modelo experimental que consiste na implantação de mini-bombas osmóticas para libertação de morfina. A avaliação dos efeitos da administração sistémica de morfina no comportamento nociceptivo foi realizada antes e 5 horas, 2, 4 e 7 dias após o implante das mini-bombas. Inicialmente, a administração de morfina produziu um efeito antinociceptivo, seguido pelo desenvolvimento de hipersensibilidade a estímulos

mecânicos e térmicos. Para estudar o envolvimento do DRt na mediação da HIO, inativou-se farmacologicamente a área com lidocaína. A administração de lidocaína no DRt reverteu totalmente a hipersensibilidade mecânica e térmica induzida pela administração de morfina. Posteriormente, avaliámos o papel de MOR no DRt durante a HIO. A administração crónica de morfina induziu um aumento do número de células a expressar MOR, sem alterar os níveis de mRNA. Em animais tratados com morfina, o *knockdown* de MOR atenuou o desenvolvimento da hipersensibilidade mecânica e térmica, enquanto que em animais tratados com soro induziu um aumento da sensibilidade à dor. Finalmente, avaliámos os efeitos da administração crónica de morfina na atividade e nas vias de sinalização de MOR no DRt. A expressão da proteína de ligação ao elemento de resposta AMPc fosforilada (pCREB), um marcador intracelular da cascata de sinalização excitatória de MOR, foi avaliada por imuno-histoquímica. A administração de morfina induziu um aumento do número de células a expressar pCREB no DRt. De seguida, avaliaram-se os efeitos da administração de DAMGO, de uma dose ultra baixa de Naloxona, que impede o acoplamento de MOR à proteína Gs excitatória, e de H-89, inibidor da PKA, na sensibilidade mecânica e térmica. O DAMGO teve um efeito antinociceptivo em animais tratados com soro e aumentou a hipersensibilidade mecânica em animais tratados com morfina. A naloxona restabeleceu o efeito antinociceptivo do DAMGO e diminuiu os níveis de pCREB em animais tratados com morfina. A administração de H-89 não teve nenhum efeito. Estes resultados indicam que a administração crónica de morfina altera o acoplamento de MOR de uma proteína Gi inibitória para uma proteína Gs excitatória no DRt, alterando a sinalização de MOR de inibitória para excitatória, resultando provavelmente no aumento da facilitação descendente do DRt.

Os resultados apresentados na presente dissertação mostram que tanto a dor neuropática como a HIO induzem diferentes adaptações no MOR a nível do DRt, o que resulta, em ambas as situações, no comprometimento da função inibitória do recetor. Tendo em consideração o importante papel dos opioides na inibição das ações facilitatórias do DRt, estas alterações contribuem provavelmente para o aumento da facilitação descendente do DRt. A alteração na sinalização de MOR em áreas supraespinais envolvidas na facilitação da transmissão nociceptiva contribui muito provavelmente para o aumento da facilitação descendente da dor na dor neuropática e na HIO. O desenvolvimento de novas estratégias terapêuticas direcionadas ao sistema opioidérgico em estruturas encefálicas deveria ser equacionado no futuro para o tratamento da dor.

Introduction

1. Pain transmission and modulation

According to the International Association for the Study of Pain (IASP), pain is defined as *"unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage*" (Loeser and Treede, 2008). Pain results from activation of sensory receptors, passage of the signal to the spinal cord and arrival to cortical areas of the brain. Therefore, the IASP definition highlights that pain is more than a sensation, it also includes perception. Pain perception is not necessarily linear to the intensity of a stimulus or similar amongst different individuals since emotional experiences can either enhance or diminish pain. Therefore, pain is an experience that results from an individualized perception of a nociceptive input influenced by the emotional state, memories and pathological, genetic, and cognitive factors (reviewed by Tracey and Mantyh, 2007).

1.1 Pain transmission

The genesis of the pain sensation occurs when thermal, mechanical or chemical stimuli of high intensity are recognized by a specific population of peripheral nerve fibers, called nociceptors. Nociceptors endings are present in all tissues and organs and can be categorized by conduction velocity, response properties and neurochemical phenotype. Aδ-fibers, responsible for the sharp or "first" pain sensation, are characterized as medium diameter fibers, thinly myelinated and conduct at intermediate velocities. C-fibers are nonmyelinated, have small cell bodies, conduct action potentials slowly and convey "second" pain. Both, 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. Other type of fibers, the Aβ-fibers, in normal conditions, solely respond

to innocuous mechanical stimuli (reviewed by Basbaum et al., 2009) but can be recruited during chronic pain (reviewed by Basbaum et al., 2009 ; Costigan et al., 2009). In fact, during chronic neuropathic pain, abnormal activity of Aβ-fibers have been implicated in mechanical allodynia, which represents a particularly prominent feature of neuropathic pain (reviewed by Costigan et al., 2009). The cell bodies of these fibers are located in the dorsal root ganglia, from which two branches emerge: a long peripheral branch and a small central branch, allowing them to function as a bidirectional signaling mechanism. In the dorsal horn, which is organized into anatomically distinct laminae, the primary afferent endings have a specific distribution pattern. In general, Aδ-fibers project to laminae I and V, C-fibers project to superficial laminae I and II and indirectly to lamina V and Aβ-fibers project to deep laminae III-VI (reviewed by Basbaum et al., 2009). Considering this distribution, superficial laminae neurons, which respond to noxious stimuli, are classified as nociceptive-specific and deep-laminae III and IV neurons that respond to innocuous stimuli are denominated as low-threshold. Finally, deep-lamina V neurons, which respond to innocuous and noxious inputs, are classified as wide dynamic range (WDR) neurons (reviewed by Basbaum et al., 2009).

Nociceptors activation usually begins with transduction, the process by which noxious stimuli are converted into electrical activity, leading to the opening of ion channels and altering the ionic flow across the cell membrane. If the stimuli are translated into a sufficiently intense electrical signal, it will result in depolarization and in action potentials that will be transmitted through peripheral afferents to the dorsal horn of the spinal cord (reviewed by Basbaum et al., 2009). Each action potential induces the release of several neurotransmitters/modulators such as, glutamate, brain-derived neurotrophic factor (BDNF), substance P (SP), calcitonin gene-related peptide (CGRP) and endomorphine-2 from axon terminals into the synapse within the spinal dorsal horn.

These neurotransmitters/modulators activate receptors on the post-synaptic nerve terminal, including N-methyl-D-aspartic acid (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), tyrosine kinase receptors, Neurokinin-1 (NK-1) receptors, calcitonin receptor-like receptor (CALCRL) and μ-opioid receptor (MOR) (reviewed by Ribeiro-da-Silva and De Koninck, 2008).

The impulses generated in the dorsal horn travel through second order neurons, which constitute the ascending pathways, to the brain through three major pathways: the spinothalamic tract, the spinoreticular tract and the spinomesencephalic tract. The spinothalamic tract carries nociceptive information to the thalamus, which is subsequently distributed to several cortical structures involved in the sensory-discriminative components and in the motivational-affective aspects of pain (reviewed by Lima, 2008). The spinoreticular tract, in which reticular formation regions project to the medial thalamus, is then connected with brain areas relevant for the emotional and cognitive dimensions of pain. Finally, the spinomesencephalic tract projects to the periaqueductal gray matter (PAG), a major antinociceptive area (reviewed by Almeida et al., 2004). The transmission of noxious stimuli by sensory neurons to second order neurons is modulated by interneurons, mainly inhibitory neurons releasing opioid peptides and γ-aminobutyric acid (GABA), which bind to opioid receptors and GABA receptors, respectively (Ribeiro-da-Silva and De Koninck, 2008). Furthermore, supraspinal descending fibers, arising from descending pain modulatory areas, are also responsible for modulating pain transmission at the spinal cord, exacerbating or inhibiting the spinal sensory transmission (reviewed by Basbaum et al., 2009; Ribeiro-da-Silva and De Koninck, 2008; Todd, 2010).

1.2. The endogenous pain control system

Nociceptive information from the whole body transmitted to the dorsal horn suffers processing by different mechanisms that enhance or inhibit its transmission to the brain and pain perception. Several supraspinal sites and pathways are involved in descending modulation, ranging from the cerebral cortex to the caudal medulla such as, the PAG - rostral ventromedial medulla (RVM) circuit, the locus coeruleus (LC), the dorsal reticular nucleus (DRt), and the caudal lateral ventrolateral medulla (VLM) (reviewed by Ren and Dubner, 2008).

The most well characterized descending pain modulatory pathway involves a circuitry linking the PAG, the RVM and the spinal cord (Figure 1). PAG stimulation results in deep analgesia (Reynolds, 1969) demonstrating that this midbrain area is able to inhibit nociceptive transmission. PAG receives direct projections from a number of forebrain areas involved in cognitive and emotional aspects of pain such as the anterior cingulate, amygdala and the hypothalamus (Beitz, 1982). The RVM has been identified as the premier relay station between the PAG and the spinal dorsal horn. Anatomical lesions or pharmacological inactivation of the RVM abolish the analgesia produced by the stimulation of the PAG (Prieto et al., 1983; Sandkühler and Gebhart, 1984). The RVM encompasses the nucleus raphe magnus and adjacent reticular formation and projects to the entire spinal dorsal horn, with lower projections to lamina III. The descending input from the RVM travels in the dorsolateral funiculus (DLF) and modulates spinal transmission. Descending pathways from the RVM can inhibit or facilitate nociceptive transmission at the spinal cord, through neurons that increase nociception (ON-cells) or inhibit nociception (OFF-cells). Both ON- and OFF-cells are modulated by opioids. ON-cells are directly inhibited by MOR agonists. OFF-cells are not directly inhibited by MOR

agonists, but are activated, via opioidergic inhibition of GABAergic inhibitory input (reviewed by Heinricher and Ingram, 2010; Heinricher and Fields, 2013). The RVM also modulates pain transmission at the spinal cord through spinal release of serotonin (5-HT), which can induce antinociception or pronociception, depending on the type of 5-HT receptor that is activated (Dogrul et al., 2009).

The LC is the main structure coordinating the central noradrenergic system (reviewed by Llorca-Torralba et al., 2016). It is involved in descending pain inhibition through the noradrenergic fibers which have terminals in the spinal cord (reviewed by Millan, 2002; Pertovaara, 2013). These have been shown to release noradrenaline that acts via α2-adrenoceptors to inhibit both primary afferents and second order projection neurons (reviewed by Millan, 2002; Pertovaara, 2013). Although LC activity has been predominantly associated with inhibition, it is now known that the LC produces bidirectional influences on thermal nociception (Hickey et al., 2014) and that it also contributes to increased descending facilitation during neuropathic pain (Martins et al., 2015b; Martins et al., 2010). It was recently demonstrated that the LC is engaged in descending inhibition of pain through its projections to the spinal cord (Hirschberg et al., 2017). Furthermore, the LC increases descending pain facilitation and spontaneous pain through projections to the DRt (Martins et al., 2015b; Martins et al., 2010) and prefrontal cortex (Hirschberg et al., 2017), respectively.

The modulation from the VLM may include facilitatory modulation (ON-like neurons), along with the well-established inhibitory effects (OFF-like neurons) (Pinto-Ribeiro et al., 2011; Tavares and Lima, 2002). Part of the inhibitory actions of the VLM is mediated by the release of noradrenaline and serotonin at the spinal cord, which result from the activation of VLM projections to the pontine A5 noradrenergic (Tavares et al., 1996) cell group and to the RVM (reviewed by Ren and Dubner, 2008). Other important

neurochemical control system at the VLM is mediated by opioids since overexpression of opioids at the VLM induces antinociception and lower nociceptive spinal neuronal activation (Martins et al., 2011). The pronociceptive effects of the VLM seems to be mediated by noradrenaline and angiotensin II, through the local activation of α2-adrenoreceptor (Cahusac and Hill, 1983) and angiotensin type 1 receptor (Marques-Lopes et al., 2009), respectively.

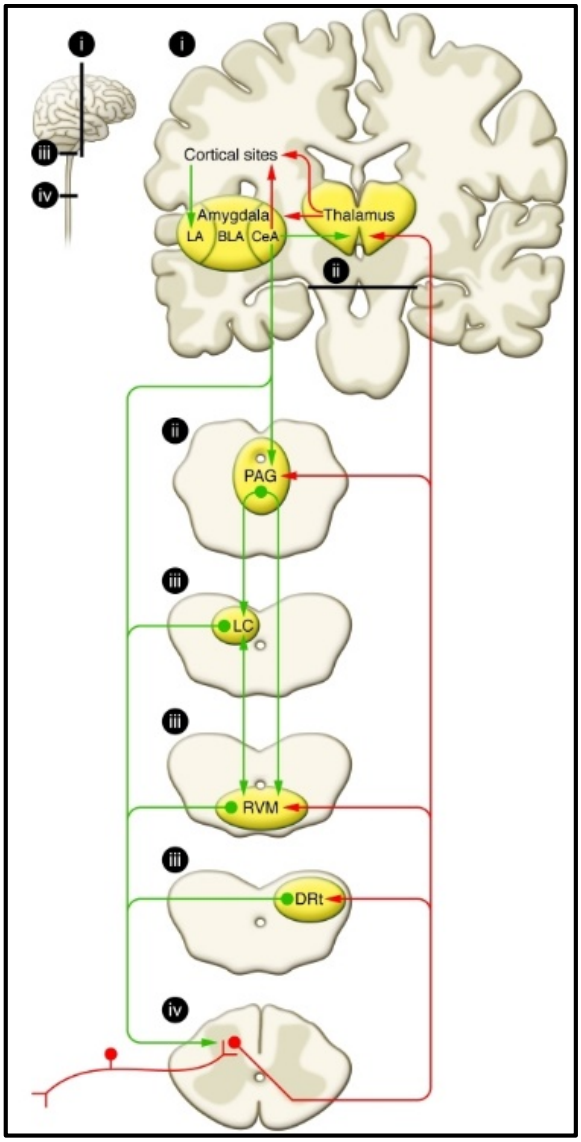

Figure 1. Representation of the pain modulatory system. Primary afferent neurons conduct nociceptive inputs to the spinal dorsal horn from where ascending projections (in red) target the thalamus, the DRt, the RVM and the PAG. Descending pain modulation is mediated through projections (in green) from cortical areas to the PAG, which communicates with the RVM and the LC, which send direct descending projections to the dorsal horn. DRt, dorsal reticular nucleus; RVM, rostral ventromedial medulla; PAG, periaqueductal gray matter; LC, locus coeruleus (Adapted from Ossipov et al., 2010)

1.3. Descending pain modulation from 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 the focus of the present thesis.

The DRt is located in the most caudal portion of the medullary dorsolateral reticular formation, more specifically, in the dorsolateral quadrant of the medulla oblongata. 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 2) (Andrezik and Beitz, 1985).

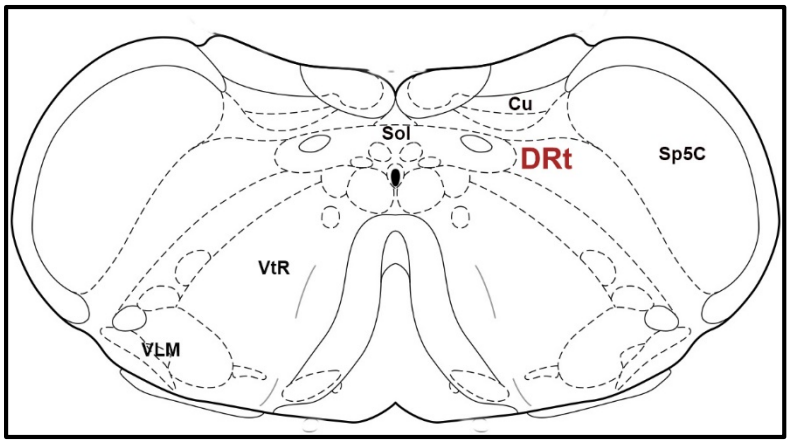

Figure 2. Diagram of a coronal section of the caudal medulla oblongata. DRt, dorsal reticular nucleus; Cu, nucleus cuneate; Sol, nucleus tractus solitaries; Sp5C, spinal trigeminal nucleus, pars caudalis; VLM, caudal ventromedial medulla; VRt-ventral reticular nucleus. Diagram of a medullary section at 5.64 mm caudal to the interaural line (Adapted from Paxinos and Watson, 2006).

The DRt has two neuronal subpopulations: total nociceptive convergent neurons that are exclusively activated by noxious stimuli conveyed by Aδ- and C-fibers from the full body, and partial nociceptive convergent neurons that are activated by noxious and innocuous stimuli conveyed by C-fibers from only some parts of the body, mainly

contralateral, and by Aδ-fibers from the whole body (Villanueva et al., 1989; Villanueva et al., 1988).

The DRt facilitates acute and chronic pain (Almeida et al., 1996; Martins et al., 2015b; Martins et al., 2010; Sotgiu et al., 2008). In fact, glutamate administration in the DRt induces a long-lasting increase in the responsiveness of spinal nociceptive neurons (Dugast et al., 2003), while pharmacological inactivation with lidocaine results in the suppression of responsiveness (Lima and Almeida, 2002).

The DRt is also involved in the diffuse noxious inhibitory control (DNIC) (Bouhassira et al., 1992; de Resende et al., 2011; Youssef et al., 2016). Commonly known as "pain inhibits pain", DNIC is a paradigm in which one noxious stimulus is used as a conditioning stimulus to induce reduction in pain perception to another spatially distant noxious stimulus (Le Bars et al., 1979). The exact mechanisms that entail the involvement of the DRt in DNIC are not known but they rely on the opioidergic modulation of the DRt (de Resende et al., 2011).

1.3.1. DRt connections with the spinal cord and the brain

The DRt is likely involved in the facilitation of pain through its direct descending projections to the spinal dorsal horn (Lima and Almeida, 2002). Indeed, the DRt receives bilateral projections from spinal cord lamina I, IV-VII and X with an ipsilateral predominance of those originated in the dorsal horn (Lima, 1990). The projections from the superficial dorsal horn travel through the dorsal funiculus whereas those from the deep dorsal horn travel in the dorsolateral fasciculus (reviewed by Lima and Almeida, 2002). DRt neurons are reciprocally connected with spinal lamina I neurons, that are characterized by excitatory synaptic contacts at both sites (Almeida et al., 2000). Lamina

I neurons projecting to the DRt convey nociceptive inputs to the DRt as they express c-Fos, a marker of spinal neuronal nociceptive activation, upon noxious stimulation (Almeida and Lima, 1997). This excitatory dorsal horn-DRt-dorsal horn loop likely forms a reverberating circuitry that leads to the amplification of the nociceptive information (Almeida et al., 2000; reviewed by Lima and Almeida, 2002).

The DRt is reciprocally connected with several brain areas involved in descending modulation such the VLM, PAG, RVM, LC and the A5 and A7 noradrenergic cell groups (Figure 3) (Almeida et al., 2002; Leite-Almeida et al., 2006). The DRt is also an important relay nucleus for descending nociceptive modulation from several brain areas such as the anterior cingulate cortex (Zhang et al., 2005), the hypothalamus (Amorim et al., 2015) and the brainstem (Leiras et al., 2015; Martins et al., 2013; Velo et al., 2013). Moreover, the DRt receives projections from cortical areas, namely from the primary and secondary somatosensory cortex, primary and secondary motor cortex and insular cortex (Almeida et al., 2002; Desbois et al., 1999). The DRt also projects to the medial thalamus and the limbic system, which suggests an integration of DRt activity with emotional aspects of pain processing. Furthermore, the DRt is connected with the extrapyramidal and orofacial motor system, which suggests an involvement of the DRt in motor reactions associated with pain (Leite-Almeida et al., 2006).

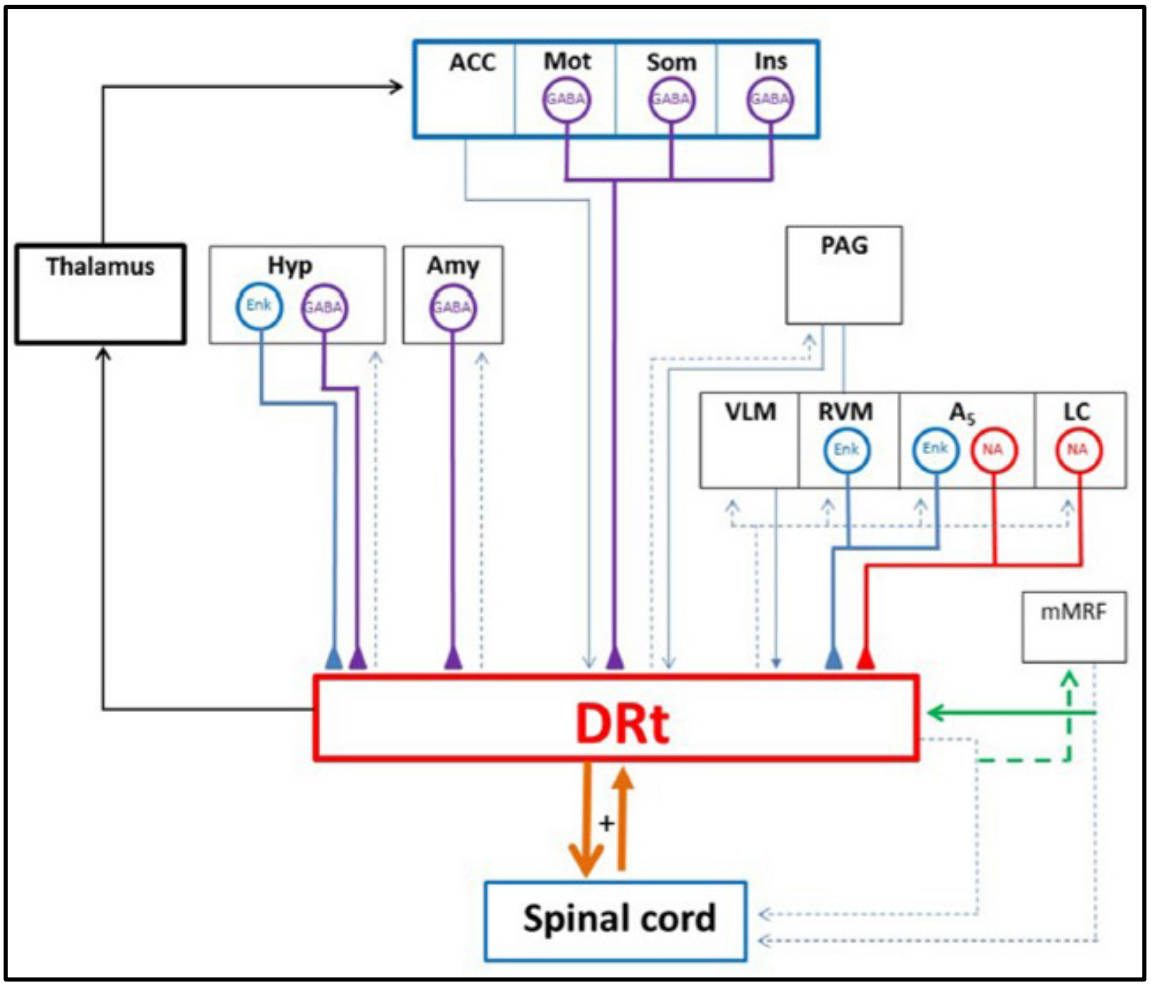

Figure 3. Schematic representation of the DRt connections with the spinal cord and several brain areas. The DRt is involved in a feedback reciprocal loop with the spinal cord (orange lines) which is involved in pain facilitation. Through its projections to the lateral ventromedial thalamus the DRt participates in a reticulo-thalamo-cortical ascending nociceptive pathway (black lines). The DRt receives afferent inputs from higher centers namely the anterior cingulate cortex (ACC), the motor (Mot), somatosensory (Som) and insular (Ins) cortices, the hypothalamus (Hyp) and the amygdala (Amy). DRt also receives afferent inputs from several brainstem areas namely the periaqueductal gray (PAG), the rostroventromedial medulla (RVM), ventrolateral medulla (VLM), the locus coeruleus (LC) and the A5 noradrenergic cell group. Afferents originated in the Mot, Som and Ins cortices, as well as the Hyp and the Amy, are GABAergic (purple lines). Hyp afferents and brainstem afferents located at the RVM and the A5 area are enkephalinergic (thick blue lines). The LC and the A5 noradrenergic cell group constitute the main source of noradrenaline (red lines) released at the DRt. A reciprocal network established between the DRt and the medial medullary reticular formation (mMRF) through collaterals (green lines) of spinally descending axons is thought to be involved in noxious sensing and nocifensive behavior. Blue thin lines represent neurochemically uncharacterized DRt afferents and dashed lines represent DRt or mMRF efferents (Adapted from Martins and Tavares, 2017).

1.3.2. Neurochemical systems that modulate DRt activity

The activity of the DRt is modulated by different neurochemical systems such as the glutamatergic (Ambriz-Tututi et al., 2013), noradrenergic (Martins et al., 2013) GABAergic (Martins et al., 2015a) and opioidergic (Martins et al., 2008; Pinto et al., 2008a).

Glutamate plays a key role in the pronociceptive actions of the DRt. Local administration of glutamate has a pronociceptive effect, whereas lesioning the DRt results in a decrease of the nociceptive responses (Almeida et al., 1996). Electrophysiological data showed that DRt activation by glutamate induces a long-lasting increase of the responses of WDR neurons to noxious electrical stimulation of the sciatic nerve (Dugast et al., 2003). Local blockade of NMDA, AMPA and mGlu1 glutamate receptors, with the respective antagonists, significantly decreases the formalin-induced pain-like behaviors and c-Fos expression both at the superficial and deep dorsal laminae (Ambriz-Tututi et al., 2013).

GABA is also involved in the mediation of pronociception from the DRt. Studies from our research group showed an increase of GABA release at the DRt in the formalin test that increases DRt pain facilitation through activation of $GABA_B$ receptors (Martins et al., 2015a). Blockage or reduction of the $GABA_B$ receptor, using pharmacological or genetic approaches, significantly attenuated formalin-induced pain while the pharmacological activation of the receptor induced the opposite (Martins et al., 2015a). The pronocipective effects of GABA are probably due to disinhibition of the DRt spinally-projecting neurons since a large proportion of $GABA_B$ receptors are expressed by local opioidergic neurons inhibiting DRt spinally projecting neurons (Martins et al., 2008; Pinto et al., 2008a).

The neurotransmitter noradrenaline is also involved in the pronociceptive actions of the DRt. The pain facilitatory actions of noradrenaline at the DRt are mediated through activation of α1-adrenoreceptors (Martins et al., 2013). Noradrenaline release at the DRt increases during the formalin test (Martins et al., 2013). The reduction of noradrenaline release at the DRt, from the noradrenergic afferents, attenuated pain behavior in the formalin test while increasing the noradrenaline levels, by inhibiting its recapture, had the opposite effect (Martins et al., 2013).

The opioidergic system represents another key modulatory system at the DRt since spinally- and non-spinally projecting neurons express MOR (Pinto et al., 2008b). The expression of MOR at DRt spinally-projecting neurons indicates that the reverberative spinal-DRt loop is under opioidergic inhibitory control. In support, antinociception occurs after opioid peptide overexpression (Martins et al., 2008) or local administration of MOR agonists (Pinto et al., 2008a). The DRt endogenous opioidergic system is also required to the DNIC modulation, since local administration of an opioid antagonist blocked DNIC (de Resende et al., 2011; Patel and Dickenson, 2019). Opioids also act at the DRt through other inhibitory mechanisms, likely by disinhibiting enkephalinergic interneurons which receive input from GABAergic interneurons expressing MOR and being presynaptically inhibited by δ –opioid receptor (DOR) expressing fibers (Pinto et al., 2008a). The opioid peptides responsible for the antinociceptive action at the DRt are mostly released from local interneurons and also from DRt afferent sources namely the RVM, the A5 noradrenergic cell group and the hypothalamus (Martins et al., 2008).

2. Opioids and Pain Modulation

2.1. Endogenous opioid peptides and opioid receptors

The endogenous opioid system plays a key role in the modulation of pain. There are four families of endogenous opioids prepropeptides that produce several receptor-specific peptides: proopiomelanocortin that produces β-endorphin, preproenkephalin that produces met- and leu-enkephalin, prodynorphin that produces dynorphins and pronociceptin that produces nociceptin (reviewed by Stein, 2016).

Currently, four genetic subtypes of opioid receptors are identified: MOR, DOR, κ- (KOR) and nociceptin- (NOP) opioid receptors, with different selectivity for the individual endogenous peptides and for opiate drugs (Chen et al., 1993; Kieffer et al., 1994; Minami et al., 1993; Mollereau et al., 1994).

MOR activation is associated with the analgesic and euphoric effects of opioids as well as the physical dependence, constipation and respiratory depression. DOR activation mediates spinal and supraspinal analgesia, convulsions and anxiolysis. KOR mediates spinal analgesia, diuresis, sedation, dysphoria and stress. NOP is involved in analgesia, stress and anxiety, feeding, learning and memory, reward/addiction and urogenital activity (reviewed by McDonald and Lambert, 2005; and Stein, 2016). β-endorphin and enkephalins produce analgesic effects acting on MOR and DOR. Dynorphins and nociceptin produce antinociceptive effects via KOR and NOP, respectively. A fifth group of opioid peptides, the endomorphins, with unknown precursors, has a high selectivity for MOR and plays an important role in pain perception (reviewed in Mogil and Pasternak, 2001).

Opioid receptors are expressed throughout the nervous system, from the central to the peripheral neurons, and by neuroendocrine, immune, and ectodermal cells (Gehdoo and Singh, 2017; Mansour et al., 1994; Neal et al., 1999; Stein, 2016; reviewed in Zöllner and Stein, 2006). In the brain, the highest expression of MOR was observed in the cerebellum, caudate nucleus and nucleus accumbens. They are also expressed in pain-modulating areas, such as the insular cortex, amygdala, LC, RVM, PAG (Pan et al., 1990; Tempel and Zukin, 1987; Vaughan and Christie, 1997) and the DRt (Pinto et al., 2008a). DOR is highly expressed in the hippocampus, cerebral cortex, putamen, caudate nucleus, nucleus accumbens and temporal lobe. The highest expression of KOR was detected in caudate nucleus, nucleus accumbens, hypothalamic nuclei and putamen (reviewed in Sobczak et al., 2014; Tempel and Zukin, 1987). NOP has a high expression in RVM, LC, PAG, amygdala and habenula (Schulz et al., 1996). All opioid receptors are expressed in the spinal cord dorsal horn and DRGs (Arvidsson et al., 1995a; Arvidsson et al., 1995b; Simonin et al., 1995).

2.2. MOR signaling

MOR is the principal pharmacological target for the opioid analgesics used in the clinical setting, such as morphine and fentanyl. Structurally, MOR belongs to the large family of seven-transmembrane G protein-coupled receptors (GPCR). Usually MOR is coupled to a Gi/o protein (Figure 4). G-proteins are composed of three subunits: α, β and γ. The binding of opioid agonists to the receptor results in a conformational change of the inhibitory Gαi/o 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 Gα subunit dissociates from the Gβγ subunits and binds

to adenylate cyclase (AC) inhibiting it and also inhibiting cyclic adenosine monophosphate (cAMP) production. Simultaneously, the Gβγ subunits interact with the different ion channels in the cell membrane. These subunits inhibit Ca^{2+} channels resulting in a decrease of intracellular Ca^{2+}, reduction of neurotransmitter release and also a decrease of the neurons excitability. In addition, the Gβγ subunits activate G protein gated inwardly rectifying potassium channels, preventing neuronal activation and propagation of action potentials (reviewed in Al-Hasani and Bruchas, 2011). Following activation, opioid receptors are phosphorylated by GPCR kinases, leading to β-arrestin (β-arr) recruitment. Arrestins are key proteins that lead to opioid receptor desensitization, by preventing G protein coupling, and promote receptor internalization via clathrin-dependent pathways. After internalization, the dephosphorylated receptor can be recycled and reintegrated in the cell surface or degraded in the lysosomes (Kovoor et al., 1997; Zhang et al., 1998). MOR phosphorylation is a crucial regulatory process related with the decrease of receptor response to agonists, i. e., tolerance to opioids, both after prolonged agonist administration (Deng et al., 2000; Schulz et al., 2004; reviewed by Stein, 2016) or during neuropathic pain (Petraschka et al., 2007). The C-terminus of MOR has multiple phosphorylation sites that are implicated in the mechanisms of receptor desensitization and trafficking. A recent preclinical study showed that the degree of phosphorylation on the C terminus has different roles in the expression of the multiple adaptive mechanisms that follow acute and long-term agonist activation (Arttamangkul et al., 2019).

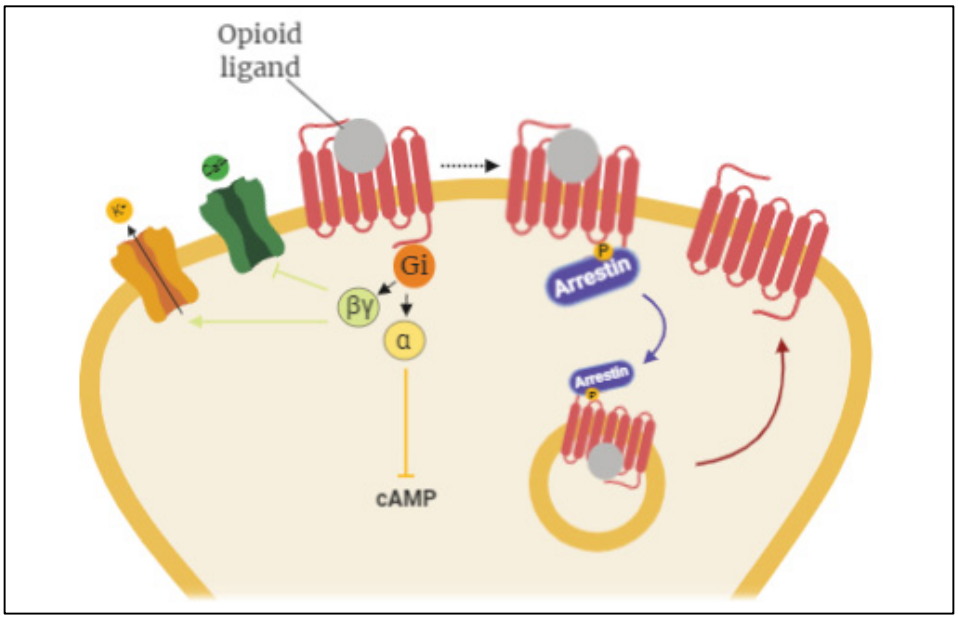

Figure 4- Summary of μ-opioid receptor signal transduction and trafficking. Receptor activation promotes distinct recruitment of G-protein and arrestins signaling cascades. While G-proteins mediate the inhibitory action of opioid signaling on neurotransmission through the stimulation of potassium efflux, decrease of intracellular calcium and inhibition of adenylyl cyclase, arrestins signaling is required for the internalization of the receptor (Adapted from Corder et al., 2018).

2.3. Opioidergic control of nociceptive pathways

At the peripheral level, immune cells produce and release opioid peptides that bind to opioid receptors in peripheral nerve terminals resulting in a reduction of cell excitability and inflammatory mediators release (reviewed by Corder et al., 2018; Dickenson and Kieffer, 2013). In the CNS, the endogenous opioid system, regulates nociceptive pathways both at spinal and supraspinal level. At the spinal level, opioids inhibit nociceptive transmission conveyed by Aδ- and C-fibers. Pre-synaptically, MOR activation inhibits Ca^{2+} channels which in turn reduces the release of excitatory molecules, such as glutamate, substance P and CGRP (reviewed by Dickenson and Kieffer, 2013 and; Stein, 2016). Post-synaptically, MOR is located on spinal neurons responsible for the integration of spino-thalamic pathway that transmits nociceptive information to supraspinal areas. At the supraspinal level, opioids inhibit nociceptive transmission from ascending pathways to the thalamus. Additionally, projections from

the thalamus to the cortex are also under opioidergic modulation(reviewed by Nadal et al., 2013).

2.4. Opioidergic control of descending pain modulatory areas

The endogenous opioid system also plays a crucial role in the modulation of descending pathways. The PAG is a key site of the analgesic activity of opioids within the CNS. GABAergic interneurons within the PAG are a critical site of action by opioids. Opioids act via postsynaptic MOR and directly inhibit GABAergic interneurons at the PAG or via presynaptic MOR to inhibit the release of GABA from nerve terminals. Under normal conditions, GABAergic interneurons exert tonic inhibition of PAG glutamatergic neurons, which are thought to be output neurons that project to the RVM. Upon MOR activation, the activity of the GABAergic neurons decreases, disinhibiting PAG projections to the RVM (Moreau and Fields, 1986). Recent studies confirmed these earlier findings by demonstrating that inhibition of GABAergic neurons or activation of glutamatergic output neurons in the PAG mimics the antinociceptive effects of opioids (Samineni et al., 2017).

At the RVM, opioids directly inhibit ON-cells (Heinricher et al., 1992) and disinhibit OFF-cells, primarily via inhibition of GABAergic inhibitory input (Heinricher et al., 1994). When MOR agonists are administered systemically or microinjected directly into the RVM, OFF-cell discharge increases while selective blockade of OFF-cell activation prevents the antinociception induced by morphine administration (Heinricher et al., 1999). Therefore, OFF-cells activation is necessary for the pain inhibitory effects of MOR agonists given systemically or supraspinally (Heinricher et al., 1997; Heinricher et al., 2001).

The DRt is also under opioidergic modulation. MOR and DOR are expressed at DRt neurons (Neto et al., 2008; Pinto et al., 2008a). In acute pain, overexpression of proenkephalin at the DRt induces analgesia, indicating that opioids inhibit DRt descending facilitation of pain (Martins et al., 2008).

The role of opioids at supraspinal pain modulatory areas has been extensively studied in acute pain settings. Nevertheless, the effects of opioids as well as the adaptive changes in the endogenous opioids peptides and opioid receptors at pain modulatory areas in other settings such as in neuropathic pain conditions and in opioid-induced hyperalgesia, remains understudied.

3. Neuropathic pain

3.1. Definition and etiology

Chronic pain is defined as pain that persists beyond the expected normal time for healing and, contrary to acute pain, offers no physiological advantage. Chronic pain involves high plasticity which leads to structural changes in the brain, ranging from the molecular to the network level. Chronic pain affects approximately 20% of the adult world population (Treede et al., 2015). In Portugal, it is estimated that 37% of the population suffers from chronic pain (Azevedo et al., 2012). Chronic pain can be divided in seven categories: chronic primary pain, chronic cancer pain, chronic posttraumatic and postsurgical pain, chronic musculoskeletal pain, chronic headache and orofacial pain, chronic visceral pain and chronic neuropathic pain (Treede et al., 2015).

Chronic neuropathic pain is defined as a direct consequence of a lesion or disease affecting the somatosensory nervous system (Loeser and Treede, 2008; Scholz et al.,

2019; Treede et al., 2015). Its prevalence ranges between 6.9% and 10% of the general population (Van Hecke et al., 2014). Neuropathic pain may be spontaneous or evoked and the two major symptoms of the later are allodynia and hyperalgesia (reviewed by Jensen and Finnerup, 2014; Woolf and Mannion, 1999). At the clinical practice, therapeutic management of neuropathic pain is challenging since drugs suggested as first-line treatments provide less than satisfactory relief in many patients (reviewed by Finnerup et al., 2015).

Lesions on the somatosensory nervous system lead to pathophysiological changes in multiple sites along the neuronal axis. These alterations include increased immune response, both peripherally and centrally, loss of synaptic connectivity and formation of new synaptic circuits, ectopic generation of action potentials and changes in descending pain control systems (reviewed by Colloca et al., 2017).

3.2. Descending pain modulation during neuropathic pain

Descending pain inhibitory and facilitatory systems function in concert, maintaining a baseline state of sensory processing. However, during neuropathic pain, these pathways exhibit a dramatic plasticity. Preclinical (Vera-Portocarrero et al., 2006; Wang et al., 2013) and clinical (Mainero et al., 2007) studies demonstrated impaired activity in the descending modulation system plays a key role in the central sensitization, allodynia and hyperalgesia detected in neuropathic pain. This is a result of a dysregulation of descending inhibition, increased facilitation, or a combination of both during neuropathic pain. Noradrenergic inhibition, via α2-adrenoceptors, appears to be suspended (Rahman et al., 2008) and the descending serotoninergic input shifts from

inhibitory to facilitatory, mainly due to the action of excitatory spinal 5-HT3 receptors (Suzuki et al., 2004; Wang et al., 2013).

Considerable evidences indicate that the maintenance of neuropathic pain depends on descending facilitation (reviewed by Bingel and Tracey, 2008; Porreca et al., 2002; Wang et al., 2013). Several studies demonstrated the importance of descending facilitation from the RVM in the neuropathic state. Pharmacological inactivation of the RVM with lidocaine abolished behavioral signs of neuropathic pain (Burgess et al., 2002; Pertovaara et al., 1996). Furthermore, surgical disruption of descending RVM projections, through lesioning the dorsolateral funiculus, eliminated behavioral signs of spontaneous and evoked neuropathic pain (Burgess et al., 2002; King et al., 2009; Ossipov et al., 2000) and prevented the increase of expression of c-Fos, a marker of spinal sensitization, in the dorsal horn (Wang et al., 2013). Also, electrophysiological studies demonstrated that RVM ON- and OFF- cells are sensitized to innocuous and noxious stimuli after nerve injury, and this is associated with behavioral hypersensitivity (Carlson et al., 2007).

The ablation of MOR expressing-cells (ON-cells) in the RVM by microinjection of dermorphin–saporin, eliminates the behavioral (Porreca et al., 2001) and molecular markers associated with this persistent pain state, including spinal c-Fos (Vera-Portocarrero et al., 2006), up-regulation of spinal dynorphin and enhanced capsaicin-evoked release of calcitonin gene-related peptide (Gardell et al., 2003). Finally, pharmacological inhibition of cholecystokinin (CCK), an important neuropeptide that acts as a pro-nociceptive agent in nerve injury, at the RVM, restores the antinociceptive potency and efficacy of opioids in the PAG, which is considerably reduced during neuropathic pain (Kovelowski et al., 2000).

Descending facilitation from the DRt is also involved in neuropathic pain. Electrophysiological studies suggest that the facilitatory role of the DRt contributes to the maintenance of spinal sensitization during neuropathic pain (Sotgiu et al., 2008). Several studies demonstrated that noradrenergic modulation of the DRt is linked to the enhancement of DRt pain facilitation during neuropathic pain. Nociceptive stimulation in neuropathic animals increased noradrenaline release at the DRt and pharmacological blockade of the α1-adrenoreceptor decreases mechanical and cold hypersensitivity. This suggests that during neuropathic pain noradrenaline enhances pain facilitation from the DRt through activation of α1-adrenoreceptor (Martins et al., 2015b). Likewise, decreasing noradrenaline release at the DRt, using viral vectors which selectively reduced noradrenaline synthesis in noradrenergic DRt afferents, significantly attenuated the behavioral manifestations of neuropathic pain (Martins et al., 2010). Further studies from our research group demonstrate that a malfunction of the inhibitory function of α2-adrenoreceptors at the DRt during neuropathic pain, likely further contributes to enhance the noradrenergic input to the DRt during neuropathic pain (Martins et al., 2015b).

3.3. Opioids and neuropathic pain

3.3.1. Altered opioidergic modulation

Opioids are regularly administered in acute and cancer pain. In chronic non-cancer pain, like neuropathic pain, their use is controversial. A recent study estimates that 30.7% of patients with chronic non-cancer pain are prescribed with opioids (reviewed by Mathieson et al., 2020). Weak and strong opioids are recommended as second and third line treatment, respectively, mainly because lack of efficacy or safety concerns (reviewed by Colloca et al., 2017; Finnerup et al., 2015; Kalso et al., 2004). However, opioid

prescription for patients with chronic non-cancer pain is common and has increased over time (reviewed by Mathieson et al., 2020).

Contrary to traditional belief, neuropathic pain is opioid responsive, although larger doses are required than those used in nociceptive pain treatment (Harke et al., 2001; Rowbotham et al., 2003; Watson and Babul, 1998). Preclinical studies examined the reasons behind the decreased effectiveness of opioids in neuropathic pain, which include alterations on MOR (Porreca et al., 1998; Rashid et al., 2004), increased spinal release of dynorphin A and CCK (Nichols et al., 1996), increased expression of spinal metabotropic glutamate receptor 1 (mGluR1) (Fundytus et al., 2001) and activation of tonic descending facilitation pathways from supraspinal areas (Kovelowski et al., 2000).

The increase of CCK after nerve injury contribute to the lower efficacy of opioids in neuropathic pain (Xu et al., 1993). The mechanism of CCK interaction with opioidergic transmission is not known but, the administration of CCK antagonists enhances the analgesic effect of opioid agonists (McCleane, 1998, 2003) while small doses of CCK reduce morphine effects (Nichols et al., 1995). The knockdown of mGluR1, at the spinal level, prevented the nerve-injury induced insensitivity to opioids (Fundytus et al., 2001). Neuropathic injury has been shown to stimulate an increased release of glutamate, which can lead to increased activation of mGluRs. The activation of mGluRs leads to an increase of the concentration of intracellular Ca^{2+} and activation of protein kinase C (PKC). Activation of PKC has been shown to phosphorylate opioid receptors, leading to the receptor desensitization and consequent decrease of the analgesic efficacy of opioid agonists (Kramer and Simon, 1999). Elevated spinal dynorphin after peripheral nerve injury may lead to sensitization of the spinal cord through its interaction with the NMDA receptor (Nichols et al., 1997). Dynorphin may enhance neuronal excitability via the action on NMDA receptor, leading to dorsal horn hyperexcitability and excessive

depolarization and excitotoxicity (Laughlin et al., 1997). The increase of spinal dynorphin levels in neuropathic pain might be a consequence of descending facilitation from RVM neurons. Lesions of the DLF or ablation of the MOR expressing cells in the RVM inhibited the spinal nerve ligation-induced neuropathic pain, as well as spinal up-regulation of dynorphin (Burgess et al., 2002; Porreca et al., 2001).

3.3.2. Altered MOR signaling

Disturbances of MOR normal mechanisms/signaling are also involved in the low efficacy of opioids during neuropathic pain. Down-regulation of MOR occurs at the DRG neurons after partial (Pol et al., 2006; Rashid et al., 2004) or complete sciatic nerve injury (Zhang et al., 1997). Likewise, decrease in MOR expression in animals with neuropathic pain was also observed at the spinal cord (Porreca et al., 1998). Positron emission tomography studies in patients with peripheral and central neuropathic pain revealed reduced MOR availability in cortical brain areas involved in pain modulation, such as the insula, striatum, PAG and thalamus (Jones et al., 2004; Maarrawi et al., 2007). A recent preclinical study confirmed the reduced availability of MOR in the cortical areas referred above in human studies, which was accompanied by reduced MOR expression (Thompson et al., 2018).

In animals, neuropathic pain was further shown to induce MOR adaptations involved in desensitization, such as reduced MOR mediated G-protein activity in the thalamus and PAG (Hoot et al., 2011) and increased MOR phosphorylation at the striatum (Petraschka et al., 2007).

The consequence of these alterations on the opioidergic system is the decrease of the analgesic efficacy of opioid agonists, which results in the use of higher doses of

opioids for the treatment of chronic pain states. However, the continuous use or the dose escalation of opioids may lead to several side effects, including tolerance, physical dependence and hyperalgesia (reviewed in Ricardo Buenaventura et al., 2008).

4. Opioid Induced Hyperalgesia

4.1. Definition

An increasing number of evidences show that opioids may cause a phenomenon often referred to as opioid induced hyperalgesia (OIH). This phenomenon is characterized by increased sensitivity to noxious and non-noxious stimuli related to opioid exposure in the absence of disease progression or opioid withdrawal (reviewed in Marion Lee et al., 2011). The overall prevalence of OIH remains indeterminate. In a clinical study with patients with cancer pain, 14% of 81 participants, the opioid treatment failed and produced pain rather than relieving it in (Mercadante et al., 2012). Other study, described that 28% of a sample of 197 patients with chronic pain receiving opioids developed more pain (Ackerman 3rd, 2006). The lack of epidemiological studies can result from the fact that OIH is often mistaken with opioid tolerance and withdrawal-associated hyperalgesia (WAH). These syndromes can manifest similar symptoms, but the physiological aetiology probably differs, and therefore, effective management of OIH requires a specific approach. Tolerance occurs when the patient seeks pain relief and increasing doses of opioids are necessary to maintain appropriate analgesia. This definition could be confused with OIH. However, in opposition to tolerance, increasing doses of opioids will only worsen pain (reviewed in Tompkins and Campbell, 2011). 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 (reviewed in Tompkins and Campbell, 2011).

4.2. Animal models and clinical evidence

Several studies suggest that humans, as well as animals, treated with opioids can develop OIH. Several preclinical studies demonstrated that administration of opioids paradoxically may increase the sensitivity to pain and potentially may aggravate preexisting pain-like behaviors in animals (Kayser et al., 1987; Mao et al., 2002; Vanderah et al., 2000). Hyperalgesia following opioid administration has been observed in three different experimental dosing paradigms including very low doses (Crain and Shen, 2001), typical analgesic maintenance doses (Celerier et al., 2001; Célèrier et al., 2000; Kayan et al., 1971), and very high doses (Woolf, 1981).

The majority of preclinical models for the study of OIH rely on the acute or chronic administration of analgesic doses of a MOR agonist. In acute protocols, the systemic administration of high doses of opioids was shown to induce a transient hyperalgesic response, within one hour, lasting for hours or days and this manifests in a dose dependent manner. In chronic administration protocols, the animals are exposed to opioids for three to twelve days via repeated subcutaneous injections, subcutaneous implantation of pellets or pumps and intermittent or continuous infusions through intrathecal catheters. The continuous administration of opioids was shown to produce antinociceptive responses in the first day, followed by the loss of this effect and a hyperalgesic state. (reviewed by Angst and Clark, 2006).

Human studies revealed that several commonly used opioid drugs including fentanyl, remifentanil, and morphine have the ability to induce OIH (Chu et al., 2006; reviewed by Colvin et al., 2019; Comelon et al., 2016; Kalaydjian et al., 2019; Lenz et al., 2011). Clinical studies have been conducted using several distinct methodologies namely: former opioid addicts on methadone maintenance therapy; in patients during the perioperative period; healthy volunteers after acute or chronic opioid exposure; and patients with chronic cancer or non-cancer pain in opioid therapy. Reports as early as 1870 mention the occurrence of OIH in morphine-addicted patients (Albutt, 1870). Clinical studies have measured pain sensitivity in former opioid addicts, treated with methadone, and this set of patients is compatible with the hypothesis that OIH, when diagnosed, is caused by chronic opioid exposure (Compton et al., 2000). At the clinical practice, OIH has a significant impact in the perioperative pain management (reviewed by Colvin et al., 2019). Patients exposed to higher doses of intraoperative opioids were associated with an increase in postoperative pain scores and higher opioid consumption (Joly et al., 2005; Richebé et al., 2011). The identification and management of OIH in these patients is crucial, because if untreated it can increase the risk of developing persistent postsurgical pain (Reviewed by Colvin et al., 2019 and; Glare et al., 2019). There are also studies describing OIH in human volunteers after acute short-term exposure to opioids (Holtman et al., 2007; Koppert et al., 2003) and the results showed aggravation of induced hyperalgesic skin lesions, expansion of the area of mechanical hyperalgesia induced by transdermal electrical stimulation (Koppert et al., 2001), 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 (Compton et al., 2003; Compton et al., 2004). Also, a small prospective study in six patients with chronic back pain, after one month of oral

morphine treatment, showed a reduction of the experimental pain threshold when compared to baseline values (Chu et al., 2006). Other study reports four cases of chronic non-malignant pain that was exacerbated by opioids, a phenomenon that was reversed by cessation of opioids use (Brodner and Taub, 1978). Finally, in a clinical study with patients with cancer pain, the opioid treatment failed and induced more pain (Mercadante et al., 2012).

4.3. Molecular mechanisms

The precise molecular mechanisms 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. Spinal cord plasticity underlying OIH has been demonstrated after both intraspinal and systemic administration of opioids. The consequence of spinal sensitization is increased transmission of noxious inputs to supraspinal sites. The influence of higher CNS centers in OIH is yet poorly studied. However, there has been an increase in the 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 during OIH (Vanderah et al., 2001).

Regarding the molecular mechanisms underlying OIH, there are evidences suggesting that after morphine binding to MOR on a post-synaptic neuron, there is activation of G-protein mediated PKC translocation and the removal of the NMDA receptor Mg^{2+} plug (Figure 5). 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 (reviewed in Deleo et al., 2004). NK-1 receptor activation by the excitatory neurotransmitter SP (King

et al., 2005), released by the pre-synaptic neuron, also contributes to the increased of intracellular Ca^{2+}(reviewed in Roeckel et al., 2016). 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. In addition, morphine is able to block the glutamate transporter resulting in an increase of glutamate in the synaptic cleft, which will then reverberate neuronal excitation.

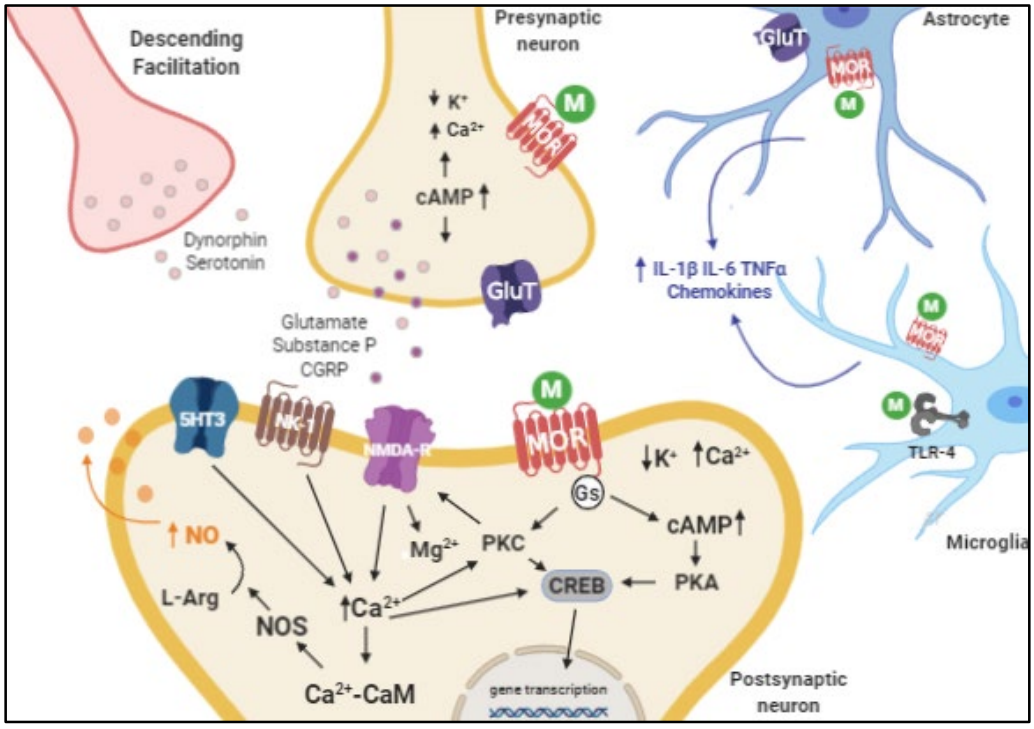

Figure 5- Molecular mechanisms proposed to be involved in the genesis of opioid hyperalgesia. Morphine (represented by M, as a representative opioid) may act on neurons and on the glial cells. Activation of neuronal MOR will increase cAMP levels, inducing an increased release of excitatory amino acids and changes in gene expression. Morphine can also block the glutamate transporter, increasing the synaptic concentrations of glutamate. The activation of PKC promote the removal of the Mg^{2+} plug from the NMDA receptor, increasing the intracellular Ca^{2+} which leads to several downstream effects, that will promote glutamate release from the pre-synaptic neuron and changes in gene expression. Activation of the 5-HT3 and NK-1 receptors are also involved in the increase of the intracellular Ca^{2+}. Increased descending facilitation, from supraspinal areas, is also involved in the spinal plasticity resulting from sustained exposure to opioids. Chronic opioid administration also acts on glial cells, increasing the production and secretion of release of pro-inflammatory factors. 5-HT3, 5-hydroxytryptamine receptor; Ca^{2+}-CaM, calcium-calmodulin; cAMP, cyclic adenosine monophosphate; CGRP. Calcitonin Gene-related Peptide; CREB, cAMP response element binding protein; GluT, glutamate transporters; IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; L-Arg, L-arginine; MOR, mu-opioid receptor; NK1, neurokinin 1 receptor; NMDA, N-methyl-D-aspartic acid receptor; NO, nitric oxide; NOS, nitric oxide synthase; PKA, protein kinase A; PKC, protein kinase C; TLR-4, Toll-like receptor 4; TNFα, Tumor Necrosis Factor alpha (Adapted from Deleo et al., 2004; Koppert and Schmelz, 2007; Roeckel et al., 2016).

As described above, opioid receptor coupled Gi/o-activation reduces cAMP levels. However, long-term activation of the receptor with the agonist can impair MOR G protein coupling. MOR can couple with Gs-protein resulting in the up-regulation of adenylate cyclase activity and increased cAMP levels (reviewed in Crain and Shen, 1998). Increased cAMP levels may via presynaptic activation increase the release of excitatory neurotransmitters at a spinal level and also induce changes in gene expression (reviewed in Koppert and Schmelz, 2007).

An increase of descending facilitation from the RVM also contributes to OIH. Morphine-induced hyperalgesia is blocked after lidocaine injection into the RVM (Vanderah et al., 2001) or after bilateral lesions of the dorsal funiculus (Gardell et al., 2002). Increased CCK at the RVM results in increased descending facilitation leading to up-regulation of spinal dynorphin and enhanced release of excitatory transmitters, like CGRP. (Gardell et al., 2002; reviewed in Koppert and Schmelz, 2007). Likewise, enhanced descending serotoninergic input from the RVM (Vera-Portocarrero et al., 2007) likely contributes to the central sensitization and enhanced pain through activation of spinal 5-HT3 receptors (Liang et al., 2011).

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 astrocytes, increasing the production and release of cytokines and chemokines, or directly on glial glutamate transporters to alter synaptic glutamate levels (Reviewed in Roeckel et al., 2016). Also, microglia activation contributes to OIH (reviewed in Watkins et al., 2009). Morphine binds to its specific receptor MOR or to the toll-like receptor 4 (TLR-4) that will activate molecular intracellular cascades triggering the glial activation and increasing the production and the release of pro-inflammatory factors (Ferrini et al., 2013; Reviewed in Grace et al., 2015;

Hutchinson et al., 2010). Once released, cytokines may act on the pre- or post-synaptic neurons, inducing abnormal spontaneous activity, or on the glial cells to promote further neuroimmune activation (reviewed in Roeckel et al., 2016).

4.4. The Role of MOR in OIH

The involvement of MOR in OIH still poorly understood. The absence of OIH in two different MOR knock-out lines of mice in both genders and with different protocols of OIH induction supports an involvement of MOR in OIH (Roeckel et al., 2017). Likewise, deletion of MOR from transient receptor potential cation channel subfamily V member 1 nociceptors prevents the development of OIH without disrupting opioid analgesia (Corder et al., 2017). This suggest that MOR expressed at primary afferent nociceptors are responsible for initiating the adverse counter-adaptations induced by opioids that lead to OIH.

As described above, opioid treatment might result in a shift of MOR coupling from Gi to Gs. The functional consequence of the switch in G protein coupling is the activation, rather than inhibition, of cell excitability (reviewed in Crain and Shen, 2000). An increase of MOR coupling to Gs at the spinal cord was observed after hyperalgesia induced by acute morphine infusion (Tsai et al., 2009). Likewise, chronic treatment with morphine altesr MOR-G protein coupling at several levels of pain circuitry, namely in sensory neurons (Crain and Shen, 2000), spinal cord (Wang et al., 2016; Wang et al., 2005) and PAG (Wang et al., 2005). It has been proposed that the inhibitory and excitatory effects of MOR might be due to specific isoforms of the receptor. MOR is a seven transmembrane domain (7TM) GPCR encoded by the OPRM1 gene. Alternative splicing mechanisms result in 7TM, 6TM and 1TM receptor isoforms. The role of these variants, their expression levels as well as distribution, have not been extensively

explored. However, morphine treatment elicited changes in mRNA levels in some isoforms and in specific brain regions (Xu et al., 2015). The 6TM variants has been studied because of their peculiar properties on nociception. A knock-out mice to all of the 6TM variants failed to develop morphine-induced hyperalgesia without altering morphine analgesia (Marrone et al., 2017). The mRNA levels of all the 6TM variants were increased in the brainstem of mice chronically treated with morphine (Xu et al., 2015). A specific 6TM isoform identified in mice, MOR-1K, induces excitatory cellular effects by activating Gs-proteins (Gris et al., 2010) and the knock-down of this isoform led to a decrease of morphine-induced hyperalgesia compared to wild-type animals (Oladosu et al., 2015). Increased mRNA levels of the 6TM isoform were observed after chronic treatment with morphine in all the brainstem, the hypothalamus and the striatum (Xu et al., 2015). Although most of the studies focus in the 6TM variants, *in vitro* studies have shown that chronic treatment with opioids also up-regulates 7TM isoforms, MOR-1B2 and MOR-1C1 (Chakrabarti et al., 2016; Verzillo et al., 2014), and their coupling to Gs-proteins (Chakrabarti et al., 2019). These observations suggest different MOR variants could be important in the excitatory effects of opioids.

5. Aims and Methodology

The opioidergic system plays a major role in the control of descending pain modulatory areas of the brain. However, there is a lack of knowledge in the alterations the system might suffer in pathological conditions such as the chronic neuropathic pain and in the paradoxical OIH phenomena, namely at descending pain facilitatory areas such as the DRt. This is especially relevant as the maintenance of neuropathic pain and OIH may rely on increased descending facilitation.

In the present dissertation we aimed at studying the opioidergic modulation of the DRt, a peculiar medullary area exerting a unique and exclusive descending facilitatory role in pain modulation, during neuropathic pain (Study I) and OIH (Study II). In order to manipulate the opioidergic system at the DRt we used pharmacological and gene transfer techniques to specifically target the MOR. In the latter technique, we used lentiviral vectors because of their ability to restrict transduction to the injection site with no retrograde transport (Snyder et al., 2010). All lentiviral vectors used carried the human synapsin promoter which allowed us to specifically manipulate the expression of MOR in neurons (Kügler et al., 2003). We focused our studies on MOR since currently the most powerful analgesic opioid drugs available act through this receptor. Furthermore, alterations on MOR function and signaling are reported both during neuropathic pain and OIH (Martínez-Navarro et al., 2019; Roeckel et al., 2016).

This dissertation includes two publications. The first study aimed at evaluating the opioidergic modulation of the DRt during neuropathic pain (***Publication I***). We used the spared nerve injury (SNI) model of neuropathic pain which induces robust and stable behavioral signs of mechanical allodynia, mechanical hyperalgesia and cold allodynia

similar to stimulus-evoked pain observed in clinical neuropathic pain syndromes (Decosterd and Woolf, 2000).

First, we performed a series of behavioral experiments in naïve animals to evaluate the role of MOR at the DRt during acute pain. For that we manipulated MOR at the DRt by using a pharmacological approach with the DAMGO agonist, or a lentiviral gene-transfer approach for MOR knockdown. We also evaluated the effects of MOR blockade at the DRt on the analgesic effects of systemic morphine. The blockade of MOR was performed by the MOR antagonist CTAP, and by lentiviral-mediated MOR knockdown. The effects of the drugs or the lentiviral-mediated MOR knockdown were assessed by the von-Frey and hotplate tests which evaluate mechanical and thermal sensitivity, respectively. Then, we measured the extracellular levels of the endogenous opioid peptides methionine- and leucine- enkephalin at the DRt of sham and SNI animals, by *in vivo* microdialysis. Afterwards, we focused on the evaluation of the expression and function of MOR in SNI animals. We used quantitative real-time PCR to quantify the expression of MOR and immunohistochemistry to quantify the numbers of MOR-expressing cells as well as the levels of phosphorylated MOR. Additionally, in SNI animals, we performed behavioral experiments to determine the potency of systemic morphine. Then, we used pharmacological and gene transfer techniques to evaluate the function of MOR at the DRt of SNI animals. This was achieved by the local injection of the MOR agonist DAMGO and by using a lentiviral vector for the overexpression of MOR. Pain assessment in SNI animals was performed using the von-Frey test to evaluate the effects of the drugs and lentiviral-mediated MOR overexpression in mechanical allodynia.

The second study aimed at evaluating the opioidergic modulation of the DRt in OIH (***Publication II***). We induced OIH via subcutaneous implantation of osmotic mini-

pumps containing morphine (Vanderah et al., 2001). Morphine is widely used to control moderate-to-severe pain and several studies have shown that morphine induced OIH in humans and in animals. Animals develop mechanical allodynia and thermal hyperalgesia which are representative symptoms of OIH in humans (Angst and Clark, 2006). We started by evaluating the behavioral effects of morphine administration at an earlier time point after mini-pumps implantation and then several days after mini-pumps implantation until day 7. For that, we used the von-Frey and the hotplate tests, which assess mechanical and thermal sensitivity, respectively. Then we evaluated the involvement of the DRt in OIH by pharmacologically blocking the DRt with lidocaine. After determining the involvement of the DRt in OIH, we performed a series of studies to evaluate the role of MOR at the DRt in OIH. We evaluated the effects of sustained morphine on MOR expression at mRNA and protein levels at the DRt by quantitative real-time PCR and immunohistochemistry, respectively. Then we determined the effects of lentiviral-mediated knockdown of MOR at the DRt in the development of OIH. Additionally, we performed several studies to evaluate the effects of chronic morphine infusion on MOR function and signaling pathways at the DRt. One of the molecular adaptations to chronic opioid exposure is the switch of MOR signaling to excitatory via coupling of MOR to Gs followed by the up-regulation of the cAMP/PKA signaling (Chakrabarti et al., 2005; Crain and Shen, 2000; Wang and Burns, 2009). To test whether this type of MOR adaptation occurs, we first evaluated the effects of chronic morphine in the expression of the phosphorylated cAMP response element binding protein (pCREB), a downstream marker of the excitatory signaling transduction pathway of MOR (Wang and Burns, 2009), by immunohistochemistry at the DRt and in the adjacent areas. Then we determined the effects of the MOR agonist DAMGO, an ultra-low dose of naloxone, which prevents MOR coupling to the Gs-protein (Crain and Shen, 2000), and the PKA

inhibitor H-89, on mechanical and thermal hypersensitivity. We also determined the effects of the ultra-low dose of naloxone on the expression of pCREB at the DRt and in adjacent areas.

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Publications

Publication I

Neuropathic Pain Induced Alterations in the Opioidergic Modulation of a Descending Pain Facilitatory Area of the Brain. Frontiers in cellular neuroscience (2019)

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 28 June 2019
doi: 10.3389/fncel.2019.00287

Neuropathic Pain Induced Alterations in the Opioidergic Modulation of a Descending Pain Facilitatory Area of the Brain

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Edited by:
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Patrick L. Sheets, Indiana University School of Medicine-South Bend, United States

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Specialty section:
This article was submitted to Cellular Neurophysiology, a section of the journal Frontiers in Cellular Neuroscience

Received: *30 January 2019*
Accepted: *13 June 2019*
Published: *28 June 2019*

Citation:
Costa AR, Carvalho P, Flik G, Wilson SP, Reguenga C, Martins I and Tavares I (2019) Neuropathic Pain Induced Alterations in the Opioidergic Modulation of a Descending Pain Facilitatory Area of the Brain. Front. Cell. Neurosci. 13:287. doi: 10.3389/fncel.2019.00287

Opioids play a major role at descending pain modulation but the effects of neuropathic pain on the brain opioidergic system remain understudied. Since descending facilitation is enhanced during neuropathic pain, we studied the opioidergic modulation of the dorsal reticular nucleus (DRt), a medullary pain facilitatory area, in the spared nerve injury (SNI) model of neuropathic pain. We first performed a series of behavioral experiments in naïve-animals to establish the role of μ-opioid receptor (MOR) in the effects of endogenous and exogenous opioids at the DRt. Specifically, we showed that lentiviral-mediated MOR-knockdown at the DRt increased sensitivity to thermal and mechanical stimuli while the MOR agonist DAMGO induced the opposite effects. Additionally, we showed that MOR-knockdown and the pharmacological blockade of MOR by CTAP at the DRt decreased and inhibited, respectively, the analgesic effects of systemic morphine. Then, we performed *in vivo* microdialysis to measure enkephalin peptides in the DRt and evaluated MOR expression in the DRt at mRNA, protein and phosphorylated form levels by quantitative real-time PCR and immunohistochemistry, respectively. SNI-animals, compared to sham control, showed higher levels of enkephalin peptides, lower MOR-labeled cells without alterations in MOR mRNA levels, and higher phosphorylated MOR-labeled cells. Finally, we performed behavioral studies in SNI animals to determine the potency of systemic morphine and the effects of the pharmacologic and genetic manipulation of MOR at the DRt. We showed a reduced potency of the antiallodynic effects of systemic morphine in SNI-animals compared to the antinociceptive effects in sham animals. Increasing MOR-cells at the DRt of SNI-animals by lentiviral-mediated MOR-overexpression produced no effects on mechanical allodynia. DAMGO induced anti-allodynia only after MOR-overexpression. These results show that MOR inhibits DRt pain facilitatory actions and that this action contributes to the analgesic effects of systemic opioids. We further show that the inhibitory function of MOR is impaired during neuropathic pain. This is

likely due to desensitization and degradation of MOR which are adaptations of the receptor that can be triggered by MOR phosphorylation. Skipping counter-regulatory pathways involved in MOR adaptations might restore the opioidergic inhibition at pain facilitatory areas.

Keywords: opioids, μ opioid receptor, neuropathic pain, descending pain modulation, dorsal reticular nucleus

INTRODUCTION

Opioids are paramount in the control of descending pain modulatory areas (Fields, 2004; Ossipov et al., 2010), but the effects of chronic neuropathic pain on the opioidergic modulation of pain control centers of the brain remain understudied. This is especially relevant as the maintenance of neuropathic pain may rely on increased descending facilitation (Kovelowski et al., 2000; Bee and Dickenson, 2008; Sotgiu et al., 2008; Martins et al., 2010, 2015b). Insights from human and rodent studies provide evidence of alterations in the supraspinal opioid system in neuropathic pain conditions. Positron emission tomography studies in patients with peripheral and central neuropathic pain revealed reduced μ opioid receptor (MOR) availability in cortical brain areas involved in pain modulation, such as the insula and the striatum, and also in the periaqueductal gray (PAG) and the thalamus (Jones et al., 2004; Willoch et al., 2004; Maarrawi et al., 2007). A recent study performed in rats with peripheral neuropathic pain confirmed a reduced availability of MOR in the cortical areas referred above in human studies and showed that this was paralleled by reduced expression of MOR (Thompson et al., 2018). In the rat, neuropathic pain was further shown to induce MOR adaptations involved in desensitization, such as reduced MOR-mediated-G-protein activity in the thalamus and PAG (Hoot et al., 2011) and increased MOR phosphorylation at the striatum (Petraschka et al., 2007).

The dorsal reticular nucleus (DRt) plays a unique role in the facilitation of pain transmission (Lima and Almeida, 2002; Martins and Tavares, 2017). The DRt establishes reciprocal excitatory connections with the spinal dorsal horn, through which it is thought to amplify pain transmission (Lima and Almeida, 2002; Martins and Tavares, 2017). Descending facilitation from the DRt is enhanced during neuropathic pain (Martins et al., 2010, 2015b) and contributes to spinal sensitization during neuropathic pain (Sotgiu et al., 2008). The opioidergic system represents a key modulatory system at the DRt since it can directly and indirectly modulate the spinal-DRt-spinal reverberative pathway. Indeed, MOR is expressed both in spinally and non-spinally projecting neurons (Pinto et al., 2008b). Opioids act through direct inhibition of DRt spinally projecting neurons and also through disinhibition of enkephalinergic interneurons which receive input from GABAergic interneurons expressing MOR (Pinto et al., 2008a). We have previously shown that opioids inhibit DRt descending facilitation (Martins et al., 2008) and that in a model of chronic inflammatory pain there is a loss of inhibitory opioidergic tone, likely produced by decreased MOR expression, which results in enhanced descending pain facilitation (Pinto et al., 2008a). The impact of neuropathic pain on the opioidergic modulation of the DRt has never been explored. In this study we sought to study the effects of neuropathic pain on the opioidergic modulation of the DRt by using the spared nerve injury (SNI) model of neuropathic pain.

We first performed a series of behavioral experiments in naïve animals which consisted on the evaluation of the effects of the pharmacological and genetic manipulation of MOR at the DRt. We also evaluated in naïve animals the effects of genetic or pharmacological blockade of MOR at the DRt on the analgesic effects of systemic morphine. Then, we evaluated, at the DRt of sham and SNI animals, the extracellular levels of the methionine- (Met) and leucine- (Leu) enkephalin peptides by *in vivo* microdialysis, the mu-opioid receptor (MOR) expression at mRNA, protein and phosphorylated form levels by quantitative real-time PCR and immunohistochemistry, respectively. Finally, in SNI animals, we also performed a series of behavioral experiments to determine the potency of systemic morphine and the effects of the pharmacological and genetic manipulation of MOR at the DRt.

MATERIALS AND METHODS

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of the University of Porto and were performed according to the ethical guidelines for pain investigation (Zimmermann, 1983). Male Wistar rats (Charles River colony, France) were maintained at 22 ± 2°C on a standard 12/12 h light/dark cycle with food and water available *ad libitum*. The animals were acclimated to the housing facility for at least 1 week before any treatment. All procedures were conducted during the light phase between 9:00 am and 5:00 pm. The subjective bias when allocating the animals to the experimental groups was minimized by arbitrarily housing the animals in pairs upon their arrival, then the animals were randomly picked from the cage for each procedure. No a priori power analysis was performed. The sample sizes were based on common practice of the research group where by default 6 animals per group are used in experiments, giving us approximately 90% power to detect large differences (2 standard deviations) between two groups, for continuous outcomes.

Lentiviral Vector Construction

Three lentiviral vectors (LV) were used, a control vector expressing EGFP (LV-EGFP) and vectors designed to knockdown (LV-MOR-R) or overexpress (LV-MOR-F) MOR. Viral vector production was performed as previously described

(Martins et al., 2015a). Briefly, to construct LV-MOR-R and LV-MOR-F, the cDNA for MOR was cloned into a lentivirus transfer vector, in antisense or sense orientation, respectively, relative to the human synapsin promoter (hSYN). This transfer vector also contains an encephalomyocarditis virus internal ribosome entry site (IRES) and the enhanced green fluorescent protein (EGFP). The virus was produced by transfection of human embryonic kidney 293T cells with the transfer vector, a packaging plasmid (pCMVΔR8.92), a plasmid encoding the rev protein (pRSV-Rev) and a plasmid encoding the vesicular stomatitis virus G glycoprotein (pMD.G). The vector LV-EGFP was constructed similarly, using a transfer vector with the hSYN promoter driving expression of EGPF in place of MOR cDNA. The titer of the vectors was determined by quantitative real-time PCR and all vectors were used at 5×10^6 TU/μL. The LV were handled under biosafety level 2 containment and operating conditions according to Biosafety In Microbiological Biomedical Laboratories [BMBL] (2009). The animals injected with the LV were housed under ABSL2 conditions for 48 h, and then housed at ABSL1.

Neuropathic Pain Induction

The SNI model of neuropathic pain was induced as described previously (Decosterd and Woolf, 2000) in rats weighing 210 to 220 g, under isoflurane anesthesia. These body weight are used to allow the animals to reach 285–315 g 2 weeks later, which is the ideal weight range for the stereotaxic surgeries. Briefly, the tibial and common peroneal components of the left sciatic nerve were carefully isolated, ligated and then sectioned. The sural nerve was maintained intact. Sham-operated animals were submitted to the same procedure except that no lesion was made. At the end of the procedure, the muscle and skin were sutured and the rats returned to their respective cages.

Stereotaxic Surgeries

Rats (naïve or subjected to SNI or sham surgery 2 weeks earlier) weighing 285 to 315 g were deeply anesthetized with an i.p. mixture of ketamine hydrochloride (60 mg/Kg) and medetomidine (0.25 mg/Kg) and placed on a kopf frame for the injection of LV or cannula implantation into the left DRt. At the end of surgery, the animals received 0.9% NaCl (0.1 ml/kg, s.c.) for rehydration followed by atipamezole hydrochloride (0.5 g/Kg, s.c.) to revert the anesthesia.

Vector Injection

Stereotaxic injections were performed for the injection of LV into the left DRt in two rostrocaudal parts of the left DRt as previously described (Martins et al., 2015a). Naïve animals were injected with 0.6 μl per site of either LV-MOR-R or the control vector LV-EGFP. SNI-animals were injected with 1 μl per site of either LV-MOR-F or LV-EGFP. A higher volume (1 μl) of LV-MOR-F, compared to LV-MOR-R, was injected at the DRt, as in preliminary experiments this volume allowed a more efficient over-expression of MOR. Nonetheless, the injection of 1 μl induced the spreading of the vector to the non-injected (contralateral) DRt in some animals. In some animals, at the completion of the lentiviral injections, a guide cannula was implanted above the left DRt, as explained below, for the injection of DAMGO.

The effects of the manipulation of MOR expression by the LV were tested before and at 7 days after stereotaxic injections. At 7 days after injection, the hSYN was previously shown to be fully active (Marques-Lopes et al., 2012; Martins et al., 2015a).

Cannula Implantation

A guide cannula was implanted into the left DRt for microdialysis or pharmacological experiments following the coordinates and experimental procedures described previously (Martins et al., 2013, 2015b).

Microdialysis Experiments

One week after stereotaxic surgery, the stylet of the guide cannula implanted in sham- and SNI-animals ($n = 6$ each) was replaced with a 2 mm open length microdialysis probe (molecular weight cutoff 45–50 kDa; Brainlink BV, Groningen, Netherlands). For stabilization purposes, the probe was perfused with Ringer's solution (140.0 mM NaCl; 4.0 mM KCl; 1.2 mM $CaCl_2$; 1.0 mM $MgCl_2$) for 2 h at a flow rate of 2.0 μl/min. Two microdialysate samples were collected in 30 min intervals, for 1 h, into mini-vials already containing 20 μl of 0.02 M formic acid. At end of each collection, the samples were immediately placed on dry ice and stored at −80°C until analysis.

Opioid Peptide Analysis

Met- and Leu-enkephalin were measured by HPLC with tandem mass spectrometry (API-5000). After collection, a mixture of BSA, ascorbic acid, acetic acid, and internal standard (Leu-Enkephalin$^{13}C_6-^{15}N$ was added to the microdialysate samples. Samples were injected by an autosampler onto a Phenomenex column (100 × 3.0 mm; 2.5 μm particle size). The gradient mobile phase contained different concentrations of acetonitrile, formic acid, and ultrapurified water, and was delivered through at a flow rate of 0.3 μl/min. Column effluent was diverted to the waste from $t = 0$–3.4 min to avoid source contamination. The quantification range was 0.5–500 pM.

Behavioral Assessment

Mechanical and thermal sensitivity were assessed by the von Frey and hot-plate tests, respectively, after a 30 min daily habituation of the animals to the experimenter and testing apparatus, for 1 week.

The von Frey test was performed by placing the animals on an elevated transparent cage with a mesh wire bottom allowing the stimulation of the plantar surface of the left hind paw with calibrated von Frey monofilaments (Stoelting, United States) with logarithmically incremental stiffness. In naïve animals, we used a series of calibrated monofilaments ranging from 0.41 to 100 g. Testing started with the 2 g filament applied perpendicular to the plantar surface for 3 s. The weakest filament that elicited a response was taken as the withdrawal threshold. Each animal was tested twice at an interval of 3 to 5 min, each value obtained was logarithmic transformed and averaged. Withdrawal thresholds were determined using the Dixon up-and-down method (Chaplan et al., 1994). In SNI- animals, which typically

develop hypersensitivity to mechanical stimuli on the injured paw (Decosterd and Woolf, 2000), the test was performed by stimulating the lateral plantar surface of the left, injured, hind paw as previously described (Tal and Bennett, 1994). Briefly, we used a series of calibrated monofilaments (Stoelting, United States), starting with the monofilament exerting the lowest force 0.008 g, in a sequence of increasing forces. The threshold was considered the lowest force that evoked a brisk withdrawal to one of five repetitive applications. Animals were also tested twice and each value was logarithmic transformed and averaged. The von Frey test we performed at the day before SNI induction or sham surgery and 14 days after surgery to confirm the development of mechanical hypersensitivity. Additionally, these animals were monitored for signs of sedation and locomotion impairments.

The hot-plate test was used to study thermal hyperalgesia in naïve animals. The test was performed on a hot-plate system (BIO-CHP Cold Hot Plate Test). 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 hot-plate surface. During the habituation period, the animals were placed on the plate set at 35°C for 15 min. On the testing day, the hot-plate was set with a surface temperature of 52°C. Nociceptive threshold was quantified as the latency (in seconds) to licking, retraction of the hind paw or jump after placement of the rat on the hot-plate. A 30 s cut-off was used to avoid tissue damage.

Pharmacological Experiments

The MOR agonist (D-ALA2,N-ME-PHE4,GLY5-OL)-enkephalin acetate (DAMGO) and MOR antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP), both obtained from Sigma-Aldrich (Portugal), were used to test the effects of MOR activation at the DRt of naïve and SNI animals. Morphine hydrochloride, generously provided by Dr. Paulo Cruz (Porto Military Hospital, Porto, Portugal), was administered s.c. All drugs were dissolved in saline.

Three sets of experiments were conducted in naïve animals. In the first set, 0.1 ng of DAMGO (n = 7) or saline (n = 6) were microinjected at the DRt. In the second set, 0.1 ng of DAMGO were microinjected at the DRt of animals previously injected with LV-MOR-R (n = 7) or LV-EGFP (n = 6). In third set, morphine (4 mg/Kg) was administered simultaneously with 0.33 μg of CTAP or saline at the DRt, saline s.c. alone or CTAP alone (n = 6 each). Two sets of experiments were conducted in SNI animals. In the first set, saline (n = 6) or DAMGO at 0.1 (n = 5), 1 (n = 6) or 10 ng (n = 7) were microinjected at the DRt. In the second set, 10 ng of DAMGO were microinjected at the DRt of animals previously injected with LV-MOR-F (n = 7) or LV-EGFP (n = 6).

DAMGO or CTAP were microinjected in a volume of 0.5 μl, infused over a period of 1 min, 1 week after cannula implantation and/or lentiviral injections, using a stainless steel needle protruding 3 mm beyond the cannula. The effects of the drugs were tested before and 15 min after injection, at their peak action (Hurley and Hammond, 2000, 2001). In morphine plus CTAP experiments, morphine was injected first followed by CTAP 15 min later and testing was performed 15 min after CTAP injection (i.e., 30 min after morphine, at its peak action (Erichsen et al., 2005). The doses of DAMGO and CTAP were determined based on previous studies performed at the DRt (Pinto et al., 2008a) and other supraspinal pain modulatory areas (Hurley and Hammond, 2000, 2001; Jongeling et al., 2009). The effects of the drugs were tested by the von Frey and hot-plate tests. The experimenter also monitored qualitatively by gross observations any behavioral changes (catatonia, agitation, ataxia, sedation), as well as levels of alertness throughout the period of testing. All tests were conducted by an experimenter blinded to the treatments.

Morphine Dose-Response Experiments

To evaluate the effect of MOR knockdown at the DRt on the analgesic potency of systemic opioids, we used naïve animals injected 1 week earlier with LV-MOR-R (n = 7) or LV-EGFP (n = 6) at the DRt. To evaluate the impact of SNI on the analgesic potency of morphine, we compared the antinociceptive potency of morphine in sham animals with the antiallodynic potency of morphine in SNI animals (n = 5 each). Naïve or sham animals were tested by the hot-plate test. SNI animals were tested by the von Frey test. The animals were injected first saline s.c. followed by incrementing doses of morphine (0.1, 1, 4, and 10 mg/Kg; s.c). Each dose of morphine was administered every 30 min immediately after testing of the previous dose. Data was converted to percent maximum possible effect (%MPE), as explained below. Dose-response curves were plotted as %MPE vs. dose and fitted with non-linear regression (variable slope model) to determine ED50 values with 95% confidence intervals (GraphPad Prism v7).

Tissue Preparation and Immunohistochemistry

The animals were deeply anesthetized with an overdose of sodium pentobarbital (150 mg/Kg i.p.) and perfused through the ascending aorta for perfusion with 200 mL of calcium-free Tyrode's solution, followed by 800 mL of a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brainstem were removed, immersed in fixative for 4 h followed by 30% sucrose in 0.1 M phosphate-buffered saline (PBS) overnight, at 4°C, and sliced at 40 μm in a freezing microtome.

Immunohistochemical Detection of MOR

In a first experimental set, MOR expression was determined in sham- and SNI- animals (n = 6 each group) at 3 weeks after SNI induction or sham-surgery. In a second experimental set, MOR expression was determined 1 week after injection of: (i) LV-MOR-R (n = 5) or LV-EGFP (n = 6) at the DRt of naïve animals; or (ii) LV-MOR-F (n = 7) or LV-EGFP (n = 6) at the DRt of SNI animals. One in every fourth section encompassing the DRt was incubated for 2 h in a blocking solution containing 0.1 M glycine and 10% normal swine serum (NSS) in 0.1 M PBS containing 0.3% Triton X-100 (PBS-T) follow by an incubation for 48 h, at 4°C, in rabbit polyclonal antibody against MOR (ref: RA10104; Neuromics, United States), diluted at 1:1000 in PBS-T containing 2% NSS. After washing with PBS-T, the sections were incubated for 1 h in a swine biotinylated

anti-rabbit serum diluted at 1:200 (Dako, Denmark) diluted in PBS-T containing 2% NSS. The sections were washed again and incubated for 1 h in PBS-T containing the avidin-biotin complex (1:200; Vector Laboratories, United States). After washing in 0.05 M Tris–HCl, pH 7.6, bound peroxidase was revealed using 0.0125% 3,3′ -diaminobenzidinetetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, United States) and 0.025% H_2O_2 in the same buffer. The sections were then dehydrated and mounted in Eukitt. Five sections encompassing the rostro-caudal extent of the DRt were randomly taken from each rat and the numbers of MOR-immunoreactive (IR) neurons were counted into the left and right DRt using the 20× objective by an experimenter blinded as to the experimental group. No differences were detected between the left and right side of the DRt for either SNI- or sham-animals (data not shown) from the first experimental set, therefore, left and right cell profile counts were summed in each tissue section from this experimental set. The DRt was delimitated in an additional set of immunoreacted sections counterstained with formol-thionin (Donovick, 1974) according to the atlas of Paxinos and Watson (1998). The specificity of the antibody anti-MOR was previously tested by blocking the antibody with a blocking peptide in immunohistochemistry and western blot analysis (Pinto et al., 2008b). We further tested antibody specificity by performing negative controls with omission of either the primary or the secondary antibodies which blocked all the immunostaining.

Immunohistochemical Detection of pMOR

The expression of pMOR was determined in sham- and SNI- animals (n = 6 each group) at 3 weeks after SNI induction or sham-surgery. One in every fourth section encompassing the DRt was processed for pMOR immunodetection, following the procedure described above; using a rabbit polyclonal antibody against MOR phosphorylated at serine 375 (Ser375) (Cell Signaling Technology, United States) diluted at 1:800 and incubated for 24 h at room temperature and 48 h at 4°C. Five sections encompassing the rostro-caudal extent of the DRt were randomly taken from each rat and the numbers of MOR-immunoreactive (IR) neurons were counted into the left and right DRt using the 20× objective by an experimenter blinded as to the experimental group. No differences were detected between the left and right side of the DRt (data not shown) therefore, left and right cell profile counts were summed in each tissue section. The specificity of the anti-pMOR was previously tested in agonist-induced phosphorylation assays in HEK293 cells expressing MOR (Schulz et al., 2004; Doll et al., 2011) or a Ser375MOR mutant (Chu et al., 2008) and by preadsorption of the pMOR antibody with an antigenic peptide in immunohistochemistry analysis (Gonzales et al., 2011). We performed additional negative controls by omission of either the primary or the secondary antibodies. No immunostaining was detected in the negative controls.

Quantitative Real-Time PCR

Three weeks after SNI- or sham- surgery rats were deeply anesthetized with an overdose of sodium pentobarbital (150 mg/kg i.p.) and sacrificed by decapitation. The brains were harvested and immediately stored at −80°C. The medulla was cut into a frozen transverse block (1 mm in depth) from −5.60 to −4.68 mm relative to the Interaural line (Paxinos and Watson, 1998) from which the DRt (left and right sides) were dissected out using a tissue micropunch (Stoeling, Chicago, IL, United States). Total RNA from the DRt was extracted using the TRI Reagent (Sigma-Aldrich, Portugal) by following the manufacturer's protocol and the RNA integrity verified by agarose gel electrophoresis. The first strand cDNA synthesis was prepared at 42°C during 1 h, from 0.5 μg of total RNA using 200 U of reverse transcriptase enzyme (Nzytech, Portugal) and 500 ng of oligo(dT)12–18 (Nzytech, Portugal). To assess for potential contaminants, a control containing all reagents except the reverse transcriptase enzyme was included for each sample. The expression levels of MOR mRNA were then quantified by the standard 2^(–delta delta CT) method using a StepOnePlus Real-Time PCR system (Applied Biosystems, United States) and a SYBR green chemistry (SYBR Select master mix, Applied Biosystems, United States). The following intron-spanning primers 5′-GCCATCGGTCTGCCTGTAAT-3′ and 5′-GAGCAGGTTCTCCCAGTAC-3′ were designed to amplify exon 2 and 3 from the MOR-1 transcript. Normalization was performed by amplification of rat GAPDH using the primers 5′-GCATGGACTGTGGTCCTCAG-3′ and 5′-CCATCACCATCTTCCAGGAG-3′. The thermal cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. Melting curve analysis of every qPCR was conducted to ensure amplicon specificity. The results were presented as relative differences to sham MOR mRNA at the DRt.

Histology

Animals used in microdialysis experiments or injected with the LV were deeply anesthetized with an overdose of sodium pentobarbital (150 mg/Kg i.p.) and sacrificed by vascular perfusion as above. The animals used in pharmacological experiments were injected 0.5 μl of 0.6% Chicago sky blue dye (Sigma, United States) through the guide cannula, and sacrificed by decapitation. The brainstems were dissected out, and the medulla oblongata was coronally sectioned at 40 μm on a cryostat. Sections collected through the entire rostrocaudal extent of the DRt were stained by the formol-thionin (Donovick, 1974) for verification of probe location or blue dye injection (**Figures 1A,B**), as previously described (Martins et al., 2010, 2015a). In LV-EGFP-injected rats the injection site was observed by direct detection of EGFP labeling (**Figure 1C**). In LV-MOR-R- and LV-MOR-F-injected rats the location of the injection tract was observed in formol-thionin stained sections because the detection of EGFP was very faint. The EGFP transgene was inserted into the expression cassette in the second position of the bicistronic constructs which might be the raison why in the LV-MOR-F vector lower levels of EGFP expression were detected. In the LV-MOR-R vector, the lower levels of EGFP might be due to the RNA interference reaction induced by antisense RNA of MOR which also degrades EGFP RNA. Only animals with vector injections, cannula or probe placement centered in the DRt were included in data analysis.

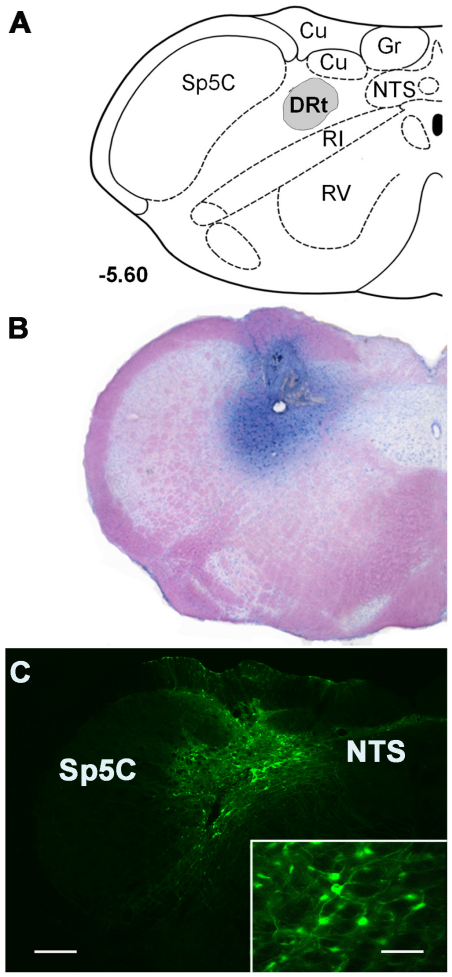

FIGURE 1 | Representative injection sites at the DRt depicted in coronal sections. **(A)** Diagram depicting the location of the dorsal reticular nucleus (DRt), at 5.60 mm caudal to the interaural line [adapted from Paxinos and Watson (1998)]. **(B)** Representative photomicrograph of a thionin-stained section illustrating an injection site in the DRt identified by the needle tract surrounded by the dye staining. **(C)** Photomicrograph of a representative injection site of the control vector LV-EGFP injected at 0.6 μl into the DRt. The injection site includes a central area located within the DRt surrounded by EGFP-labeled neurons (better depicted at higher magnification in the insert). Scale bar in **C:** 200 μm (**B** is at the same magnification), scale bar of the enlargement: 50 μm. Abbreviations: Cu, cuneate nucleus; Gr, nucleus gracilis; IR, intermediate reticular nucleus; NTS, nucleus of the solitary tract; Sp5C, spinal trigeminal nucleus, pars caudalis; VR, ventral reticular nucleus.

Calculation of MPE and Statistical Analysis

To enable the comparison of DAMGO or morphine effects after MOR knock down at the DRt (**Figures 3**, **4**) and also the comparison of morphine effects in sham and SNI animals (**Table 2**), raw data was converted to percent maximum possible effect (%MPE) according to the equation: % MPE = (Post drug value−Pre-drug value)/(Ceiling value−Pre-drug value) × 100. In **Figures 3**, **4**, "D7" (i.e., 7 days after LV injection) was taken as "Pre-drug value." Paw withdrawal thresholds obtained in the von Frey test were log transformed for the estimation of MPE. To determine the %MPE from the data obtained in the von Frey test performed in naïve animals (**Figure 3**), the ceiling value (i.e., the maximum stimulus applied) was 100 g after DAMGO injection. To determine the %MPE from the data obtained in the von Frey test performed in SNI animals (**Table 2**), the ceiling value was the pre-operative (i.e., before SNI induction) value which was 15 g. To determine the %MPE from the data obtained in the hot-plate test, a cutoff latency of 30 s was taken as the ceiling value. %MPE values are presented as mean ± SD.

The behavioral effects of DAMGO, CTAP or the vectors, obtained in the hot-plate and von Frey test, and the %MPE of morphine in sham and SNI animals were analyzed by a two-way mixed ANOVA for repeated measurements. Mechanical threshold responses, obtained in the von Frey test, were logarithmic transformed to enable ANOVA analysis. In case of a significant interaction between group and time, we proceeded with pairwise comparisons using Tukey's correction to adjust p-values for multiple testing. The effects of the LV on the number of MOR-IR cells in the left-injected (ipsilateral) and right (contralateral) DRt was analyzed by a two-way mixed ANOVA for repeated measurements (with the LV as a between factor and DRt sides as a within factor) followed by pairwise comparisons using Tukey's correction. The unpaired *t*-test was used to compare the number of MOR- IR cells, pMOR-IR cells and MOR-mRNA levels between SNI- and sham-animals, the %MPE of DAMGO in LV-EGFP and LV-MOR-R and the ED50 of morphine in LV-EGFP and LV-MOR-R. The normality assumption was checked by inspection of the distribution of the variables both with q-q plots and histograms. However, we must acknowledge that the sample size limits the ability to detect departures from normality. The statistical analysis was performed by GraphPad Prism v7 and SPSS v24. The significance level was set at 0.05 and all statistical tests were two-tailed.

RESULTS

MOR Expression at the DRt Produces Antinociception

The effects of MOR activation at the DRt of naïve animals were studied by using pharmacological and gene transfer approaches. The effects of both experimental approaches were tested by the von Frey and hot-plate tests.

In the first approach, we tested the effects of MOR activation by microinjection of DAMGO at 0.1 ng (n = 7) and saline (n = 6) into the left DRt. The analysis of the effects of DAMGO in the von Frey test revealed a significant interaction between treatment and time ($F_{1,11}$ = 7.57, p = 0.019; **Figure 2A**). DAMGO increased withdrawal thresholds (1.5 ± 0.3) compared to before the injection (i.e., T0: 1.2 ± 0.2; p = 0.026) and saline (1.1 ± 0.1; p = 0.004; **Figure 2A**). Saline produced no significant effects (**Figure 2A**). Withdrawal thresholds before DAMGO and saline injections were not different (**Figure 2A**). The analysis of the effects of DAMGO in the hot-plate test revealed a significant interaction between treatment and time ($F_{1,11}$ = 5.53, p = 0.038; **Figure 2B**). DAMGO increased latencies (13.3 ± 3.4 s) compared

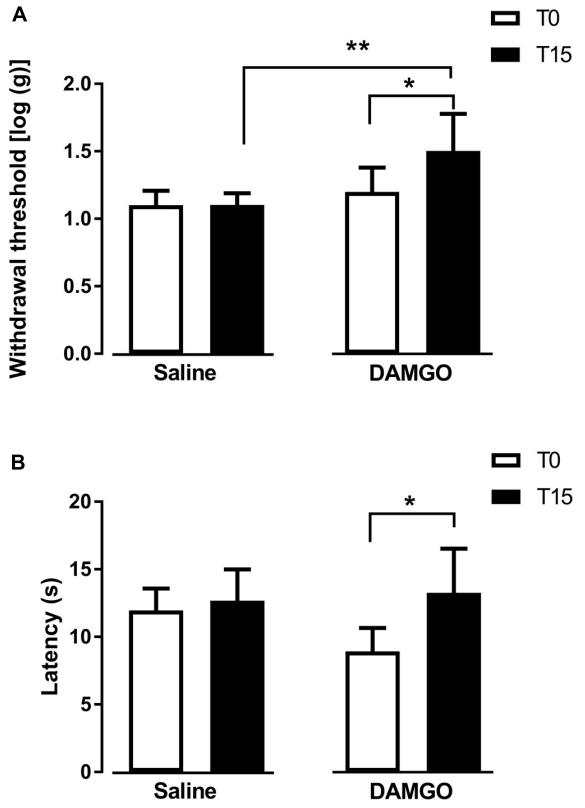

FIGURE 2 | MOR activation at the DRt by the agonist DAMGO decreases mechanical and thermal sensitivity in naïve animals. Saline or DAMGO were injected at the DRt and their effects were assessed before (T0) and 15 min after (T15) injection by the von Frey **(A)** and the Hot plate **(B)** tests which evaluate mechanical and thermal sensitivity, respectively. Data are presented as mean ± SD (Saline $n = 6$; DAMGO $n = 7$). *$p < 0.05$, **$p < 0.01$.

to before the injection (8.9 ± 1.9 s; $p = 0.012$; **Figure 2B**). Saline produced no significant effects (**Figure 2B**). Latencies before DAMGO and saline injections were not different (**Figure 2B**). No visible signs of sedation or ataxia were observed after DAMGO injection and the rats remained alert throughout the testing period.

In the second approach, we used a lentiviral vector (LV-MOR-R) to knockdown the expression of MOR at the DRt. The effects were tested before (D0) and 7 days (D7) after DRt injections. The analysis of the numbers of MOR-IR neurons after the injection of LV-EGFP ($n = 6$) and LV-MOR-R ($n = 5$) at the left DRt revealed a significant interaction between vectors and DRt sides ($F_{1,9} = 8.32$, $p = 0.018$; **Figures 3A–C**). LV-MOR-R decreased the number of MOR-IR neurons in the left ipsilateral-injected DRt (20.7 ± 2.0) compared to the contralateral DRt (29.1 ± 2.7; $p = 0.004$) or compared to the ipsilateral LV-EGFP injected DRt (36.9 ± 2.3; $p < 0.001$; **Figure 3C**). The numbers of MOR-IR neurons were not different between the ipsilateral and contralateral DRt after LV-EGFP injection (**Figure 3C**). The analysis of the behavioral data obtained in the von Frey test revealed a significant interaction between vectors and time ($F_{1,9} = 21.06$, $p = 0.001$; **Figure 3D**). LV-MOR-R decreased withdrawal thresholds (0.7 ± 0.1) compared to before vector injection (i.e., D0: 1.1 ± 0.1; $p < 0.001$) and to LV-EGFP (0.9 ± 0.1; $p < 0.001$, **Figure 3D**). LV-EGFP also decreased withdrawal thresholds (0.9 ± 0.1) compared to D0 (1.1 ± 0.1; $p = 0.004$; **Figure 3D**). Withdrawal thresholds before LV-EGFP and LV-MOR-R injections were not different (**Figure 3D**). The analysis of the behavioral data obtained in the hot-plate test revealed a significant interaction between vectors and time ($F_{1,9} = 11.21$, $p = 0.008$; **Figure 3G**). LV-MOR-R decreased latencies (6.2 ± 0.2 s) compared to before vector injection (8.5 ± 0.8 s; $p = 0.008$) and to LV-EGFP (8.1 ± 1.6 s; $p = 0.024$, **Figure 3G**). LV-EGFP produced no significant effects (**Figure 3G**). Latencies before LV-EGFP and LV-MOR-R injections were not different (**Figure 3G**).

We also tested whether MOR knockdown at the DRt reduced the antinociceptive effects of the MOR agonist DAMGO injected at the DRt. We used a different set of animals simultaneously injected with LV-EGFP ($n = 6$) or LV-MOR-R ($n = 7$) and implanted with a guide cannula into the left DRt. One week later, we injected DAMGO at 0.1 ng, through the guide cannula. The effects were tested before (D0) and 7 days (D7; i.e., before the injection of DAMGO) after injection of the LV and 15 min after the injection of DAMGO (D7 + DAMGO). The analysis of the behavioral data obtained in the von Frey test revealed a significant interaction between vectors and time ($F_{2,22} = 45.06$, $p = < 0.001$; **Figure 3E**). In the LV-MOR-R group, DAMGO increased withdrawal thresholds (1.07 ± 0.2), compared to before the injection (0.7 ± 0.08; $p < 0.001$), to values similar to D0 (1.06 ± 0.08; **Figure 3E**). In the LV-EGFP group, DAMGO increased withdrawal thresholds (1.75 ± 0.07) compared to before the injection (1.15 ± 0.05; $p < 0.001$) and D0 (1.16 ± 0.04; $p < 0.001$; **Figure 3E**). The MPE of DAMGO in the von Frey test was lower in the LV-MOR-R group (33.8 ± 12.6%) compared to the LV-EGFP group (95.0 ± 12.3%; $p < 0.001$; **Figure 3F**). The analysis of the behavioral data obtained in the hot-plate test revealed a significant interaction between vectors and time ($F_{2,22} = 11.47$, $p < 0.001$; **Figure 3H**). In the LV-MOR-R group, DAMGO increased latencies (10.1 ± 1.8 s), compared to before the injection (5.9 ± 1.1 s; $p = 0.04$), to values similar to D0 (8.2 ± 0.7 s; **Figure 3H**). In the LV-EGFP group, DAMGO increased latencies (15.6 ± 1.6 s), compared to before the injection (10.2 ± 2.0 s; $p < 0.001$) and to D0 (9.3 ± 0.8 s; $p < 0.001$; **Figure 3H**). The MPE of DAMGO in the hot-plate test was marginally lower in the LV-MOR-R group (17.3 ± 7.4%) compared to the LV-EGFP group (27.0 ± 8.8%; $p < 0.053$; **Figure 3I**).

MOR Expression at the DRt Contributes to the Analgesic Effects of Systemic Morphine

To evaluate whether the expression of MOR at the DRt of naïve animals is relevant for the analgesic effects of opioids administered systemically, we determined the effects of s.c. morphine after genetic MOR knockdown or pharmacological blockade of MOR at the DRt. The analgesic effects of morphine in both experimental approaches were tested by the hot-plate test.

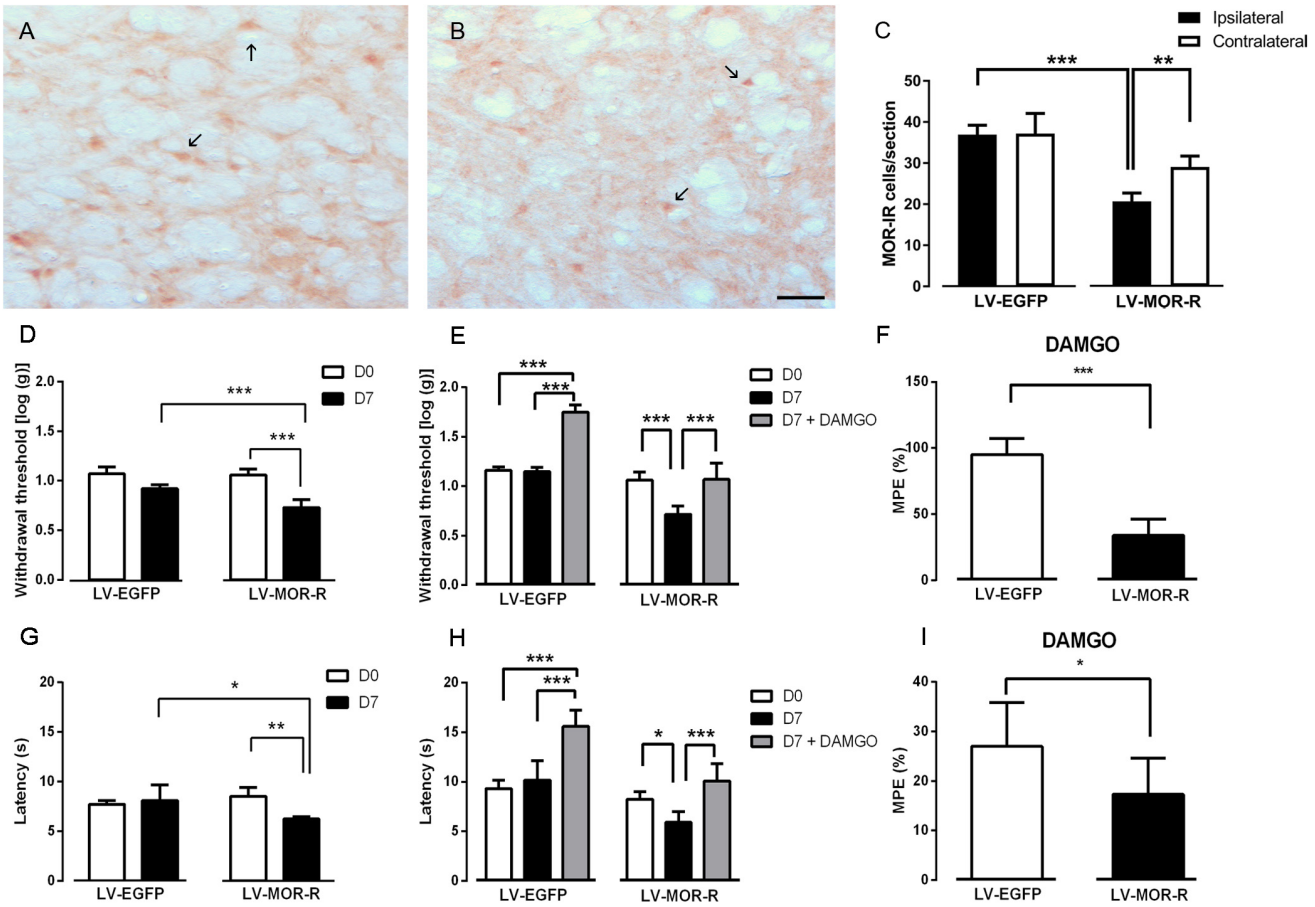

FIGURE 3 | Lentiviral-mediated MOR knockdown at the DRt increases mechanical and thermal sensitivity in naïve animals. Representative photomicrographs of MOR-immunoreactive (IR) cells at the DRt of naïve animals injected with LV-EGFP **(A)** and LV-MOR-R **(B)**. Typical MOR immunolabeling is marked by arrows. Scale bar in **(B)**: 100 μm (A is at the same magnification). Data in **(C)** represents the number of MOR immunoreactive (IR) cells after lentiviral vectors injection into the DRt at the injected (ipsilateral) and contralateral side. LV-EGFP (n = 6) or LV-MOR-R (n = 5) were injected at the DRt and their effects were assessed before (D0) and 7 days (D7) after injection by the von Frey **(D)** and hot-plate **(G)** which evaluate mechanical and thermal sensitivity, respectively. An additional group of animals injected with LV-EGFP (n = 6) or LV-MOR-R (n = 7) into the DRt, was administrated 0,1 ng of DAMGO at the DRt. The effects of DAMGO were assessed before (D7) and 15 min after injection (D7+DAMGO) by the von Frey **(E)** and hot-plate **(H)**. Data in **(F)** and **(I)** represents the effects of DAMGO converted to percent maximum possible effect (%MPE) on the von Frey and hot-plate tests, respectively. Data are presented as mean ± SD.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

In the first experimental approach, we administered saline or morphine s.c. in a cumulative dosing procedure (0.1, 1, 4, and 10 mg/Kg) at 1 week after the injection of LV-EGFP (n = 6) or LV-MOR-R (n = 7) into the left DRt. The analysis of the behavioral data obtained in the hot-plate test revealed that morphine increased latencies in a concentration-dependent manner ($F_{4,44}$ = 153.8, $p < 0.001$) and that latencies were lower in the LV-MOR-R group ($F_{1,11}$ = 28,21, $p < 0.001$) consistently for all morphine doses, i.e., no interaction was detected between groups and morphine doses ($F_{4,44}$ = 1.86, p = 0.135; **Figure 4A**). The morphine dose that produced 50% of the MPE, i.e., the ED50 in the LV-MOR-R group (ED50 = 5.24 mg/Kg (95% CI: 4.24–6.48 mg/Kg)) was 2-fold greater than in the LV-EGFP group (ED50 = 2.64 mg/Kg (95% CI 1.97–3.53 mg/Kg); t_{11} = 10.15; $p < 0.001$; **Figure 4B**) which indicates a reduction of the analgesic potency of morphine in the LV-MOR-R group.

In the second experimental approach, we determined the effects of morphine s.c. at 4 mg/Kg in animals simultaneously injected with the MOR antagonist CTAP at the DRt. The animals were treated either with saline s.c. alone (n = 6), morphine s.c. plus injection of saline or CTAP at the DRt (n = 6 each), or injected with CTAP alone at the DRt (n = 6). The analysis of the data obtained in the hot-plate test revealed a significant interaction between treatments and time ($F_{3,20}$ = 21.52, $p < 0.001$; **Figure 5**). Morphine plus saline at the DRt increased latencies (20.6 ± 4.4 s) compared to baseline (9.7 ± 1.6 s) and to s.c. saline (9.8 ± 0.9 s; $p < 0.001$; **Figure 5**). The latencies of morphine plus CTAP at the DRt after treatment were not different from baseline (**Figure 5**). Morphine plus CTAP at the DRt (9.9 ± 1.9 s) significantly prevented the elevation of latencies induced by morphine plus saline at the DRt ($p < 0.001$; **Figure 5**). The injection of CTAP alone at the DRt produced no effects compared to baseline

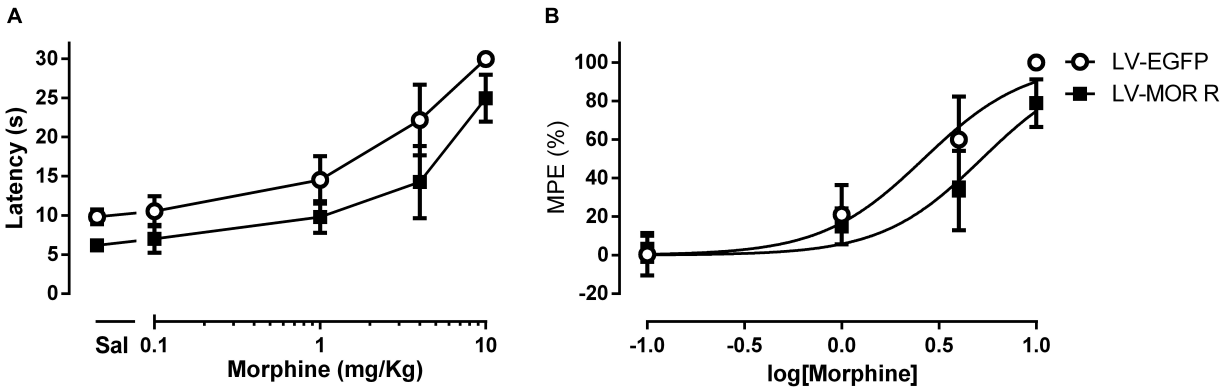

FIGURE 4 | Lentiviral-mediated MOR knockdown at the DRt reduces the analgesic potency of systemic morphine in naïve animals. **(A)** Withdrawal latencies in the hot-plate test after saline administration followed by incrementing doses of morphine (0.1, 1, 4, and 10 mg/Kg) at 1 week after LV-EGFP or LV-MOR-R injection into the DRt. **(B)** Cumulative dose response curves of systemic morphine plotted as percentage of maximum possible effect (% MPE) and fitted by non-linear regression. The ED50 of morphine was 5.24 mg/Kg (95% CI: 4.24–6.48 mg/Kg) in the LV-MOR-R group and 2.64 mg/Kg (95% CI 1.97–3.53 mg/Kg) in the LV-EGFP group. Data are presented as mean ± SD (LV-EGFP, n = 6; LV-MOR-R, n = 7).

TABLE 1 | Met- and Leu-Enkephalin levels at the DRt of SNI animals.

	Met-Enkephalin (pM)	Leu-Enkephalin (pM)
Rat# 800	4.02	N.D.
Rat# 900	5.42	1.22
Rat# 1000	1.59	N.D.
Rat# 2200	3.53	0.89
Rat# 2900	5.73	1.17
Rat# 3000	4.94	1.39
Mean ± SD	**4.20 ± 1.53**	**1.17 ± 0.20**[a]

[a]*N.D., not detected – values below the lower limit of quantification. Mean calculated using values within the limit of quantification.*

TABLE 2 | Effects of morphine in sham and SNI animals.

Morphine (mg/Kg)	% MPE	
	Sham	SNI
0.1	7.2 ± 6.9	−6.6 ± 4.6
1	24.4 ± 9.7	11.2 ± 16.3
4	58.0 ± 13.4	51.7 ± 14.7
10	100 ± 0.0	93.4 ± 3.9
ED50 mg/Kg (95%CI)	**2.5 (1.9–3.3)**	**3.7 (2.9–4.5)**

% MPE values are presented as mean ± SD.

(**Figure 5**). Latencies at baseline were not different between the groups (**Figure 5**).

Effects of SNI on Endogenous Opioid Peptide Levels and the Expression and Phosphorylation of MOR at the DRt

Extracellular levels of Met- and Leu-enkephalin at the DRt of sham- and SNI-animals (n = 6 each) were calculated by averaging the values of two consecutive DRt microdialysates obtained through the course of 1 h. In SNI-animals, an average of 4.20 ± 1.53 pM of Met-enkephalin and 1.17 ± 0.20 pM of Leu-enkephalin were measured in microdialysate samples (**Table 1**). Leu-enkephalin levels were below the limit of detection in two SNI-animals (**Table 1**). In sham-operated animals, Met- and Leu-enkephalin peptides could not be measured as they were below the limit of quantification.

We studied the effects of SNI induction on the expression of MOR at the DRt by evaluating MOR mRNA levels and also the number of MOR-IR cells. The analysis of MOR mRNA levels at the DRt of sham (n = 3) and SNI (n = 5) animals revealed no significant differences between the two groups (t_6 = 1.22; p = 0.268; **Figure 6D**). The analysis of the number of MOR-IR neurons at the DRt of sham- and SNI animals (n = 6 each) showed significantly lower numbers of MOR-IR neurons in SNI-animals (48.7 ± 5.8) compared to sham-animals (67.6 ± 4.3; t_{10} = 6.37; p < 0.001; **Figures 6A–C**).

The effects of neuropathic pain on the phosphorylation of MOR was analyzed by evaluating the number of pMOR-IR at the DRt of sham- and SNI animals (n = 6 each). The analysis of the number of pMOR-IR cells showed significantly higher numbers of pMOR-IR cells in SNI-animals (58.1 ± 6.3) compared to sham-animals (44.6 ± 6.2; t_{10} = 3.71; p = 0.004; **Figures 7A–C**).

The Antiallodynic Potency of Systemic Morphine in SNI Animals Is Reduced Compared to the Antinociceptive Effects in Naïve Animals

We evaluated the potency of systemic morphine in sham and SNI animals by the administration of morphine s.c. in a cumulative dosing procedure (0.1, 1, 4, and 10 mg/Kg). The effects of morphine were determined by the hot-plate test, in the sham group, and by the von Frey test, in the SNI group, and the results were converted in %MPE to enable the comparison. The MPEs of the antinociceptive and antiallodynic effects of

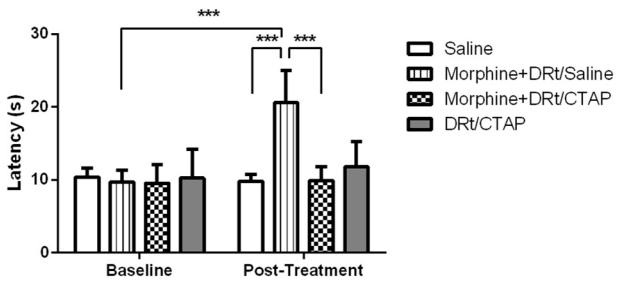

FIGURE 5 | Pharmacological blockade of MOR at the DRt inhibits the analgesic effects of systemic morphine in naïve animals. Morphine was administered s.c. at 4 mg/Kg or saline in animals simultaneously injected with the MOR antagonist CTAP at the DRt. The animals were treated either with saline s.c. alone, morphine s.c. at 4 mg/Kg plus the injection of saline or 0.33 μg of the MOR antagonist CTAP at the DRt, or injected with 0.33 μg of CTAP alone at the DRt. Morphine was injected first followed by CTAP 15 min later. Withdrawal latencies were measured in hot-plate test before s.c. injections and 15 min after saline or CTAP injection at the DRt. Data are presented as mean ± SD (n = 6 each group). ***$p < 0.001$.

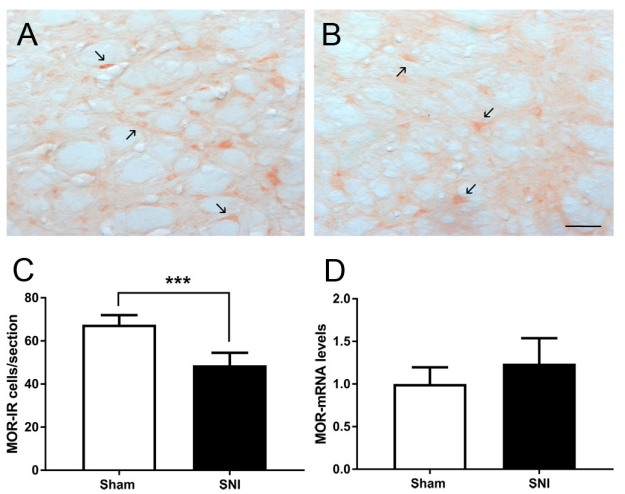

FIGURE 6 | Effects of SNI induction on the expression of MOR at the DRt. Representative photomicrographs of MOR-immunoreactive (IR) cells at the DRt of sham- **(A)** and SNI- **(B)** animals. Typical MOR immunolabeling is marked by arrows. Scale bar in **(B)**: 100 μm (**A** is at the same magnification). Data in **(C)** represents the number of MOR-IR cells at the DRt of sham and SNI animals (n = 6/group). Data in **(D)** represents MOR mRNA levels at the DRt of sham (n = 3) and SNI (n = 5) animals, the results are presented as relative differences to sham MOR mRNA at the DRt. Data in C and D are presented as mean ± SD. ***$p < 0.001$.

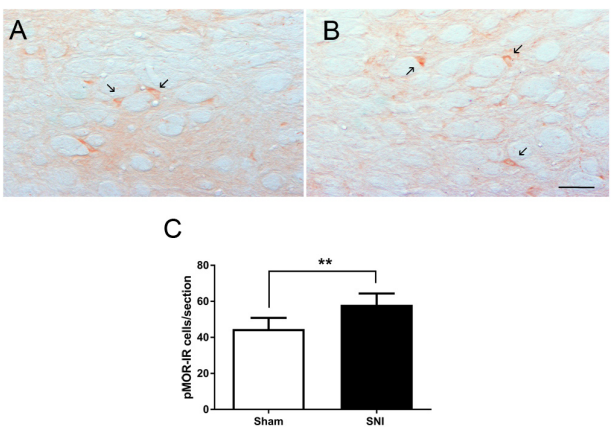

FIGURE 7 | Neuropathic pain increases pMOR expression at the DRt. Representative photomicrographs of pMOR-immunoreactive (IR) cells at the DRt of sham- **(A)** and SNI- **(B)** animals. Typical pMOR immunolabeling is marked by arrows. Scale bar in **(B)**: 100 μm (A is at the same magnification). Data in **(C)** represents the number of pMOR-IR cells at the DRt of sham- and SNI- animals (n = 6/group). Data in **C** are presented as mean ± SD. **$p < 0.01$.

morphine are reported in **Table 2**. The overall analysis revealed that the antinociceptive and antiallodynic MPEs in sham and SNI animals, respectively, increased in a concentration-dependent manner (morphine doses effect: $F_{3,24}$ = 218.5, $p < 0.001$) and that the MPEs of morphine in SNI animals were lower than the MPEs of morphine in sham animals (group effect: $F_{1,8}$ = 5.97, p = 0.040) for all doses tested as indicated by the absence of interaction between the MPEs of the groups and morphine doses (groups × morphine doses interaction: $F_{3,24}$ = 0.50, p = 0.685; **Table 2**). The ED50 of the antiallodynic effect (ED50 = 3.7 mg/Kg (95% CI: 2.9–4.5 mg/Kg) was nearly 1.5-fold greater than the ED50 of the antinociceptive effect (ED50 = 2.5 mg/Kg (95% CI: 1.9–3.3 mg/Kg); t_8 = 5.29; $p < 0.001$; **Table 2**) which indicates a reduced potency of morphine against SNI-induced pain behavior.

SNI Induces an Impairment of MOR Function at the DRt

We performed two sets of experiments to determine the effects of SNI in MOR function at the DRt. In the first set of experiments we aimed at determining the effects of MOR activation. In the second set of experiments, since the number of MOR-IR cells was significantly decreased at the DRt of neuropathic animals, we aimed at testing the effects of restoring the number of MOR-IR cells by overexpressing MOR at the DRt. The effects of both approaches were tested by the von Frey test. Following SNI, the animals developed signs of mechanical allodynia in a manner similar to previous studies (Decosterd and Woolf, 2000; Martins et al., 2010), as shown in the von Frey test by the decrease of withdrawal threshold compared to age-matched naïve animals (**Figures 8**, **9**). In the first approach, the effects of MOR activation were tested in SNI animals at 3 weeks after SNI induction by microinjection of saline (n = 6) or DAMGO at several doses [0.1 ng (n = 5), 1 ng (n = 6) and 10 ng (n = 7)] into the DRt. The overall analysis showed no effect of treatment ($F_{3,20}$ = 2.34, p = 0.104), nor time ($F_{1,20}$ = 3.16 p = 0.091) or interaction (treatment × time: $F_{3,20}$ = 0.16, p = 0.922; **Figure 8**). No behavioral changes were detected after injection of each dose of DAMGO and the levels of alertness also remained unchanged after each injection.

In the second approach, to determine the effects of restoring the number of MOR-IR cells at the DRt of SNI animals, we used a lentiviral vector (LV-MOR-F) to overexpress MOR. LV-MOR-F

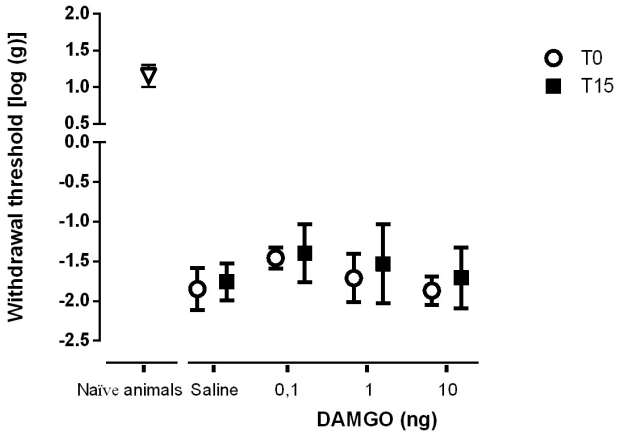

FIGURE 8 | MOR activation at the DRt by the agonist DAMGO produces no effects on mechanical allodynia in SNI animals. Saline (n = 6) or DAMGO at 0.1 ng (n = 5), 1 ng (n = 6) or 10 ng (n = 7) were injected into the DRt and their effects were assessed before (T0) and 15 min after (T15) injection by the von Frey test which evaluates mechanical sensitivity. At T0 (i.e., 3 weeks after SNI induction), all SNI animals presented a marked mechanical hypersensitivity, indicative of mechanical allodynia, as shown by the decreased withdrawal thresholds compared to the withdrawal thresholds of the age-matched naïve animals (n = 13) used in **Figure 2** for the injection of saline or DAMGO. The withdrawal thresholds of naïve animals correspond to the values obtained before the injection of saline or DAMGO in those animals. Data are presented as mean ± SD.

(n = 7) or the control vector LV-EGFP (n = 6) were injected into the left DRt 2 weeks after SNI induction. The effects of the vectors were tested before (D0; i.e., at 2 weeks after SNI induction) and 7 days (D7) after injection, on the von Frey test. The analysis of the numbers of MOR-IR cells revealed that LV-MOR-F increased the number of MOR-IR cells regardless of DRt sides ($F_{1,11}$ = 15.8, p = 0.002), no differences were found between the ipsilateral and contralateral DRt ($F_{1,11}$ = 0.64, p = 0.439) nor a significant interaction (vectors × DRt sides $F_{1,11}$ = 0.001, p = 0.973; **Figure 9C**). MOR-IR cells after LV-MOR injection (ipsilateral: 39.8 ± 8.9; contralateral: 37.8 ± 6.1) were higher compared to LV-EGFP injection (ipsilateral: 28.5 ± 5.8; contralateral: 26.7 ± 3.9; **Figure 9C**). The analysis of the behavioral data obtained in the von Frey test revealed only a significant effect of time ($F_{1,11}$ = 22.02, p < 0.001), but no effect of vectors ($F_{1,11}$ = 0.25, p = 0.629) nor a significant interaction (vectors × time: $F_{1,11}$ = 3.28, p = 0.097; **Figure 9D**). Withdrawal thresholds at D7 (LV-EGFP: −2.0 ± 0.2; LV-MOR-F: −1.9 ± 0.2) dropped compared to D0 (LV-EGFP: −1.5 ± 0.2; LV-MOR-F: −1.7 ± 0.3; **Figure 9D**).

In order to confirm whether virally expressed MOR was functional, we used an additional set of animals that were simultaneously injected with LV-EGFP (n = 6) or LV-MOR-F (n = 7) and implanted with a guide cannula, at the left DRt, 2 weeks after SNI induction. Seven days later, we injected DAMGO at 10 ng, through the guide cannula, and tested its effects on the von Frey test. Overall, the analysis of the data revealed a significant interaction between vectors and time ($F_{1,11}$ = 52.68, p < 0.001; **Figure 9E**). DAMGO increased withdrawal thresholds in LV-MOR-F-injected animals (−0.8 ± 0.4) compared to before the injection (−1.9 ± 0.2; p < 0.001) and compared to LV-EGFP (−2.0 ± 0.2; p < 0.001, **Figure 9E**). In contrast, the injection of DAMGO in LV-EGFP-injected animals produced no significant alterations (**Figure 9E**). Withdrawal thresholds before the injection of DAMGO were not significantly different between LV-EGFP- and LV-MOR-F-injected animals (**Figure 9E**).

DISCUSSION

We show, for the first time, the effects of neuropathic pain on the opioidergic modulation of the DRt, a major pain facilitatory area of the brain. Our main results indicate that MOR plays a key role in the analgesic effects of systemic opioids, which becomes impaired following SNI. Our results show that SNI increases extracellular-enkephalinergic peptides at the DRt, alongside with a reduction of the number of MOR-IR cells without alterations in MOR gene transcription. We further show that SNI increases the number of phosphorylated MOR-IR cells at the DRt. Given the involvement of MOR phosphorylation in the degradation and desensitization of the receptor, it is likely that the impairment of MOR in SNI-animals might be due this post-translational modifications of MOR. Taken together these alterations might contribute to a loss of inhibition of pain facilitation from the DRt which may underlie the imbalance of pain modulation toward pain facilitation during chronic pain and also impact on the efficacy of exogenous opioids in the treatment of neuropathic pain (Finnerup et al., 2015).

The present study shows that during SNI there is increased release of the endogenous opioid peptides Met- and Leu-enkephalin at the DRt. These peptides are likely released from local enkephalinergic interneurons and also from DRt afferent sources namely the RVM, the A_5 noradrenergic cell group and the hypothalamus (Martins et al., 2008). Because these peptides were not detected in sham-animals we were not able to quantify the magnitude of this increase. This increase is consistent with the role of the endogenous opioids in the regulation of nociceptive transmission (Zubieta et al., 2001). Furthermore, a regional release of endogenous opioids has been shown in cortical and sub-cortical brain areas of patients with persistent pain of neuropathic origin (Jones et al., 1999; Willoch et al., 2004; Harris et al., 2007; Maarrawi et al., 2007). Studies performed in the rat with persistent pain of inflammatory and neuropathic origin showed up-regulation of Met-enkephalin at the spinal cord (Cesselin et al., 1980; Faccini et al., 1984; Noguchi et al., 1992; Sommer and Myers, 1995; Hossaini et al., 2014). In supraspinal pain modulatory areas, chronic inflammatory pain in the rat, increased enkephalin peptides at several brainstem nuclei including the PAG and RVM (Williams et al., 1995; Hurley and Hammond, 2001).

We found a reduction in the number of MOR-IR cells at the DRt of SNI-animals. This seems to be a common effect of neuropathic pain at pain modulatory areas. Neuropathic pain induced by peripheral nerve section reduces MOR immunostaining in the cell bodies of primary sensory neurons

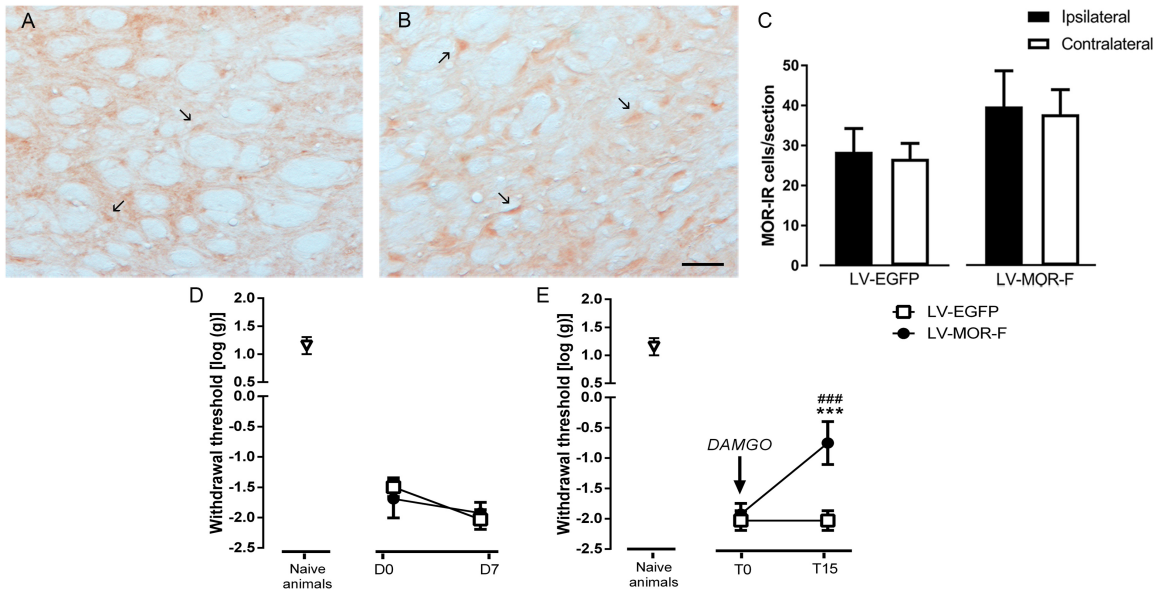

FIGURE 9 | Effects of lentiviral-mediated MOR overexpression at the DRt on mechanical allodynia in SNI-animals. Representative photomicrographs of MOR-immunoreactive (IR) cells at the DRt of SNI animals injected with LV-EGFP **(A)** and LV-MOR-F **(B)**. Typical MOR immunolabeling is marked by arrows. Scale bar in **(B)**: 100 μm (A is at the same magnification). Data in **(C)** represent the number of MOR immunoreactive (IR) neurons after lentiviral vectors injection into the DRt at the injected (ipsilateral) and contralateral side. LV-EGFP (n = 6) or LV-MOR-F (n = 7) were injected at the DRt and their effects were assessed before (D0; i.e., 2 weeks after SNI induction) and 7 days (D7) after injection, by the von Frey test **(D)** which evaluates mechanical sensitivity. In an additional group of animals injected into the DRt with LV-EGFP (n = 6) or LV-MOR-F (n = 7), DAMGO at 10 ng was administrated at the DRt and its effects were assessed before (T0; i.e., 7 days after vectors injection) and 15 min (T15) after injection by the von Frey test **(E)**. At D0 (i.e., 2 weeks after SNI induction; **D**) and D7/T0 (i.e., 3 weeks after SNI induction; **D,E**), all SNI animals presented a marked mechanical hypersensitivity, indicative of mechanical allodynia, as shown by the decreased withdrawal thresholds compared to the withdrawal thresholds of the age-matched naïve animals (n = 13) used in **Figure 2** for the injection of saline or DAMGO. The withdrawal thresholds of naïve animals correspond to the values obtained before the injection of saline or DAMGO in those animals. Data in **C,D,E** are presented as mean ± SD. $^{***}p < 0.001$ vs. T0; $^{###}p < 0.001$ vs. LV-EGFP.

in DRGs and at their central terminal in the dorsal horn (deGroot et al., 1997; Goff et al., 1998; Porreca et al., 1998; Kohno et al., 2005; Sumizono et al., 2018) and also in cortical structures involved in pain modulation (Thompson et al., 2018). The reduction of the number of MOR-IR cells found in our study, contrary to the reduction of MOR immunostaining in primary sensory neurons (Kohno et al., 2005), is not because of down-regulation of MOR gene expression at the DRt since we found no alterations in MOR mRNA levels between sham- and SNI-animals. One possible explanation is that counter-regulatory adaptations may lead to increased traffic of MOR to degradative intracellular pathways. Indeed, MOR can be down-regulated by increased targeting to degradation in lysosomes (Law et al., 2000) which has also been observed to occur in a neuropathic pain model (Mousa et al., 2013). The phosphorylation of MOR is a post-translational modification which plays a major role in the regulation of MOR function after acute or prolonged exposure to agonists (Zhang et al., 2009; Williams et al., 2013; Allouche et al., 2014). An important mechanism triggered by MOR phosphorylation is the internalization of the receptor. Upon phosphorylation, MOR is internalized after what it can either be recycled back to the cell membrane or trafficked to lysosomes (Johnson et al., 2005). Here, we found increased pMOR-IR cells at the DRt of neuropathic animals. Furthermore, we detected MOR phosphorylation at the ser^{375} residue which represents a major phosphorylation site involved in MOR internalization (El Kouhen et al., 2001). Therefore, it is likely that the reduction of MOR-IR cells at the DRt of neuropathic animals could result from increased phosphorylation of MOR followed by internalization of the receptor and, ultimately, increased degradation in lysosomes.

The pharmacological and gene transfer studies in the DRt of naïve animals show that opioids modulate noxious thermal (heat) as well as and non-noxious mechanical (tactile) sensitivity through their actions at local MOR. The results are consistent with the activation of MOR resulting in inhibition of DRt facilitatory actions on both sensory modalities. Our results set for the first time a role for the DRt in the modulation of non-noxious mechanical sensitivity and the involvement of MOR in such actions. Additionally, the reduction of withdrawal thresholds to tactile stimuli, observed upon MOR knockdown, is indicative of the development of mechanical allodynia, i.e., a painful sensation caused by non-noxious mechanical stimuli. This increased mechanical sensitivity is likely due to decreased inhibition of tonic DRt descending facilitation. In line with this, it was shown that tactile allodynia is integrated predominantly at supraspinal brainstem nuclei (Saade et al., 2006), and the down-regulation of MOR at descending pain modulatory pain areas, induced by neonatal inflammation, was associated to the development of mechanical allodynia (Yan and Kentner, 2017). Additionally, tonic descending facilitation was shown to be

involved in the mediation of mechanical allodynia after nerve injury (Ossipov et al., 2000).

To explore the effects of the alterations of the opioidergic system on MOR function at the DRt of SNI-animals, we determined the effects of the MOR agonist DAMGO on mechanical allodynia which is robustly developed after SNI induction. Additionally, mechanical allodynia constitutes a representative symptom of neuropathic pain in humans (Woolf and Mannion, 1999). We did not test thermal hyperalgesia, as changes in heat thresholds are difficult to measure in the SNI model (Decosterd and Woolf, 2000). We detected a decrease of the antinociceptive effects induced by DAMGO on mechanical allodynia, which is likely due to the reduction of MOR-IR cells at the DRt in neuropathic animals. Hence, based on the effects of MOR knockdown on mechanical sensitivity abovementioned, the reduction of MOR-IR cells at the DRt likely induces an impairment of the opioidergic inhibition of DRt descending facilitation in neuropathic animals. Incidentally, a reduction in the numbers of MOR-IR cells has also been shown in a model of chronic inflammatory pain, but this resulted in DAMGO-induced hyperalgesic effects at the DRt (Pinto et al., 2008a). This effect was likely caused by increased GABAergic input to the DRt, which is probably due to diminished opioidergic inhibition since local GABAergic interneurons express MOR, and GABA during inflammatory pain contributes to increasing descending pain facilitation (Martins et al., 2015a).

The loss of effect of DAMGO on mechanical allodynia suggests an impairment of the opioidergic inhibition of DRt descending facilitation in SNI-animals. Nonetheless, the impairment of MOR inhibitory actions cannot be solely explained by the reduction of MOR-IR cells since increasing the MOR protein at the DRt by lentiviral-mediated MOR gene expression did not alter mechanical allodynia. In these experiments, the MOR protein was efficiently up-regulated, as demonstrated by increased MOR-IR cells. Of note, the up-regulation of MOR was observed both at the ipsilateral (injected side) and contralateral side due to spreading of the vector which was injected at a higher volume than LV-MOR-R. MOR was also correctly trafficked and folded to the cell membrane, since microinjection of DAMGO at the DRt produced antiallodynic effects. However, it is worth noting that the effects of DAMGO in MOR-overexpression experiments were only partial since mechanical sensitivity did not revert to naïve thresholds, and the antiallodynic effects were obtained with a high dose of DAMGO. Therefore, based on the latter experiments with DAMGO together with the absence of effects of MOR up-regulation on mechanical allodynia, in spite of the high levels of endogenous enkephalin peptides, we suggest that neuropathic pain might also induce desensitization of MOR function at the DRt. The phosphorylation of MOR plays a major role in desensitization and the fact that MOR phosphorylation is increased at the DRt of neuropathic animals further argues in favor of this hypothesis. The effects of MOR knockdown on DAMGO effects in naïve animals, suggest that the remaining MOR at the DRt of naïve animals are still sensitive and therefore not phosphorylated, while, the absence of antiallodynic effects after MOR-overexpression in SNI-animals, further reinforces that MOR in SNI-animals might be highly subject to phosphorylation. The evaluation of pMOR levels after MOR knockdown and MOR-overexpression as well as the manipulation of MOR phosphorylation by increasing and decreasing the phosphorylation of MOR after MOR knockdown and MOR-overexpression, respectively, should confirm the role of MOR phosphorylation in MOR desensitization at the DRt. Phosphorylation induces desensitization of MOR by blocking the interaction of proteins with previously accessible regions of the receptor and changing the types of G protein the receptor interacts with and through which it mediates intracellular signaling (Johnson et al., 2005; Allouche et al., 2014). Increasing evidences of MOR desensitization induced by neuropathic pain include reduced MOR-mediated-G-protein activity in the thalamus and PAG (Hoot et al., 2011) and also increased MOR phosphorylation at the spinal dorsal horn (Narita et al., 2004) and the striatum (Petraschka et al., 2007). We propose that the mechanisms triggered by increased MOR phosphorylation, desensitization and increased targeting of MOR to degradation, could be due to prolonged activation of MOR by the high levels of endogenous opioid peptides found at the DRt of SNI-animals. Indeed, endogenous opioid peptide ligands, such as as enkephalins and endorphins induce robust desensitization and endocytosis (Llorente et al., 2012; Allouche et al., 2014) and the sustained release of endogenous peptides in the brain of neuropathic mice has been shown to induce desensitization of MOR and opioid tolerance (Petraschka et al., 2007).

In summary, the present study shows that induction of a model of neuropathic pain is associated with alterations in the opioidergic system at the DRt and that these alterations likely impact on downstream intracellular pathways that regulate MOR function. These alterations likely contribute to a loss of inhibition of pain facilitation from the DRt further enhancing descending facilitation during neuropathic pain. The treatment of neuropathic pain could benefit from the development of new compounds which can skip pathways involved in counter-regulatory mechanisms (Siuda et al., 2017).

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983). The protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of the University of Porto.

AUTHOR CONTRIBUTIONS

AC participated in the study design, performed stereotaxic surgeries for lentiviral injections and pharmacological experiments, immunohistochemical procedures and blinded cell counting of MOR and pMOR labeling, and manuscript drafting. PC performed microdialysis experiments. GF participated in the design of the microdialysis study, opioid peptide analysis, and writing of the manuscript. SW provided the viral vectors

produced in his laboratory. CR participated in real-time PCR experiments and writing of the manuscript. IM and IT participated in the study design, discussion of the results, and writing of the manuscript. All authors have read and approved the final version of the manuscript finalized by IM.

FUNDING

This work was supported by the FCT (PTCD/SAU-NSC/110954/2009 FEDER/COMPETE) and ESF (NORTE-08-5369-FSE-000026).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publication II

Shift of μ-opioid receptor signaling in the dorsal reticular nucleus is implicated in morphine-induced hyperalgesia in male rats. Anesthesiology. (2020)

ANESTHESIOLOGY

Shift of μ-opioid Receptor Signaling in the Dorsal Reticular Nucleus Is Implicated in Morphine-induced Hyperalgesia in Male Rats

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ANESTHESIOLOGY 2020; XXX:00–00

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- The phenomenon of opioid-induced hyperalgesia is supported by descending pain facilitation from brainstem nuclei
- The dorsal reticular nucleus is modulated by opioids and mediates descending pain facilitation in some settings

What This Article Tells Us That Is New

- Using a rat model of opioid-induced hyperalgesia, it was shown that reducing dorsal reticular activity with lidocaine blocked nociceptive sensitization from opioid infusion
- Knockdown of μ-opioid receptors or prevention of excitatory signaling using naloxone in the dorsal reticular nucleus prevented and reversed opioid-induced hyperalgesia

ABSTRACT

Background: Increased descending pain facilitation accounts for opioid-induced hyperalgesia, but the underlying mechanisms remain elusive. Given the role of μ-opioid receptors in opioid-induced hyperalgesia in animals, the authors hypothesized that the dorsal reticular nucleus, a medullary pain facilitatory area, is involved in opioid-induced hyperalgesia through altered μ-opioid receptor signaling.

Methods: The authors used male Wistar rats (n = 5 to 8 per group), chronically infused with morphine, to evaluate in the dorsal reticular nucleus the expressions of the μ-opioid receptor and phosphorylated cAMP response element-binding, a downstream marker of excitatory μ-opioid receptor signaling. The authors used pharmacologic and gene-mediated approaches. Nociceptive behaviors were evaluated by the von Frey and hot-plates tests.

Results: Lidocaine fully reversed mechanical and thermal hypersensitivity induced by chronic morphine. Morphine-infusion increased μ-opioid receptor, without concomitant messenger RNA changes, and phosphorylated cAMP response element-binding levels at the dorsal reticular nucleus. μ-opioid receptor knockdown in morphine-infused animals attenuated the decrease of mechanical thresholds and heat-evoked withdrawal latencies compared with the control vector (von Frey [mean ± SD]: −17 ± 8% *vs.* −40 ± 9.0%; $P < 0.001$; hot-plate: −10 ± 5% *vs.* −32 ± 10%; $P = 0.001$). μ-opioid receptor knockdown in control animals induced the opposite (von Frey: −31 ± 8% *vs.* −17 ± 8%; $P = 0.053$; hotplate: −24 ± 6% *vs.* −3 ± 10%; $P = 0.001$). The μ-opioid receptor agonist (D-ALA2,N-ME-PHE4,GLY5-OL)-enkephalin acetate (DAMGO) decreased mechanical thresholds and did not affect heat-evoked withdrawal latencies in morphine-infused animals. In control animals, DAMGO increased both mechanical thresholds and heat-evoked withdrawal latencies. Ultra-low-dose naloxone, which prevents the excitatory signaling of the μ-opioid receptor, administered alone, attenuated mechanical and thermal hypersensitivities, and coadministered with DAMGO, restored DAMGO analgesic effects and decreased phosphorylated cAMP response element-binding levels.

Conclusions: Chronic morphine shifted μ-opioid receptor signaling from inhibitory to excitatory at the dorsal reticular nucleus, likely enhancing descending facilitation during opioid-induced hyperalgesia in the rat.

(ANESTHESIOLOGY 2020; XXX:00–00)

Opioids are the gold standard for the treatment of moderate to severe pain.[1] Notwithstanding, repeated opioid administration produces a paradoxical effect, known as opioid-induced hyperalgesia.[2,3] Opioids produce analgesia *via* activation of the μ-opioid receptor. However, it has also been shown that μ-opioid receptor is required for opioid-induced hyperalgesia.[4,5] Acute μ-opioid receptor activation induces μ-opioid receptor coupling to inhibitory guanine nucleotide-binding proteins inhibiting nociceptive transmission.[6] Chronic exposure to opioids switches μ-opioid receptor coupling to a stimulatory guanine nucleotide-binding protein,[7] which leads to the activation of the adenyl cyclase/cAMP pathway, upregulation of protein kinase A,[8] and activation by phosphorylation of the cAMP response element-binding protein.[9] Blocking the excitatory

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

Submitted for publication October 7, 2019. Accepted for publication May 15, 2020. From the Department of Biomedicine–Unit of Experimental Biology, Faculty of Medicine (A.R.C., M.S., C.R., I.T., I.M.), I3S–Institute for Research and Innovation in Health (A.R.C., M.S., C.R., I.T., I.M.), and IBMC–Institute for Molecular and Cell Biology (A.R.C., M.S., C.R., I.T., I.M.), University of Porto, Porto, Portugal; Department of Pharmacology, Physiology, and Neuroscience, University of South Carolina School of Medicine, Columbia, South Carolina (S.P.W.); Sydney School of Public Health, Faculty of Medicine and Health, The University of Sydney, Sydney, Australia (A.T.-P.).

 Anesthesiology 2020; XXX:00–00. DOI: 10.1097/ALN.0000000000003412

<zdoi;. DOI: 10.1097/ALN.0000000000003412>

signaling by ultra-low doses of antagonists increased analgesia, attenuated tolerance, and reversed paradoxical pain sensitization.[8,10–12] The cellular alterations induced by chronic opioids in μ-opioid receptor signaling have been observed at several levels of the pain circuitry.[8,11,13] An enhancement of descending pain facilitation from the brainstem has been shown to account for opioid-induced hyperalgesia.[14–16] Given the major regulatory role of opioids in descending pain modulatory pathways,[17,18] altered μ-opioid receptor signaling could be involved in the enhancement of descending pain facilitation,[19] but this has never been evaluated.

The dorsal reticular nucleus belongs to the endogenous pain control system with a unique pain-facilitatory role.[20] The dorsal reticular nucleus establishes reciprocal excitatory connections with the spinal dorsal horn, which constitute a reverberative circuit through which it is thought to amplify pain transmission at the spinal cord.[20,21] Opioids play a key role at the dorsal reticular nucleus. μ-opioid receptor is expressed in dorsal reticular nucleus-spinally and nonspinally projecting neurons,[22] and its activation plays a fundamental inhibitory role at the dorsal reticular nucleus,[23] which accounts for the analgesic effects of systemic opioids.[24] However, the effects of sustained opioid treatment on μ-opioid receptor signaling at the dorsal reticular nucleus has never been studied. Here, after establishing the involvement of the dorsal reticular nucleus in a model of opioid-induced hyperalgesia induced by sustained systemic morphine infusion,[14] we explored the effects of sustained morphine on μ-opioid receptor function and signaling at the dorsal reticular nucleus. The involvement of the dorsal reticular nucleus in opioid-induced hyperalgesia was determined by dorsal reticular nucleus blockade with lidocaine. We determined the effects of sustained morphine on μ-opioid receptor expression at messenger RNA (mRNA) and protein levels by quantitative real-time polymerase chain reaction and immunohistochemistry, respectively, at the dorsal reticular nucleus. We also evaluated the expression of the phosphorylated cAMP response element binding protein, a downstream marker of the excitatory signaling transduction pathway of the μ-opioid receptor,[9] by immunohistochemistry at the dorsal reticular nucleus. Then, we determined the effects of lentiviral-mediated knockdown of μ-opioid receptor at the dorsal reticular nucleus in the development of opioid-induced hyperalgesia. Finally, we performed pharmacologic studies to evaluate the effects of the injection, at the dorsal reticular nucleus, of the μ-opioid receptor agonist (D-ALA2,N-ME-PHE4,GLY5-OL)-enkephalin acetate (DAMGO), an ultra-low-dose naloxone, which prevents μ-opioid receptor coupling to a stimulatory guanine nucleotide-binding protein,[8] and H-89, a specific protein kinase A inhibitor, on opioid-induced hyperalgesia.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of the University of Porto (Porto, Portugal) and were performed in accordance with the European Community Council Directive (2010/63/EU) and the ethical guidelines for pain investigation.[25] Pathogen-free adult male Wistar rats (Charles River colony, France) were maintained under controlled temperature (22 ± 2°C) and light (12/12h light/dark cycle, lights on between 8:00 h and 20:00 h) conditions with *ad libitum* access to food and water. We did not use female animals because previous studies found no sex-dependency for μ-opioid receptor implication in opioid-induced hyperalgesia.[4,5] The animals were allowed to acclimate to the housing facility for at least one week before any procedure. All experiments were conducted during the light phase. The subjective bias when allocating the animals to the experimental groups was minimized by arbitrarily housing the animals in pairs upon their arrival, then the animals were randomly picked from the cage for each procedure. After stereotaxic surgeries, the animals were housed individually. No *a priori* power analysis was performed. The sample sizes were based on common practice of the research group where, by default, six animals per group are used in experiments, giving us approximately 90% power to detect large differences (two standard deviations) between two groups, for continuous outcomes. There were no missing data; all values from animals correctly injected/implanted into the dorsal reticular nucleus and from the animals with misplaced "out sites" injections included in the analysis were available for the analysis. Also, no outliers were detected, and all the values were included in the analysis.

Lentiviral Vectors

The lentiviral vectors used were produced as previously described.[24] Briefly, the cDNA for the μ-opioid receptor was cloned into a lentiviral transfer vector, inserted in antisense orientation relative to the human synapsin promoter which restricts transgene expression to neurons.[26] This transfer vector, which is the vector for μ-opioid receptor knockdown, also contained an encephalomyocarditis virus internal ribosome entry site, the enhanced green fluorescent protein, and the woodchuck hepatitis virus posttranscriptional regulatory element. The virus was produced by transfection of human embryonic kidney 293T cells with the transfer vector, a packaging plasmid, a plasmid encoding the rev protein, and a plasmid encoding the vesicular stomatitis virus G glycoprotein. The control vector was constructed similarly, using a transfer vector with the human synapsin promoter driving the expression of the enhanced green fluorescent protein. The titer of the vectors was determined by quantitative real-time polymerase chain reaction, and both vectors were used at 5×10^6 transducing units per microliter.

Opioid-induced Hyperalgesia Induction

Opioid-induced hyperalgesia was induced in the rats by the continuous subcutaneous infusion of morphine

hydrochloride (generously provided by Dr. Paulo Cruz, Porto Military Hospital, Porto, Portugal) at 45 μg · μL^{-1} · h^{-1} for 7 days as described previously.[14] ALZET® osmotic minipumps (model 2001; USA) were used for the delivery of morphine or the saline vehicle solution in control animals at 1 μl/h pump infusion rate for 7 days. The minipumps were implanted in animals weighting 285 to 315 g, and, unless otherwise indicated, the subcutaneous implantation was performed under isoflurane anesthesia.

Stereotaxic Surgeries

Rats weighting 285 to 315 g were deeply anesthetized with an intraperitoneal mixture of ketamine hydrochloride (0.06 g/Kg) and medetomidine (0.25 g/Kg) and held in a stereotaxic frame (David Kopf Instruments, USA) for the implantation of a cannula or the injection of lentiviral vectors into the left dorsal reticular nucleus. Immediately after the stereotaxic procedures, the animals were also implanted with osmotic minipumps filled with morphine or saline as described for the induction of opioid-induced hyperalgesia. Upon completion of the latter procedure, the animals received 0.9% NaCl (0.1 ml/kg, subcutaneous) for rehydration followed by atipamezole hydrochloride (0.5 g/Kg, subcutaneous) to revert the anesthesia.

Cannula Implantation. A guide cannula was implanted into the left dorsal reticular nucleus for pharmacologic experiments following the coordinates, determined according to the rat brain atlas[27] relative to the interaural line (Anterior-Posterior: −6.0 mm; Medial-Lateral: −1.4 mm; Dorsal-Ventral: −1.5 mm), and procedures described previously.[24]

Lentiviral Vector Injection. Stereotaxic injections were performed for the injection of the vector for μ-opioid receptor knockdown in morphine- (n = 6) and saline-infused animals (n = 5) or the injection of the control vector in morphine- (n = 6) and saline-infused animals (n = 7) in two rostrocaudal parts of the left dorsal reticular nucleus following the coordinates of the atlas Paxinos and Watson[27] (first injection: Anterior-Posterior: −6.0 mm; Medial-Lateral: −1.4 mm; Dorsal-Ventral: −1.5 mm; second injection: Anterior-Posterior: −6.4 mm; Medial-Lateral: −1.3 mm; Dorsal-Ventral: −1.7 mm), as described previously.[24] A total of 0.6 μl was injected per site. The effects of the lentiviral vectors on nociceptive behaviors were assessed by the von Frey and hot-plate tests as described below (see Nociceptive Behavior section) before and 7 days after the stereotaxic injections. The human synapsin promoter was previously shown to be fully active at day 7 after injection at the dorsal reticular nucleus.[24] All tests were conducted by an experimenter blinded to the treatments. The primary outcome measures in the studies using lentiviral vectors were mechanical and thermal sensitivities evaluated by the von Frey and hot plate tests, respectively. The results obtained in the von Frey test, presented graphically, were reported as mean withdrawal thresholds (± SD) and as mean percentage of baseline (*i.e.*, before lentiviral injections) ± SD. The results obtained in the hot-plate test were reported as mean withdrawal latency (± SD) and as mean percentage of baseline (*i.e.*, before lentiviral injections) ± SD.

Nociceptive Behavior

The sustained administration of morphine at the dose regimen used typically induces hypersensitivity to mechanical and thermal stimulation.[14] The von Frey and hotplate tests were used to evaluate mechanical and thermal sensitivity, respectively, in the rats. The animals were habituated to the experimenter and the experimental environment for a period of one week. The von Frey test was performed by placing the animals on an elevated transparent cage with a mesh wire bottom allowing the stimulation of the plantar surface of the left hind paw with calibrated von Frey monofilaments (Stoelting, USA) with logarithmically incremental stiffness ranging from 0.4 g to 60 g. Testing started with the 2-g filament applied perpendicularly to the plantar surface for 3 s. Withdrawal thresholds were determined using the Dixon up-and-down method.[28] The hotplate test was performed by placing the animals on a hotplate system (BIO-CHP Cold Hot Plate Test), with a surface temperature of 52°C. The nociceptive threshold was quantified as the latency (in seconds) to licking, retraction of the hind paw, or jump after placement of the rat on the hotplate. A 30-s cut off was used to avoid tissue damage.

All animals were behaviorally evaluated before and 7 days after the implantation of minipumps filled with morphine or saline to confirm the development of mechanical and thermal hypersensitivity. The animals were also monitored for signs of sedation. All animals implanted with morphine minipumps developed mechanical and thermal hypersensitivity. Nonetheless, despite all efforts to maintain the same experimental and environmental conditions throughout the study, basal nociceptive thresholds within saline- and morphine-infused animals were not always consistent. This is likely because the experimental groups were not always performed in the same exact period, owing to the high number of animals, and rodent behavior variation from animal to animal further strengthened this variability.

One experimental group, performed in response to peer review, was performed with the aim of evaluating the analgesic effects of morphine in the early times after the implantation of morphine-minipumps. For that, the animals were behaviorally evaluated before the implantation of saline or morphine minipumps (n = 4 rats each) and at several time points after minipump implantation (5 h and 2, 4, and 7 days).

Motor Activity

The rotarod test was used to evaluate the effects of lidocaine at the dorsal reticular nucleus on the motor performance of the rats. The test was performed on naïve animals after training once a day for two consecutive days. Training

consisted on placing the rats on a rotating rod (Ugo Basile, Italy) with the rate of rotation set at 10 revolutions per minute, until they fell off or until reaching a cutoff time set at 180 s. The animals that remained on the rod for 180 s were injected with either lidocaine (4% wt/vol) or saline (n = 7 each) at the dorsal reticular nucleus and the test was performed 30 min later. Animals that did not remain on the rod for 180 s were considered to have motor impairments.[14] The test was conducted by an experimenter blinded to the treatment. The results were reported as mean time of permanence on the rod (± SD).

Pharmacologic Experiments

The animals were injected at the dorsal reticular nucleus with either 4% (wt/vol) of lidocaine hydrochloride, 0.1 ng of the μ-opioid receptor agonist DAMGO, 1.5 ng (*i.e.*, an ultra-low-dose) of naloxone hydrochloride or 0.5 μg of N-[2-(4-Bromocinnamylamino)ethyl]-5-isoquinoline (H-89). All drugs were obtained from Sigma-Aldrich (Portugal) and dissolved in saline. Lidocaine or saline was injected at the dorsal reticular nucleus of morphine- (n = 6 per group) or saline-infused animals (lidocaine n = 5; saline n = 6). The animals of this experimental set were tested before and 30 min after injection. The dose and timing of lidocaine action were chosen based on previous studies.[14] In a second set of animals, DAMGO or saline were injected at the dorsal reticular nucleus of morphine- (DAMGO n = 5; saline n = 6) or saline-infused animals (DAMGO n = 7; saline n = 6). The animals were tested before and 15 min after injection. The dose and timing of DAMGO action were chosen based on previous studies performed at the dorsal reticular nucleus and another medullary area.[24,29,30] In a third set of animals, naloxone was injected in saline- (n = 7) or morphine-infused animals (n = 8). The animals were tested before and 30 min after the injection of naloxone. Immediately after testing, morphine-infused animals were further injected with DAMGO, at the dorsal reticular nucleus, and tested 15 min later. The effects of naloxone + DAMGO were compared with the effects of DAMGO injected alone at the dorsal reticular nucleus of morphine-infused animals from the second experimental set (n = 5). In a separate group of morphine-infused animals, that were not behaviorally tested, naloxone (n = 7) or saline (n = 5) were injected 30 min before DAMGO at the dorsal reticular nucleus, and 15 to 20 min later the animals were euthanized for immunodetection of phosphorylated cAMP response element-binding (as described in the Tissue Preparation and Immunohistochemistry section). The ultra-low dose of naloxone and timings of action were chosen based on previous studies.[31] In a fourth set of animals, performed in response to peer review, H-89 was injected in morphine-infused animals (n = 6). The animals were tested before and 40 min after the injection of H-89. The effects of H-89 were compared with the effects of saline injected alone at the dorsal reticular nucleus of morphine-infused animals from the first experimental set (n = 6). The dose and timing of H-89 were chosen based on previous studies.[32]

The injections were performed 7 days after guide cannula/minipumps implantation using a stainless-steel needle protruding 3 mm beyond the cannula and a volume of 0.5 μl was infused over a period of 1 min. All tests were conducted by an experimenter blinded to the treatments. The primary outcomes in the pharmacologic studies were mechanical and thermal sensitivities evaluated by the von Frey and hot plate tests, respectively. The results obtained in the von Frey test, presented graphically, were reported as mean withdrawal thresholds (± SD). The results obtained in the hot-plate test were reported as mean withdrawal latency (± SD).

Tissue Preparation and Immunohistochemistry

After the last behavioral evaluation or drug injection, the animals were deeply anesthetized with an overdose of sodium pentobarbital (70 mg/kg intraperitoneal) and perfused through the ascending aorta with 100 ml of calcium free Tyrode's solution, followed by 750 ml of a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brainstems were removed, immersed in fixative for 4 h followed by 30% sucrose in 0.1 M phosphate-buffered saline overnight, at 4°C, and sliced at 40 μm in coronal orientation in a freezing microtome.

Immunodetection of the μ-opioid Receptor. Two different groups of animals were used: one group of animals which was implanted with morphine- or saline-minipumps (n = 6 each) and a second group of animals which was implanted with morphine- or saline minipumps and further injected with lentiviral vectors into the dorsal reticular nucleus (saline: control vector n = 7; vector for μ-opioid receptor knockdown n = 5; morphine: control vector and vector for μ-opioid receptor knockdown n = 6 each). One in every fourth section encompassing the dorsal reticular nucleus was incubated for 2 h in a blocking solution containing 0.1 M glycine and 10% normal swine serum in 0.1 M phosphate-buffered saline containing 0.3% Triton X-100 follow by an incubation for 48 h, at 4°C, in rabbit polyclonal antibody against μ-opioid receptor (ref: RA10104; Neuromics, USA), diluted at 1:1,000. After washing, the sections were incubated for 1 h in a swine biotinylated anti-rabbit serum diluted at 1:200 (Dako, Denmark). The sections were washed again and incubated for 1 h with the avidin-biotin complex (1:200; Vector Laboratories, USA). After washing in 0.05 M Tris-HCl, pH 7.6, bound peroxidase was revealed using 0.0125% 3,3'-diaminobenzidinetetrahydrochloride (Sigma-Aldrich, USA) and 0.025% H_2O_2 in the same buffer. The antibodies were diluted in 0.1 M phosphate-buffered saline containing 0.3% Triton X-100 and 2% normal swine serum. The avidin-biotin complex was diluted in the same solution without serum. The sections were then dehydrated and mounted in Eukitt. The primary outcome of the immunohistochemical detection of the μ-opioid receptor

was the number of μ-opioid receptor–immunoreactive neurons. Five sections encompassing the rostro-caudal extent of the dorsal reticular nucleus were randomly taken from each rat and the numbers of μ-opioid receptor–immunoreactive neurons occurring into the left and right dorsal reticular nucleus using the ×20 objective were counted by an experimenter blinded as to the experimental group. No differences were detected between the left and right side of the dorsal reticular nucleus for either saline- or morphine-infused animals (data not shown); therefore, left and right cell profile counts were summed in each tissue section from this experimental group. In the second group of animals, which was implanted with morphine or saline minipumps and further injected with lentiviral vectors into the left dorsal reticular nucleus, the left and right cell profile counts were also summed in each tissue section. The dorsal reticular nucleus was delimitated in an additional set of immunoreacted sections counterstained with formol-thionin[33] according to the atlas of Paxinos and Watson.[27] The specificity of the anti–μ-opioid receptor antibody was previously tested by blocking the antibody with a blocking peptide in immunohistochemistry and western blot analysis.[22] We further tested antibody specificity by performing negative controls with omission of either the primary or the secondary antibodies which blocked all the immunostaining.

Immunodetection of Phosphorylated cAMP Response Element-binding. Two separate experimental settings were used. In the first experimental setting, phosphorylated cAMP response element-binding expression was evaluated in morphine- and saline- infused animals (n = 6 each). In the second experimental setting, performed in response to peer review, phosphorylated cAMP response element-binding expression was evaluated in morphine-infused animals, pretreated with an ultra-low dose of naloxone (n = 7) or saline (n = 5) at the dorsal reticular nucleus before DAMGO injection at the dorsal reticular nucleus. One in every fourth section encompassing the dorsal reticular nucleus was incubated for 2 h in a blocking solution, as explained above for the immunodetection of the μ-opioid receptor, followed by an incubation for 48 h, at 4°C, in rabbit polyclonal antibody against phosphorylated cAMP response element-binding (ref:06-519; Millipore, USA), diluted at 1:1,000. After washing, the sections were incubated for 1 h in a swine biotinylated anti-rabbit serum diluted at 1:200 (Dako, Denmark). The sections were washed again and incubated for 1 h with the avidin-biotin complex (1:200; Vector Laboratories, USA). After washing in 0.05 M Tris-HCl, pH 7.6, bound peroxidase was revealed using 0.0125% 3,3'-diaminobenzidinetetrahydrochloride (Sigma-Aldrich, USA) and 0.025% H_2O_2 in the same buffer. The antibodies and the avidin-biotin complex were diluted in the solutions described above for the immunodetection of the μ-opioid receptor. The sections were then dehydrated and mounted in Eukitt. The primary outcome of the immunohistochemical detection of phosphorylated cAMP response element-binding was the number of phosphorylated cAMP response element-binding positive nuclei. Five sections encompassing the rostro-caudal extent of the dorsal reticular nucleus were taken from each animal and photomicrographs were taken using a Zeiss light microscope with a high-resolution digital camera. The number of phosphorylated cAMP response element-binding positive nuclei was calculated using an automated cell counter plugin of the ImageJ software. In the first experimental setting, because no differences were detected between the left and right side of the dorsal reticular nucleus for either saline- or morphine-infused animals (data not shown), left and right numbers of phosphorylated cAMP response element-binding positive nuclei were summed in each tissue section. In the second experimental setting, phosphorylated cAMP response element-binding positive nuclei were counted in the left-ipsilateral dorsal reticular nucleus injected with the drugs. To verify whether drug-induced effects were restricted to the dorsal reticular nucleus, in both the first and second experimental setting, the numbers of phosphorylated cAMP response element-binding positive nuclei were additionally counted in the following medullary areas adjacent to the dorsal reticular nucleus, the *cuneate* nucleus, the nucleus of the solitary tract and the trigeminal subnucleus *caudalis*. We tested antibody specificity by performing negative controls with omission of either the primary or the secondary antibodies which blocked all the immunostaining.

Quantitative Real-time Polymerase Chain Reaction

Seven days after morphine- or saline-minipump implantations (n = 9 per group), rats were deeply anaesthetized with an overdose of sodium pentobarbital (150 mg/kg intraperitoneal) and euthanized by decapitation. The brains were harvested and immediately stored at −80°C. The medulla oblongata was cut into a frozen transverse block (1 mm in depth) from −5.60 to −4.68 mm relative to the Interaural line[27] from which the dorsal reticular nucleus (left and right sides) were dissected out using a tissue micropunch (Stoeling, USA). Each sample (n = 3 per group) was prepared by pooling the dorsal reticular nucleus from three animals. Total RNA from the dorsal reticular nucleus was extracted using the PureLink RNA Mini Kit (Thermofisher Scientific, Portugal) by following the manufacturer's protocol and the RNA integrity verified by agarose gel electrophoresis. The first strand cDNA synthesis was prepared at 42°C during 1h, from 0.8 μg of total RNA using 200 U of reverse transcriptase enzyme (Nzytech, Portugal) and 500 ng of oligo(dT)12-18 (Nzytech, Portugal). To assess for potential contaminants, a control containing all reagents except the reverse transcriptase enzyme was included for each sample. The expression levels of μ-opioid receptor mRNA were then quantified by the standard 2^(–delta delta CT) method using a StepOnePlus Real Time polymerase chain reaction system (Applied Biosystems) and a SYBR green chemistry (SYBR Select master mix,

Applied Biosystems). The following intron-spanning primers 5'-GCCATCGGTCTGCCTGTAAT-3' and 5'-CCAGATTTTCTAGCTGGTGGTTAG-3' were designed to amplify exon 2 and the junction of exon 3a/4 from the canonical μ-opioid receptor-1 transcript. Normalization was performed by amplification of rat GAPDH using the primers 5´-GCATGGACTGTGGTCCTCAG-3´ and 5´-CCATCACCATCTTCCAGGAG-3´. The thermal cycling conditions included an initial denaturation step at 95°C for 15 s, followed by 45 cycles at 95°C for 15 s, 53°C for 30 s, and 72°C for 1 min. Melting curve analysis of every quantitative polymerase chain reaction was conducted to ensure amplicon specificity. The results were presented as relative differences to μ-opioid receptor mRNA of saline-infused animals at the dorsal reticular nucleus.

Histology

After the last behavioral evaluation, the animals used in the pharmacologic experiments were administered 0.5 μl of 0.6% Chicago sky blue dye (Sigma, Portugal) through the guide cannula and euthanized by decapitation, whereas the animals injected with the lentiviral vectors were killed by vascular perfusion for verification of the injection site as previously described.[24] In control vector-injected rats, the injection site was identified by direct detection of the enhanced green fluorescent protein labeling (fig. 1,A and B). In rats injected with the vector for μ-opioid receptor knockdown, the injection tract was detected by the formol-thionin staining[33] because the expression of the enhanced green fluorescent protein was faint. In this vector the enhanced green fluorescent gene was inserted into the expression cassette in the second position of the bicistronic lentiviral construct, after an internal ribosome entry site element, and in this type of constructs the enhanced green fluorescent gene expression is lower compared with a vector just containing a promoter and the reporter gene, as observed previously.[24] Additionally, the destruction of the antisense RNA, placed in the first position of the bicistronic construct, also likely degrades the enhanced green fluorescent mRNA. We analyzed injection sites encompassing the dorsal reticular nucleus (Supplemental Digital Content, fig. S1, http://links.lww.com/ALN/C402) and for the purpose of control injections, we also analyzed misplaced injections outside the dorsal reticular nucleus, termed here "out sites," which were located either dorsally in the *cuneate* nucleus or laterally in the spinal trigeminal nucleus.

A total of three morphine- and three saline-infused animals received lidocaine injections placed outside the dorsal reticular nucleus. A total of four morphine- and four saline-infused animals received DAMGO injection in the out sites. A total of three saline- and four morphine-infused animals received ultra-low dose naloxone injections in out sites. The latter four morphine-infused animals further received an injection of DAMGO 30 min after the microinjection of the ultra-low dose naloxone. In the H-89 experimental group, three animals were injected in out sites with saline and four animals were injected in out sites with H-89. A representative distribution of drug injection sites within the dorsal reticular nucleus and in out sites is depicted in Supplemental Digital Content, figure S2 (http://links.lww.com/ALN/C403).

Control vector injections were placed in out sites in two saline- and two morphine-infused animals. Injections performed with the vector for μ-opioid receptor knockdown were placed in out sites in three saline- and one morphine-infused animals.

Statistical Analysis

The behavioral effects of the drugs located in the dorsal reticular nucleus and in adjacent sites (termed out sites), the effects of lentiviral vectors located in the dorsal reticular nucleus, and the effects of the vector for μ-opioid receptor knockdown in out sites, obtained in the von Frey and hot plate tests, were analyzed by a two-way mixed ANOVA for repeated measurements. Mechanical threshold responses, obtained in the von Frey test, were logarithmic transformed because of their skewed distribution. In case of a significant interaction between group and time, we proceeded with pairwise comparisons using Tukey's correction for multiple testing. The behavioral results of lentiviral vectors injections located in out sites are shown in the Supplemental Digital Content, figure S3 (http://links.lww.com/ALN/C404); however, no statistical analysis was performed, except for the injections with the vector for μ-opioid receptor knockdown, because of the small number of animals for each lentiviral vector (described in the Histology section). The results from the other lentiviral out site's injections were inspected from individual value plots. *t* tests for independent samples were used to compare the mean number of μ-opioid receptor-immunoreactive cells, phosphorylated cAMP response element-binding$^+$ nuclei and μ-opioid receptor-mRNA levels between saline- and morphine-infused animals and the mean numbers of phosphorylated cAMP response element-binding$^+$ nuclei in Saline+DAMGO- and Naloxone+DAMGO-injected animals. The normality assumption was checked by inspection of the distribution of the variables both with q-q plots and histograms. However, we must acknowledge that the sample size limits the ability to detect departures from normality. The statistical analysis was performed by GraphPad Prism version 7 and SPSS version 24. The significance level was set at 0.05, and all statistical tests were two-tailed.

Results

Dorsal Reticular Nucleus Activation Is Involved in Opioid-induced Hyperalgesia

The subcutaneous administration of morphine at 45 μg · $\mu L^{-1} \cdot h^{-1}$ initially produced antinociception (5 h; fig. 1,A and B), followed by the development of a marked hypersensitivity to mechanical stimuli, from day 4 onwards (fig. 1A), and to thermal stimuli detected at day 7 (fig. 1B), as previously

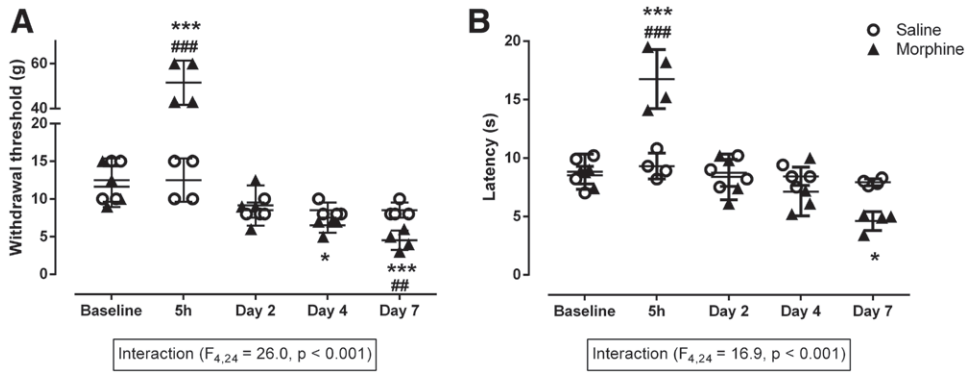

Fig. 1. Time-course analysis of the effects of morphine delivered subcutaneously at 45 μg · $μL^{-1}$ · h^{-1} on mechanical and thermal sensitivity evaluated by the von Frey (*A*) and hot-plate (*B*) tests, respectively. The tests were performed before and after saline or morphine minipumps implantation (n = 4 each) at 5 h and 2, 4, and 7 days. The interactions between groups and time are presented at the bottom of the *x* axis. Each symbol represents individual animal values, and the error bars represent mean ± SD. **P* < 0.05, ****P* < 0.001 *versus* baseline; ##*P* < 0.01, ###*P* < 0.001 *versus* saline-infused animals.

shown.[14] The injection of lidocaine, but not saline, into the dorsal reticular nucleus significantly reversed mechanical (fig. 2, A and C) and thermal hypersensitivity (fig. 2, B and D). Lidocaine injections in out sites produced no effects, except for an increase of mechanical thresholds which did not fully reverse mechanical hypersensitivity (Supplemental Digital Content, fig. S4, A and B, http://links.lww.com/ALN/C405). The administration of lidocaine into the dorsal reticular nucleus did not interfere with the motor function of the animals as shown in the rotarod test (Supplemental Digital Content, fig. S5, http://links.lww.com/ALN/C423).

Chronic Morphine Increased the Levels of μ-opioid Receptor and Phosphorylated cAMP Response Element-binding at the Dorsal Reticular Nucleus

We next determined the effects of sustained morphine administration in the expression of the μ-opioid receptor by evaluating μ-opioid receptor mRNA levels and the number of μ-opioid receptor-immunoreactive cells at the dorsal reticular nucleus. No significant differences were found in mRNA levels between morphine- and saline-infused animals (fig. 3D). The numbers of μ-opioid receptor-immunoreactive cells were significantly higher in morphine- (103 ± 26 cells) compared with saline-infused animals (63 ± 9 cells; fig. 3, A–C).

We also evaluated the expression of phosphorylated cAMP response element-binding at the dorsal reticular nucleus by analyzing the numbers of positively labeled nuclei. Higher numbers were found in the dorsal reticular nucleus of morphine- (1,329 ± 315 nuclei) compared with saline-infused animals (935 ± 218 nuclei; fig. 4, A–C). To evaluate whether this effect was restricted to the dorsal reticular nucleus, we also analyzed its adjacent medullary areas. No differences were found between morphine- and saline-infused animals (Supplemental Digital Content, fig. S6, A–G, http://links.lww.com/ALN/C406).

DAMGO Injection at the Dorsal Reticular Nucleus Enhances Morphine-induced Hypersensitivity

We next determined the effects of μ-opioid receptor activation by the agonist DAMGO injected at the dorsal reticular nucleus in saline- and morphine-infused animals. In morphine-infused animals, DAMGO increased mechanical hypersensitivity and did not alter thermal hypersensitivity (fig. 5, A and B). In saline-infused animals, DAMGO reduced mechanical and thermal sensitivity (fig. 5, C and D). DAMGO injection in out sites produced no significant effects (Supplemental Digital Content, fig. S4, C and D, http://links.lww.com/ALN/C405).

Downregulation of μ-opioid Receptor Expression at the Dorsal Reticular Nucleus Attenuates the Development of Opioid-induced Hyperalgesia

Then we determined whether μ-opioid receptor expression was involved in the development of opioid-induced hyperalgesia by evaluating the effects of knocking down the receptor at the dorsal reticular nucleus. In animals injected with the control vector, morphine-infusion increased the numbers of immunoreactive cells compared with saline-infusion. The injection of the vector for μ-opioid receptor knockdown reduced the numbers of immunoreactive cells both in morphine- and saline-infused animals (fig. 6, A–C). Morphine-infused animals injected with the control vector showed decreased mechanical thresholds and heat-evoked withdrawal latencies indicative of the development of developed mechanical and thermal hypersensitivity, respectively (fig. 7, A and B). In saline-infused animals, the control vector

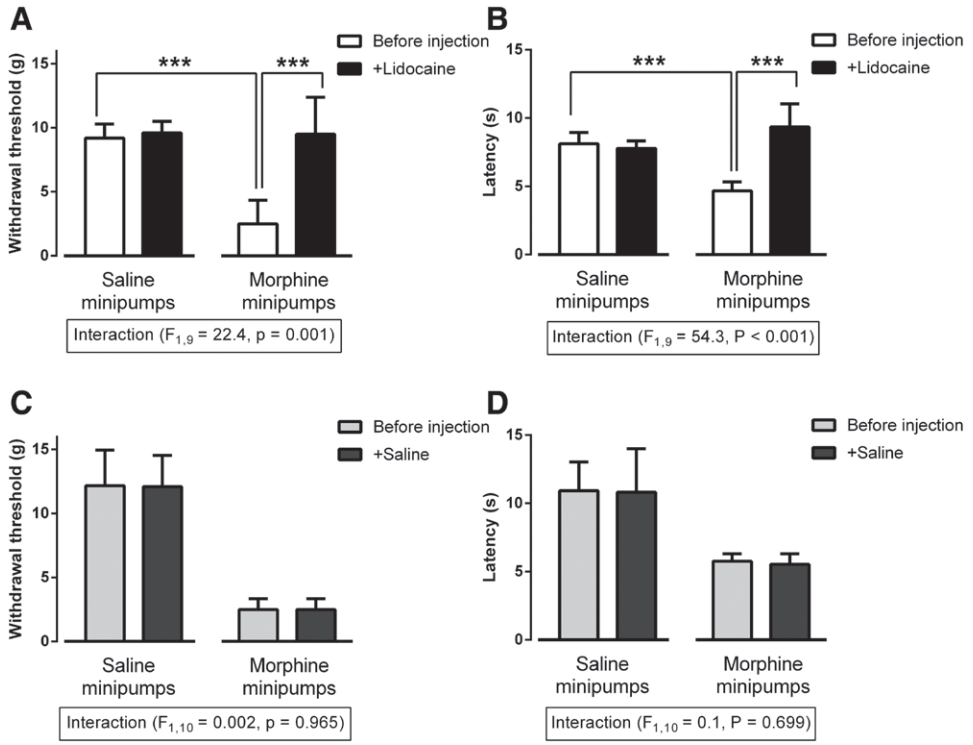

Fig. 2. Blockade of the dorsal reticular nucleus with lidocaine fully reversed mechanical and thermal hypersensitivity induced by chronic morphine. The dorsal reticular nucleus was injected with lidocaine or saline 7 days after the implantation of saline- or morphine-minipumps. The von Frey (*A* and *C*) and hot-plate (*B* and *D*) tests, which evaluate mechanical and thermal sensitivity, respectively, were performed before and 30 min after the injection of lidocaine (+Lidocaine) or saline (+Saline). The interactions between groups and time are presented at the bottom of the *x* axis. The injection of saline produced no effects in the von Frey ($F_{1,10}$ = 0.002, P = 0.965; *C*) and hot plate ($F_{1,10}$ =1.2, P = 0.303; *D*) tests. Data are presented as mean ± SD (Saline-infused animals: lidocaine n = 5, saline n = 6; Morphine-infused animals: lidocaine n = 6, saline n = 6). ***P < 0.001.

produced no effects except for a reduction of mechanical sensitivity (fig. 7, A and B). The knockdown of the μ-opioid receptor attenuated the reduction of mechanical thresholds and heat-evoked withdrawal latencies in morphine-infused animals and produced the opposite in saline-infused animals (fig. 7, C and D). In the morphine group, the magnitude of reduction of mechanical thresholds (−17 ± 8% *vs.* −40 ± 9%) and heat-evoked withdrawal latencies (−10 ± 5% *vs.* −32 ± 2%) was lower compared with the control vector (fig. 7, E and F). In the saline group, receptor-knockdown decreased mechanical (31 ± 8% *vs.* −17 ± 8%) and heat-evoked withdrawal latencies (−24 ± 6% *vs.* −2 ± 10%) compared with the control vector (fig. 7, E and F). Vector injections in out sites produced no effects (Supplemental Digital Content, fig. S3, http://links.lww.com/ALN/C404).

Ultra-low Dose of Naloxone Attenuates Opioid-induced Hyperalgesia

An ultra-low dose of naloxone administered into the dorsal reticular significantly attenuated mechanical (fig. 8A) and thermal hypersensitivity in morphine-infused animals (fig. 8B). Naloxone produced no effects in saline-infused animals (fig. 8). Saline-vehicle injections were not performed as, in the same experimental conditions, it produced no effects (fig. 2, C and D). Naloxone injections in out sites produced attenuated morphine-induced mechanical and thermal hypersensitivity (Supplemental Digital Content, fig. S4, E and F, http://links.lww.com/ALN/C405). The latter results suggest naloxone diffusion to adjacent areas upon injection.

Ultra-low Dose of Naloxone Restores the Analgesic Effects of DAMGO and Decreases Phosphorylated cAMP Response Element-binding Levels at the Dorsal Reticular Nucleus after Chronic Morphine

We next determined the effects of the pretreatment with an ultra-low dose of naloxone on the effects of DAMGO in morphine-infused animals. For that, we compared morphine-infused animals injected with DAMGO alone at the dorsal reticular nucleus with morphine-infused animals that were injected with an ultra-low dose of naloxone at the dorsal reticular nucleus 30 min before DAMGO injection. DAMGO after pretreatment with naloxone, contrary

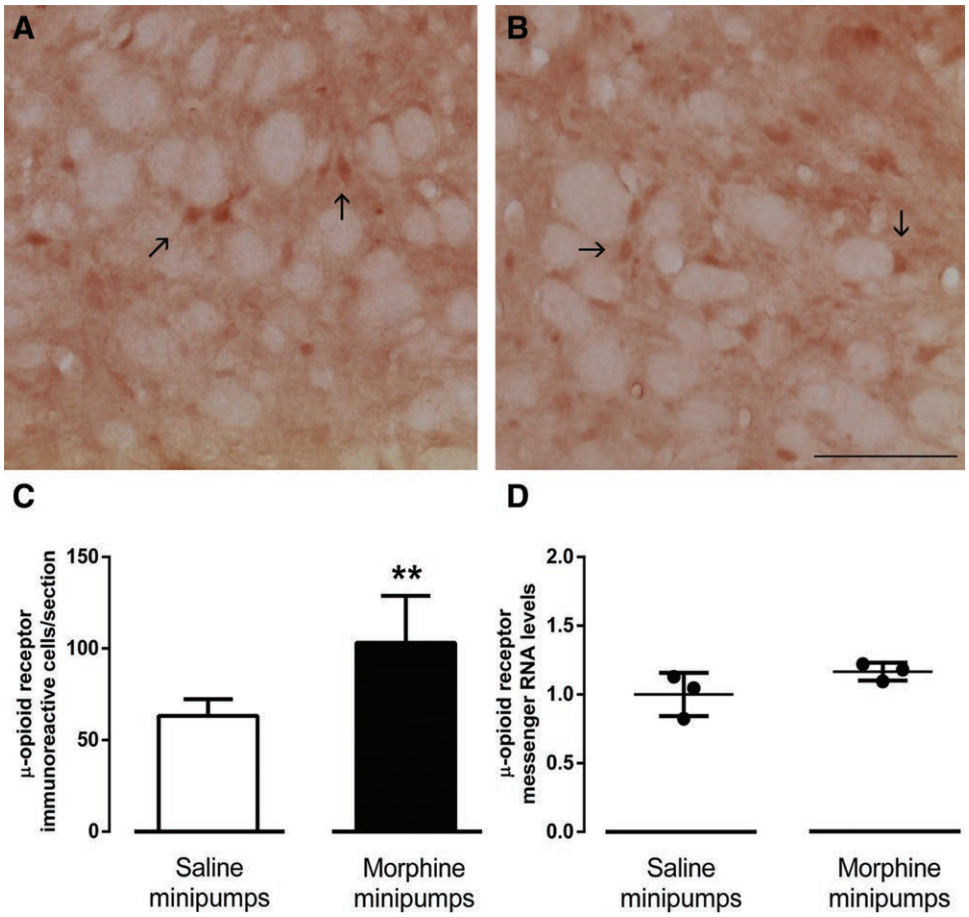

Fig. 3. Effects of chronic morphine infusion on the expression of μ-opioid receptor at the dorsal reticular nucleus. Representative photomicrographs of μ-opioid receptor–immunoreactive cells at the dorsal reticular nucleus of saline- (*A*) or morphine- (*B*) infused animals. Typical μ-opioid receptor immunolabeling is marked by arrows. Scale bar in panel *B*, 100 μm (*A* is at the same magnification). The numbers of immunoreactive cells in morphine-infused were significantly higher compared with saline-infused animals (n = 6 per group; $t_{10} = 3.6$, $P = 0.005$; *C*). No differences were found in μ-opioid receptor messenger RNA levels between saline- and morphine-infused animals (3 samples per group and n = 3 animals each sample; $t_4 = 1.7$; $P = 0.167$; *D*). The data in *D* are presented as relative differences to saline-infused animals. Data in *C* and *D* are presented as mean ± SD.

to DAMGO alone, fully reversed mechanical and thermal hypersensitivity (fig. 9, A and B). Injection of naloxone followed by DAMGO in out sites also showed antinociceptive effects for DAMGO (Supplemental Digital Content, fig. S4, G and H, http://links.lww.com/ALN/C405).

We further evaluated the effects of ultra-low dose naloxone in the expression of phosphorylated cAMP response element-binding in morphine-infused animals injected with saline or an ultra-low dose of naloxone into the dorsal reticular nucleus 30 min before the microinjection of DAMGO. Naloxone pretreatment showed significantly lower numbers of positively labeled nuclei (909 ± 77 nuclei) compared with saline (1,099 ± 163 nuclei; fig. 9, C–E) at the dorsal reticular nucleus. Additionally, because naloxone likely diffuses from the injection site, we also evaluated the expression of phosphorylated cAMP response element-binding in medullary areas adjacent to the dorsal reticular nucleus. No differences were found at the adjacent areas (Supplemental Digital Content, fig. S6, H–J, http://links.lww.com/ALN/C406).

Protein Kinase A Inhibition at The Dorsal Reticular Nucleus Produces No Effects in Opioid-induced Hyperalgesia

To investigate the involvement of the protein kinase A signaling pathway at the dorsal reticular nucleus, we tested the effects of the protein kinase A inhibitor H-89 at the dorsal reticular nucleus of morphine-infused animals. The injection of H-89 into the dorsal reticular nucleus (fig. 10) or in out sites produced no effects (Supplemental Digital Content, fig. S4, I and J, http://links.lww.com/ALN/C405).

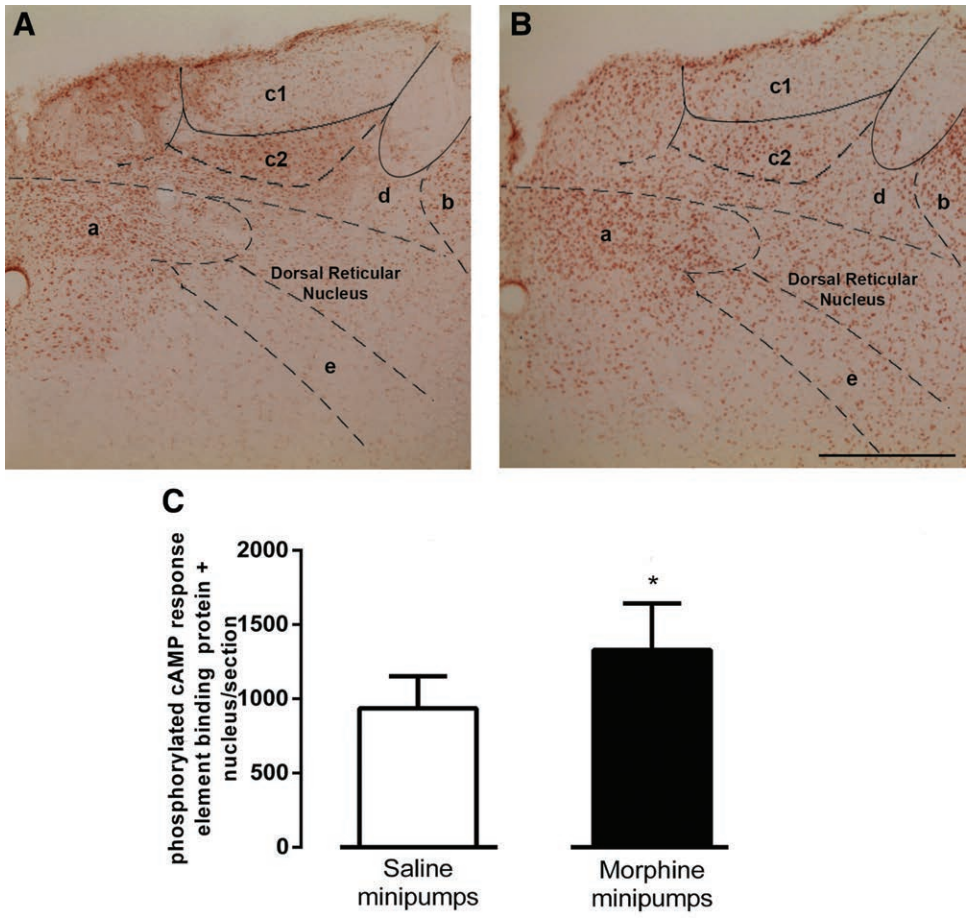

Fig. 4. Effects of chronic morphine infusion on the expression of phosphorylated cAMP response element-binding protein at the dorsal reticular nucleus. Representative photomicrographs of positively labeled nuclei in saline- (*A*) or morphine- (*B*) infused animals. Scale bar in *B*, 500 μm (*A* is at the same magnification). The numbers of positively labeled nuclei in morphine-infused animals were significantly higher compared with saline-infused animals (n = 6 per group; $t_{10} = 2.5$, $P = 0.030$; *C*). Letters in *A* and *B*: a, nucleus of the solitary tract; b, trigeminal subnucleus caudalis; c1, cuneate fasciculus; c2, cuneate nucleus; d, matrix region of the medulla; e, intermediate reticular nucleus. Data in *C* are presented as mean ± SD.

Discussion

This study shows that the dorsal reticular nucleus, a major descending pain facilitatory area of the brain, is involved in the mediation of opioid-induced hyperalgesia. We show that chronic morphine infusion increases the levels of the μ-opioid receptor and phosphorylated cAMP response element-binding, a downstream marker of the excitatory signaling of μ-opioid receptor, at the dorsal reticular nucleus. We further show that μ-opioid receptor activation by DAMGO at the dorsal reticular nucleus enhances opioid-induced hyperalgesia, whereas μ-opioid receptor knockdown produces the opposite. Furthermore, we demonstrate that preventing μ-opioid receptor excitatory signaling attenuates opioid-induced hyperalgesia, restores the analgesic effects of DAMGO, and decreases phosphorylated cAMP response element-binding levels at the dorsal reticular nucleus. Taken together, our results indicate that chronic morphine infusion induces a switch in μ-opioid receptor signaling from inhibitory to excitatory at the dorsal reticular nucleus, which is likely one of the underlying cellular mechanisms of increased descending pain facilitation during opioid-induced hyperalgesia.

The complete reversal of mechanical and thermal hypersensitivity induced by lidocaine at the dorsal reticular nucleus indicates that descending pain facilitation from this area is involved in opioid-induced hyperalgesia. The activation of descending pain facilitatory systems[14,15] is involved in opioid-induced hyperalgesia likely through an enhancement of spinal dorsal excitability.[19,34] The activation of descending pain facilitation from another medullary area, the rostral ventromedial medulla, has been previously shown to be involved in the mediation of opioid-induced hyperalgesia induced by acute[35] and sustained opioid administration[14] through local

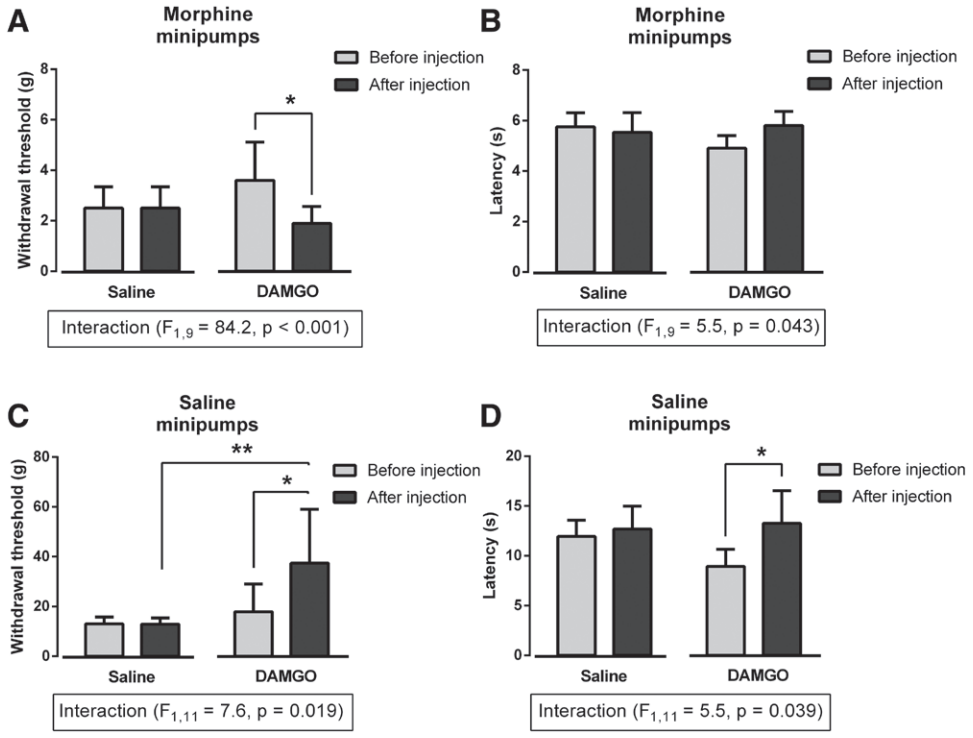

Fig. 5. μ-opioid receptor activation at the dorsal reticular nucleus by the agonist DAMGO enhances morphine-induced hypersensitivity. The dorsal reticular nucleus was injected with DAMGO or saline 7 days after the implantation of saline or morphine minipumps. The von Frey (*A* and *C*) and hot-plate (*B* and *D*) tests, which evaluate mechanical and thermal sensitivity, respectively, were performed before and 15 min after injections. The interaction between treatment and time is presented at the bottom of the *x* axis. In morphine-infused animals, in the hot plate test, no significant effects of treatment (DAMGO *vs.* saline; $F_{1,9}$ = 1.0, P = 0.336) or time ($F_{1,9}$ = 2.1, P = 0.184; *B*) were found. Data are presented as mean ± SD (saline-infused animals: DAMGO n = 7, saline n = 6; morphine-infused animals: DAMGO n = 5, saline n = 6). *P < 0.05, **P < 0.01.

enhanced endogenous cholecystokinin activity and activation of cholecystokinin$_2$ receptors.[16] We show that one of the mechanisms underlying the involvement of the dorsal reticular nucleus is the activation of the μ-opioid receptor. Whereas μ-opioid receptor expressed in sensory neurons was shown to be critical to the initiation of opioid-induced hyperalgesia,[5] the sustained activation of the receptor in the dorsal reticular nucleus might contribute to its maintenance by triggering descending facilitatory influences to the spinal dorsal horn. Mechanisms independent of the μ-opioid receptor might also interplay in the dorsal reticular nucleus, because lidocaine fully blocks opioid-induced hyperalgesia whereas μ-opioid receptor knockdown or the ultra-low dose naloxone only produced an attenuation. These mechanisms could involve the NMDA receptor-glutamatergic system[4,20,21] and microglia-to-neuron signaling.[36]

The absence of alterations in μ-opioid receptor mRNA levels is consistent with other studies showing the lack of effect of chronic morphine in the transcriptional regulation of the receptor in the brain.[37,38] This infers that the high number of cells expressing the receptor after morphine infusion could be attributable to increased expression at the protein level[39] or a change in the trafficking of the receptor resulting in its accumulation at the plasma membrane. The latter is supported by the fact that morphine is a poor internalizing agonist and the internalization of the receptor is crucial for targeting the receptor to degradation.[6] Chronic morphine was shown to induce different effects in the density of the receptor with either downregulation, no change, or upregulation.[40–42] Tolerance has been associated, although not exclusively, with downregulation.[6] At the dorsal reticular nucleus, downregulation of the receptor was associated with tolerance or diminished responsiveness to the inhibitory action of opioids.[24] In the present work, the upregulation is likely linked to a switch of signaling of the receptor from inhibitory to excitatory, as suggested by our cellular and behavioral data. At the cellular level, the shift to excitatory signaling is suggested by the increased expression of phosphorylated cAMP response element-binding, and decreased expression after ultra-low dose naloxone treatment, which prevents μ-opioid receptor coupling to a stimulatory guanine nucleotide-binding protein and

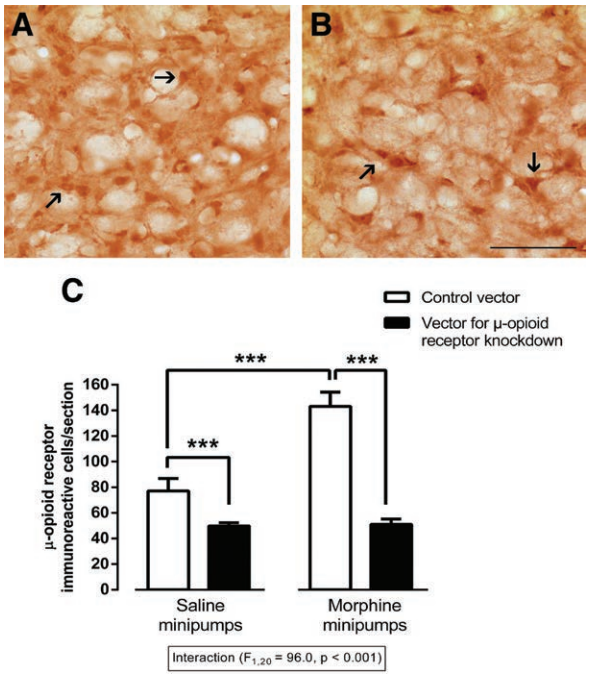

Fig. 6. Lentiviral-mediated knockdown of μ-opioid receptor expression in the dorsal reticular nucleus. Representative photomicrographs of μ-opioid receptor–immunoreactive cells in the dorsal reticular nucleus of morphine-infused animals after injection of the control vector (*A*) or the vector for μ-opioid receptor knockdown (*B*). Typical μ-opioid receptor immunolabeling is marked by arrows. Scale bar in *B*, 100 μm (*A* is at the same magnification). The interaction between groups and vectors is presented at the bottom of the *x* axis. Data in *C* are presented as mean ± SD (saline-infused animals: control vector n = 7, vector for μ-opioid receptor knockdown n = 5; morphine-infused animals: control vector n = 6, vector for μ-opioid receptor knockdown n = 6). ***$P < 0.001$.

restores coupling of the receptor to an inhibitory guanine nucleotide-binding protein.[9] The best-established molecular adaptation to chronic opioid exposure is up-regulation of the cAMP/cAMP-dependent protein kinase A signaling. Additionally, cAMP response element-binding is activated by phosphorylation predominantly by protein kinase A.[7–9] However, the absence of effects of H-89 discards upregulation of this pathway at the dorsal reticular nucleus. Hence, other signaling pathways might be involved; for example, the protein kinase C and extracellular signal-regulated kinase pathways.[3,4,43–45]

At the behavioral level, DAMGO enhanced mechanical allodynia in morphine-infused animals and induced antinociceptive effects in saline-infused animals. Of note, we did not observe an enhancement of thermal hyperalgesia in the hot plate test, after the administration of DAMGO in morphine-infused animals, probably because of methodologic limitations. In the hot plate test, the heat intensity is usually set up to observe responses within 5 to 10 s.[46] Accordingly, in saline-infused animals, which showed withdrawal latencies on average near to 10 s, increased or decreased withdrawal latencies were observed after DAMGO or μ-opioid receptor knockdown, respectively, but in morphine-infused animals which showed withdrawal latencies on average near to 5 s, shorter latencies, indicative of an enhancement of hyperalgesia, are not likely detected. The antinociceptive effects of DAMGO are likely yielded through the inhibition of dorsal reticular nucleus descending facilitation. Further corroborating this, knockdown of the μ-opioid receptor at the dorsal reticular nucleus of saline-infused animals increased sensitivity to mechanical and thermal stimuli as previously shown.[24] It is important to point out that the control vector, although with less magnitude than the vector for μ-opioid receptor knockdown, selectively increased mechanical sensitivity in saline-infused animals. This effect is likely due to the enhanced green fluorescent protein expressed from the vector rather than the lentiviral transduction, because this protein is dose-dependently toxic to some neuronal cells.[47] The vector for μ-opioid receptor knockdown also carries this reporter gene, but its expression is almost null, therefore the effects of the vector are likely exclusively due to receptor knockdown. The pronociceptive effects of DAMGO in morphine-infused animals are likely mediated through μ-opioid receptor excitatory effects on dorsal reticular nucleus descending facilitation, which is further supported by the attenuation of opioid-induced hyperalgesia by knockdown of the receptor. Finally, the behavioral data are consistent with μ-opioid receptor being expressed on dorsal reticular nucleus-spinally projecting neurons,[22] which are excitatory and engaged in a reciprocal circuitry responsible for pain amplification.[20,21]

The switch of μ-opioid receptor signaling from inhibitory to excitatory is further suggested by the effects of ultra-low dose naloxone on phosphorylated cAMP response element-binding levels selectively at the dorsal reticular nucleus. Ultra-low dose naloxone attenuated morphine-induced hyperalgesia after injection into the dorsal reticular nucleus as well as in adjacent (out) sites. Morphine-infusion increased phosphorylated cAMP response element-binding levels in the dorsal reticular nucleus but not in adjacent areas, and ultra-low dose naloxone pretreatment in morphine-infused animals decreased phosphorylated cAMP response element-binding levels in the dorsal reticular nucleus while it had no effect in adjacent areas. These results indicate the selective involvement of the dorsal reticular nucleus and that the effects of naloxone injected in out sites are likely due to its diffusion to the dorsal reticular nucleus. Additionally, DAMGO in the dorsal reticular nucleus, but not in adjacent areas, produced an enhancement of mechanical hypersensitivity. This is consistent with the alterations of the μ-opioid receptor selectively at the dorsal reticular nucleus. Pretreatment with ultra-low dose naloxone restored the analgesic effects of DAMGO injected in the dorsal reticular

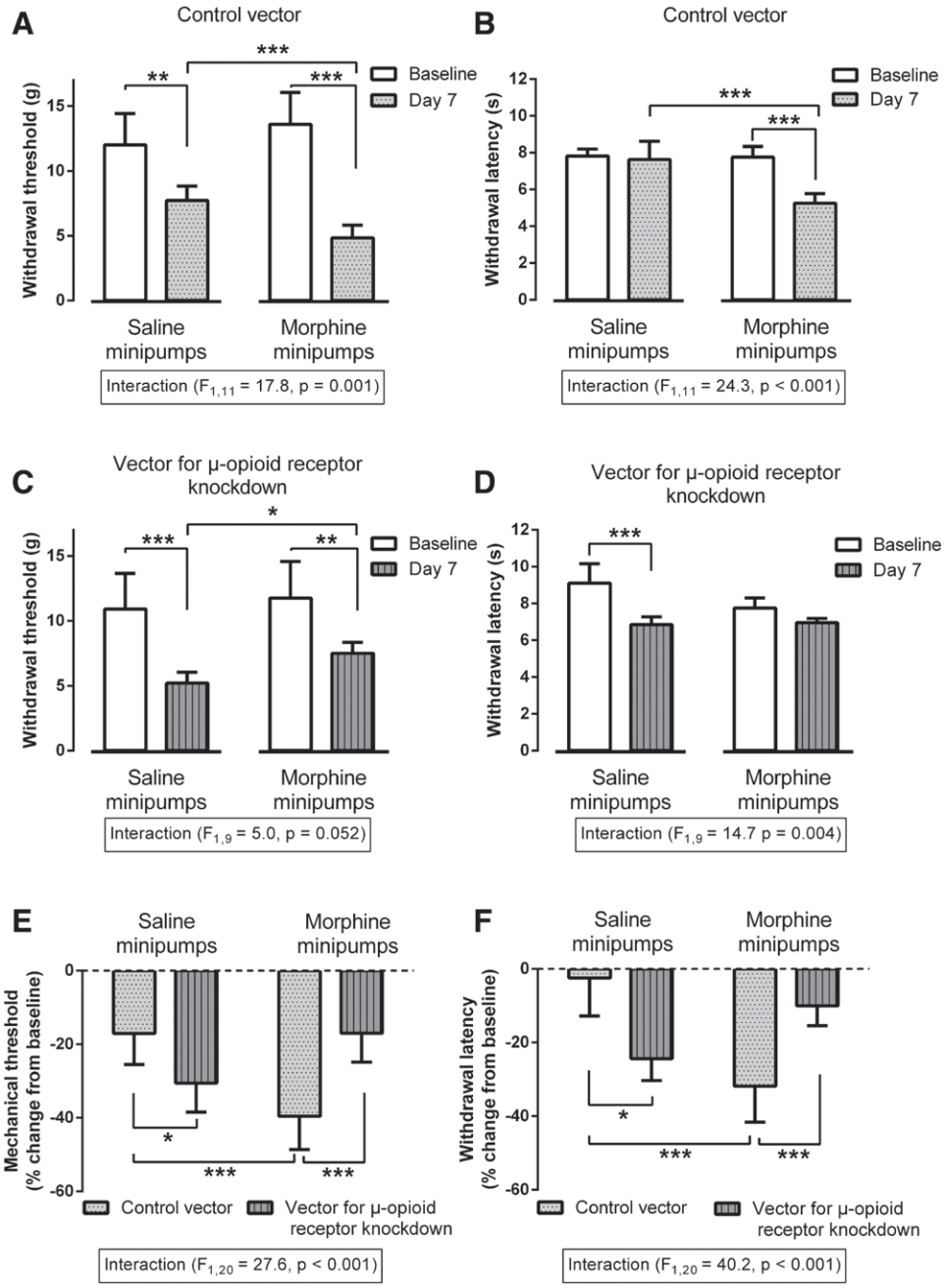

Fig. 7. Lentiviral-mediated μ-opioid receptor knockdown at the dorsal reticular nucleus attenuates the development of the mechanical and thermal hypersensitivity induced by chronic morphine. The control vector or the vector for μ-opioid receptor knockdown were injected at the dorsal reticular nucleus immediately after the implantation of saline or morphine minipumps and their effects were assessed before (*i.e.*, at baseline) and 7 days after injection by the von Frey (*A*, *C*, and *E*) and hot-plate (*B*, *D*, and *F*) tests, which evaluate mechanical and thermal sensitivity, respectively. Data in *E* and *F* represent the percentage of change from baseline (*i.e.*, before the injection of lentiviral vectors). The interactions between groups and time or groups and vectors are presented at the bottom of the *x* axis. Data are represented as mean ± SD (saline-infused animals: control vector n = 7, vector for μ-opioid receptor knockdown n = 5; morphine-infused animals: control vector n = 6, vector for μ-opioid receptor knockdown n = 6). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

nucleus as well as in out sites. Unlike at the dorsal reticular nucleus where morphine potentiates the coupling of the receptor to a stimulatory guanine nucleotide-binding protein, in adjacent areas the coupling of the receptors to inhibitory and stimulatory guanine nucleotide-binding proteins likely occurs dynamically.[8,9] Naloxone injected in

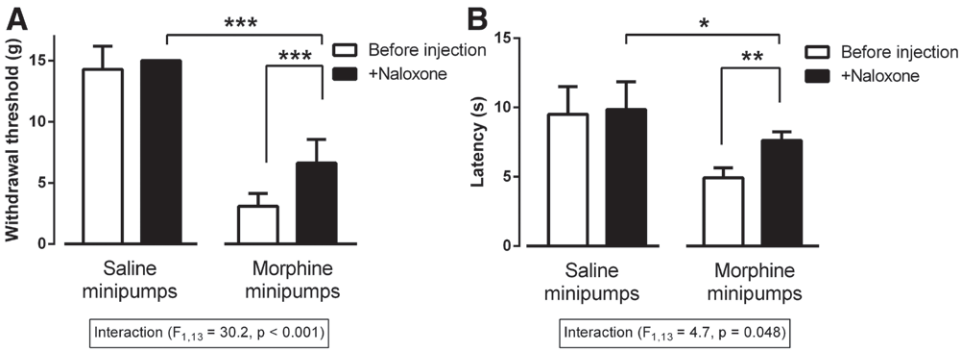

Fig. 8. Ultra-low dose naloxone at the dorsal reticular nucleus attenuates mechanical and thermal hypersensitivity induced by chronic morphine. The dorsal reticular nucleus was injected with naloxone 7 days after the implantation of saline or morphine minipumps. The von Frey (*A*) and hot-plate (*B*) tests, which evaluate mechanical and thermal sensitivity, respectively, were performed before and 30 min after naloxone (+Naloxone) injection. The interactions between groups and time are presented at the bottom of the *x* axis. Data are presented as mean ± SD (saline-infused animals n = 7; morphine-infused animals n = 8). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

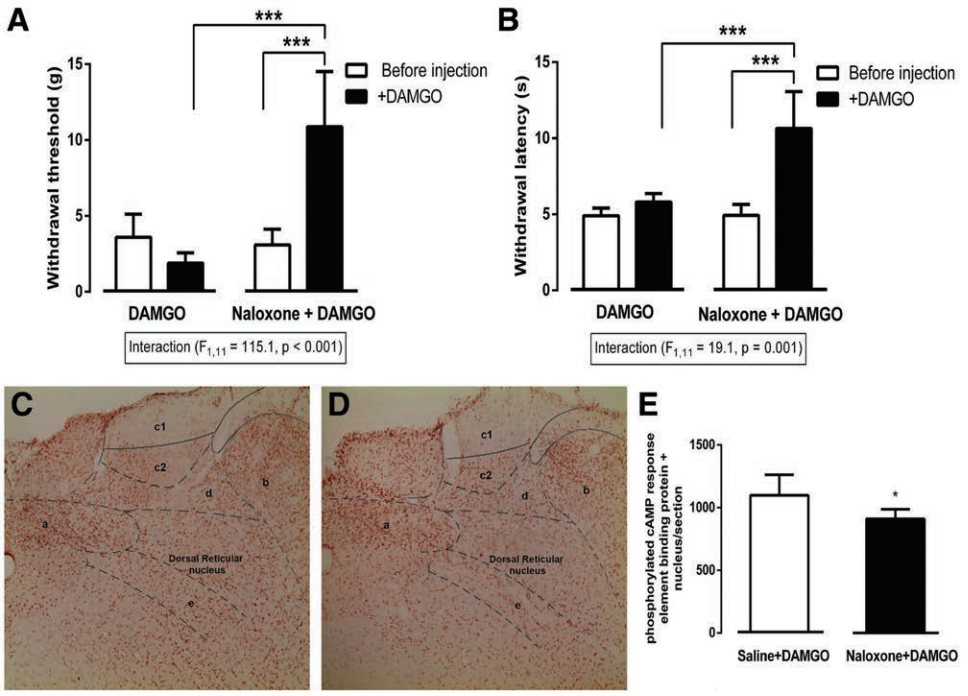

Fig. 9. Pretreatment with an ultra-low dose naloxone restores the analgesic effects of DAMGO and decreases phosphorylated cAMP response element-binding protein levels at the dorsal reticular nucleus after chronic morphine infusion. The dorsal reticular nucleus was injected with DAMGO alone (DAMGO; n = 5) or an ultra-low dose of naloxone followed 30 min later by DAMGO (Naloxone + DAMGO; n = 8), and the von Frey (*A*) and hot-plate (*B*) tests, which evaluate mechanical and thermal sensitivity, respectively, were performed before the injection of the drugs and 15 min after the injection of DAMGO (+DAMGO). The interactions between treatments and time are presented at the bottom of the *x* axis. Representative photomicrographs of positively labeled nuclei at the dorsal reticular nucleus after local injection of saline + DAMGO (*C*) or Naloxone + DAMGO (*D*). Scale bar in *D*, 500 μm (*C* is at the same magnification). The numbers of positively labeled nuclei at the dorsal reticular nucleus after injection of Naloxone + DAMGO (n = 5) were significantly lower compared with saline + DAMGO (n = 7) injection (t_{10} = 2.7, *P* = 0.022-0K; *E*). Data in *A*, *B*, and *E* are presented as mean ± SD. ****P* < 0.001. Letters in *C* and *D*: a, nucleus of the solitary tract; b, trigeminal subnucleus caudalis; c1, cuneate fasciculus; c2, cuneate nucleus; d, matrix region of the medulla; e, intermediate reticular nucleus.

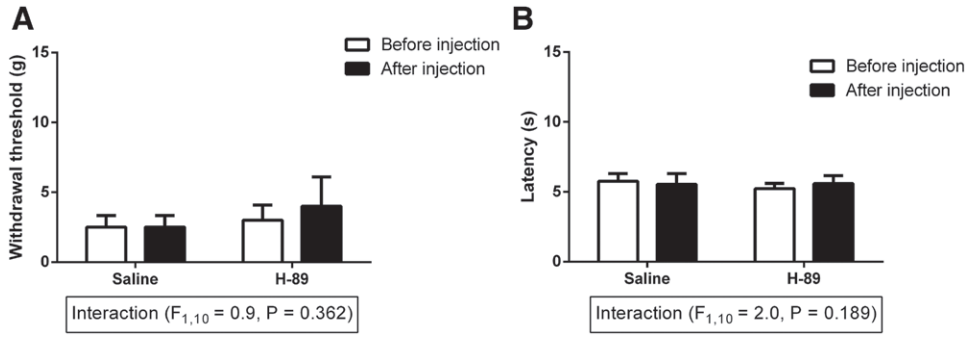

Fig. 10. Protein kinase A inhibition at the dorsal reticular nucleus produces no effects in mechanical and thermal hypersensitivity induced by chronic morphine. The dorsal reticular nucleus was injected with the protein kinase A inhibitor H-89 or saline 7 days after the implantation of morphine-minipumps. The von Frey (*A*) and hot-plate (*B*) tests, which evaluate mechanical and thermal sensitivity, respectively, were performed before and 40 min after injection. The interactions between groups and time are presented at the bottom of the *x* axis. No significant effects were detected between treatments (saline *vs.* H-89) both in the von Frey ($F_{1,10}$ = 2.1, P = 0.175; *A*) and hot plate ($F_{1,10}$ = 0.7, P = 0.414; *B*) tests. Data are presented as mean ± SD (saline n = 6; H-89 n = 6).

out sites likely shifts this balance toward increased inhibitory coupling. Together with naloxone diffusion to the dorsal reticular nucleus, this might explain the analgesic effects of DAMGO in out sites. It is unlikely that the effects of the ultra-low dose of naloxone might be due to the spread of naloxone to the rostral ventromedial medulla, since opioid-sensitive neurons in this area were shown to be resistant to the development of cellular adaptations after local repeated morphine microinjections.[48] The involvement of the rostral ventromedial medulla in opioid-induced hyperalgesia has been shown to be mediated through cholecystokinin.[16] Furthermore, μ-opioid receptor alterations in the rostral ventromedial medulla and dorsal reticular nucleus, in response to various types of pain, seem to be different.[24,30,49] More generally, in supraspinal pain modulatory areas, μ-opioid receptor seems to also adapt to different types of pain by diverse mechanisms that are nucleus specific.[50] This study shows that μ-opioid receptor–dependent mechanisms are involved in the activation of descending pain facilitation in opioid-induced hyperalgesia. To better envisage the translational perspectives of this study, future studies should be performed to confirm that the alterations found in μ-opioid receptor function and signaling are the same in female animals. Notwithstanding, uncovering the mechanisms involved in opioid-induced activation of descending facilitatory pathways may offer opportunities for developing new approaches to improve opioid analgesia and prevent paradoxical hyperalgesia.

In summary, our results show that descending pain facilitation from the dorsal reticular nucleus is involved in opioid-induced hyperalgesia and that it entails upregulation of μ-opioid receptor and altered μ-opioid receptor signaling pathways in dorsal reticular nucleus neurons. Because the μ-opioid receptor plays an important inhibitory function at the dorsal reticular nucleus, which accounts for the analgesic effects of systemic opioids,[24] the shift to excitatory signaling is likely in the genesis of increased descending pain facilitation.

Research Support

Support for this study was provided by an International Association for the Study of Pain (Washington, D.C.) Early Career Research Grant; grant NORTE-08-5369-FSE-000026 from European Social Fund (Porto, Portugal); and grant PTCD/SAU-NSC/110954/2009- FEDER/COMPETEFCT from the Portuguese National Funding agency for science (FCT; Lisbon, Portugal).

Competing Interests

The authors declare no competing interests.

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Discussion and Conclusions

The present thesis focuses on the study of the opioidergic modulation of the DRt in two different pain conditions, neuropathic pain, using the SNI model, and OIH. We focused our studies on MOR, since most therapeutic and side effects of the opioid analgesics are mediated by this receptor. During the development of this work we demonstrate that different pain conditions lead to different functional alterations of MOR in the DRt.

We show that MOR at the DRt plays a key role in the analgesic effects of systemic opioids, and that MOR is impaired following SNI. We further show that MOR impairment at the DRt in SNI-animals might be due to the increased phosphorylation of the receptor. The present thesis further shows the involvement of the DRt in the mediation of OIH. Chronic morphine infusion, leading to OIH, induces a switch in MOR signaling from inhibitory to excitatory at the DRt.

Together these results show that different painful conditions, induce different alterations on the opioidergic system at the DRt. These alterations likely contribute to the impairment of the opioidergic inhibition of DRt facilitatory actions, which may underlie the imbalance of pain modulation in these pain conditions towards pain facilitation. Since increased descending pain facilitation is one of the underlying mechanisms of both neuropathic pain and OIH, the impairment of opioidergic modulation at the DRt, is likely one of the major events contributing to the development of these pain conditions.

1. Opioidergic modulation of the DRt during acute pain

In our studies, we show for the first time that MOR at the DRt contributes to the analgesic effects of systemic opioids in naïve animals (***Publication I***). Using pharmacological and gene transfer techniques to block MOR at the DRt, we found that MOR knockdown reduced morphine analgesic effects and the pharmacological blockade of MOR, with the opioid antagonist CTAP, completely blocked the effects of morphine.

Furthermore, the results obtained in naïve animals also show that opioids at the DRt modulate noxious thermal as well as and non-noxious mechanical sensitivity through their actions at local MOR (***Publication I***). The results are in agreement with the activation of MOR resulting in the inhibition of DRt facilitatory actions. We show, for the first time, that the DRt plays a role in the modulation of non-noxious mechanical sensitivity and the involvement of MOR in such actions. The reduction of withdrawal thresholds to tactile stimuli, observed upon MOR knockdown, is indicative of the development of mechanical allodynia. This increased mechanical sensitivity is likely due to decreased inhibition of tonic DRt descending facilitation. In line with this, it was shown that tactile allodynia is integrated predominantly at supraspinal brainstem nuclei (Saadé et al., 2006). Furthermore, down-regulation of MOR within the descending pain network, induced by a model of neonatal inflammation, was associated to the development of mechanical allodynia (Yan and Kentner, 2017).

2. Opioidergic modulation of the DRt during neuropathic pain

Opioid peptides and their receptors are typically involved in the mechanisms that reduce pain, but functional and biochemical alterations in the opioidergic system appear to play a critical role in the development and maintenance of neuropathic pain. Our results presented in ***Publication I*** show that in SNI animals there is an increased release of the endogenous opioid peptides Met- and Leu-enkephalin at the DRt. These peptides are likely released from local enkephalinergic interneurons and also from afferent areas, namely the RVM, the A5 noradrenergic cell group and the hypothalamus (Martins et al., 2008). Both animal and human studies have indicated that the activation of the endogenous opioid system by nociceptive stimuli induce an increase of endogenous opioid peptides release (Bencherif et al., 2002; Sprenger et al., 2006; Zangen et al., 1998; Zubieta et al., 2001). Release of endogenous opioids has been shown in cortical and sub-cortical brain areas of patients with neuropathic pain (Harris et al., 2007; Jones et al., 1999; Maarrawi et al., 2007; Willoch et al., 2004) and also in patients with inflammatory pain (Jones et al., 1994). Studies performed in animals with neuropathic pain showed up-regulation of enkephalins or their precursors at the spinal cord (Hossaini et al., 2014; Rojewska et al., 2018; Sommer and Myers, 1995) and at the nucleus accumbens (Wawrzczak-Bargieła et al., 2020). In inflammatory pain models, up-regulation of enkephalins were observed at the spinal cord (Cesselin, 1980; Faccini et al., 1984; Noguchi et al., 1992) and in brainstem nuclei, including the PAG and RVM (Hurley and Hammond, 2000; Sapio et al., 2020; Williams et al., 1995).

We found a decrease in the number of MOR-IR cells at the DRt of SNI-animals. Neuropathic pain induced by peripheral nerve section reduces MOR immunostaining in the cell bodies of primary sensory neurons in the DRGs and at their central terminal in

the dorsal horn (Goff et al., 1997; Kohno et al., 2005; Porreca et al., 1998; Sumizono et al., 2018) and also in supraspinal areas involved in pain modulation, like the caudate–putamen and insula (Thompson et al., 2018). The reduction of the number of MOR-IR cells found in our study is not a consequence of the down-regulation of MOR gene expression at the DRt since we found no alterations in MOR mRNA levels between control and SNI-animals. One possible explanation is that counter-regulatory adaptations may lead to increased traffic of MOR to degradative intracellular pathways. MOR can be down-regulated by increased targeting to degradation in lysosomes (Hislop et al., 2011; Law et al., 1984), which was described in a model of diabetic neuropathy (Mousa et al., 2013). MOR phosphorylation is a post-translacional modification that plays a major role in the regulation of MOR function after acute or prolonged exposure to agonists (Williams et al., 2013; Zhang et al., 2009b). An important mechanism triggered by MOR phosphorylation is the internalization of the receptor. Upon phosphorylation, MOR is internalized and delivered to the cytoplasmic compartment of early endosomes. There, it can be recycled back to the plasma membrane in a fully sensitized state or delivered to lysosomes for degradation (Johnson et al., 2005). We found an increase of phosphorylated MOR-IR cells (pMOR) at the DRt of SNI animals. The C-terminal tail of MOR contains 11 serine and threonine residues capable of being phosphorylated (Kliewer et al., 2019). We detected MOR phosphorylation at the ser375 residue which represents a major phosphorylation site involved in MOR internalization (El Kouhen et al., 2001). Therefore, it is likely that the reduction of MOR-IR cells at the DRt of neuropathic animals could result from increased phosphorylation of the receptor, followed by internalization and, ultimately, increased degradation in lysosomes.

At the DRt of SNI animals, the antinociceptive effects of the MOR agonist DAMGO are lost. We only determined the effects of DAMGO on mechanical allodynia,

which is a representative symptom of neuropathic pain in humans (Woolf and Mannion, 1999), since changes in heat thresholds are difficult to measure in the SNI model (Decosterd and Woolf, 2000). The loss of DAMGO antinociceptive effect is likely due to the reduction of MOR-IR cells at the DRt in neuropathic animals. Therefore, and based on the effects of MOR knockdown on naïve animals, the reduction of MOR-IR cells at the DRt likely induces an impairment of the opioidergic inhibition of DRt descending facilitation in SNI animals. A reduction in the numbers of MOR-IR cells at the DRt has also been shown in a model of chronic inflammatory pain, but this resulted in a DAMGO-induced hyperalgesic effect (Pinto et al., 2008a). The hyperalgesic effects of DAMGO at the DRt during inflammatory pain might be due to a different MOR adaptation, probably a switch of MOR coupling to a Gs-protein. Indeed, it has been shown that the DRt is involved in the antinociceptive effects produced by systemic administration of a low-dose of naloxone during inflammatory pain (Tsuruoka et al., 1997). The ultra-low dose of naloxone prevents MOR coupling to a Gs-protein and restores MOR coupling to a Gi-protein (Wang and Burns, 2009), restoring thus the inhibitory signaling of MOR at the DRt, and in the study by Tsuruoka et al., the antinociceptive effects of naloxone were prevented by DRt lesion. In supraspinal pain modulatory areas, MOR seems to adapt to different types of pain by diverse mechanisms that are nucleus specific (Dickenson et al., 2020). For example, at the RVM, MOR antinociceptive effects are enhanced after local injection of an opioid agonist both in neuropathic and inflammatory models of pain (Dickenson et al., 2020; Zhang and Hammond, 2010).

The loss of DAMGO effects in SNI-animals cannot be totally explained by the reduction of MOR-IR cells since the lentiviral mediated increase of MOR expression did not alter mechanical allodynia, but a high dose of DAMGO induced an antiallodynic effect in MOR-overexpression experiments. Therefore, we suggest that neuropathic pain

might also induce desensitization of MOR function at the DRt. The phosphorylation of MOR plays a major role in desensitization and the fact that MOR phosphorylation is increased at the DRt of neuropathic animals further argues in favor of this hypothesis. Increasing evidences of MOR desensitization induced by neuropathic pain include reduced MOR-mediated-G-protein activity in the thalamus, PAG, raphe magnus and amygdala (Hoot et al., 2011; Kanbara et al., 2014; Llorca-Torralba et al., 2020; Narita et al., 2006) and also increased MOR phosphorylation at the DRG (Mousa et al., 2016), spinal dorsal horn (Narita et al., 2004) and the striatum (Petraschka et al., 2007). As shown in Figure 6, we propose that the high levels of endogenous opioid peptides found at the DRt of neuropathic animals increased MOR phosphorylation, desensitization and increased targeting of MOR to degradation. Indeed, endogenous opioid peptide ligands, such as enkephalins and endorphins induce robust desensitization and endocytosis (Allouche et al., 2014; Llorente et al., 2012). Prolonged exposure to opioids uncouples MOR from Gi/o proteins and this process is mediated by agonist-dependent phosphorylation of specific intracellular serine and threonine residues (Williams et al., 2013). Ligand-activated receptors can be phosphorylated by specific GPCR kinases (GRKs) or second-messenger-dependent protein kinases, like c-Jun amino-terminal kinase, PKC, PKA, calcium calmodulin kinase, and mitogen-activated protein kinases (Koch and Höllt, 2008; Williams et al., 2013). The phosphorylated receptor can then bind to β-arr, leading to a series of events that include activation of intracellular signaling mechanisms as well as clathrin-induced internalization (Williams et al., 2013). In LC neurons, MOR desensitization induced by met-enkephalin is mediated by at least two distinct mechanisms, one mediated by GRK2 and β-arr-2 interaction and other involving the extracellular-signal-regulated kinases 1 and 2 (ERK1/2) activity, while inhibition of each mechanism individually failed to prevent MOR desensitization (Dang

et al., 2009). Under pathophysiological conditions, sustained release of endogenous peptides in the brain of neuropathic animals has also been shown to induce desensitization of MOR and opioid tolerance (Petraschka et al., 2007).

Based on previous data of our research group (Pinto et al., 2008b) at the DRt, MOR is expressed both in spinally- and non-spinally projecting neurons. The impairment of MOR inhibitory function in the spinally-projecting neurons, involved in the reverberative spino-medullary excitatory loop (Lima and Almeida, 2002), probably contributes to the amplification of pain transmission. MOR is also expressed by GABAergic interneurons which inhibit enkephalinergic interneurons. The loss of opioidergic inhibition in GABAergic interneurons should decrease the release of enkephalins at the DRt. However, this effect does not counteract the increase of endogenous enkephalins at the DRt, probably due to the impairment of the GABAergic inhibitory function during neuropathic pain (Li et al., 2019).

In spite of our results showing that alterations on MOR are essential for the loss of opioidergic inhibition of pain facilitation from the DRt, we cannot exclude the intervention of other receptors since opioid receptors do not necessarily function independently and can exist as heterodimers that modulate their pharmacology (George et al., 2000). Indeed, MOR-DOR heterodimerization has been shown to play an important role in the loss of opiate analgesic potential as well as in the development of tolerance (Bailey and Connor, 2005; Rozenfeld et al., 2007). Bivalent ligands with MOR agonist and DOR antagonist profiles have shown analgesic activity with comparatively decreased development of analgesic tolerance (Daniels et al., 2005). MOR and DOR are usually localized in different cell compartments, where MOR is found mainly on the cell surface, whereas DOR is almost exclusively intracellular (Arvidsson et al., 1995; Cahill et al., 2001a; Cahill et al., 2001b; Pinto et al., 2008a), but prolonged opioid stimulation

increases the recruitment of intracellular DOR to the cell surface (Cahill et al., 2001b). Additionally, DOR is also expressed at the DRt. The involvement of other opioid receptors in relation to MOR deserves to be evaluated in the future.

The study presented in ***Publication I*** shows that neuropathic pain is associated with alterations in the opioidergic system at the DRt. These alterations likely impact negatively DNIC. DNIC is an endogenous form of inhibitory control that occurs when a distant painful conditioning stimulus is used to alleviate another noxious stimulus and the DRt is a key area involved in DNIC (Bouhassira et al., 1992; Youssef et al., 2016). It has been shown that blocking the opioidergic inhibition of the DRt blocks DNIC (de Resende et al., 2011; Patel and Dickenson, 2019). During neuropathic pain, both in animal models and in the clinic, there is a loss of DNIC (Bannister et al., 2015; Kennedy et al., 2016; Yarnitsky et al., 2012). It would be interesting to evaluate, in future studies, whether the impairment of MOR inhibitory function at the DRt is involved in the loss of DNIC during neuropathic pain. These studies are important inasmuch as DNIC is used in clinical settings where it is known as conditioned pain modulation. It is used as a reliable indicator of the integrity of the descending pain modulatory system (Kennedy et al., 2016; Youssef et al., 2016). In clinical settings, DNIC has also been used as a tool to predict the susceptibility to chronic pain as it has been shown that patients with decreased DNIC have higher susceptibility of developing chronic pain (Kennedy et al., 2016; Villanueva, 2009). But mechanistically, DNIC remains understudied.

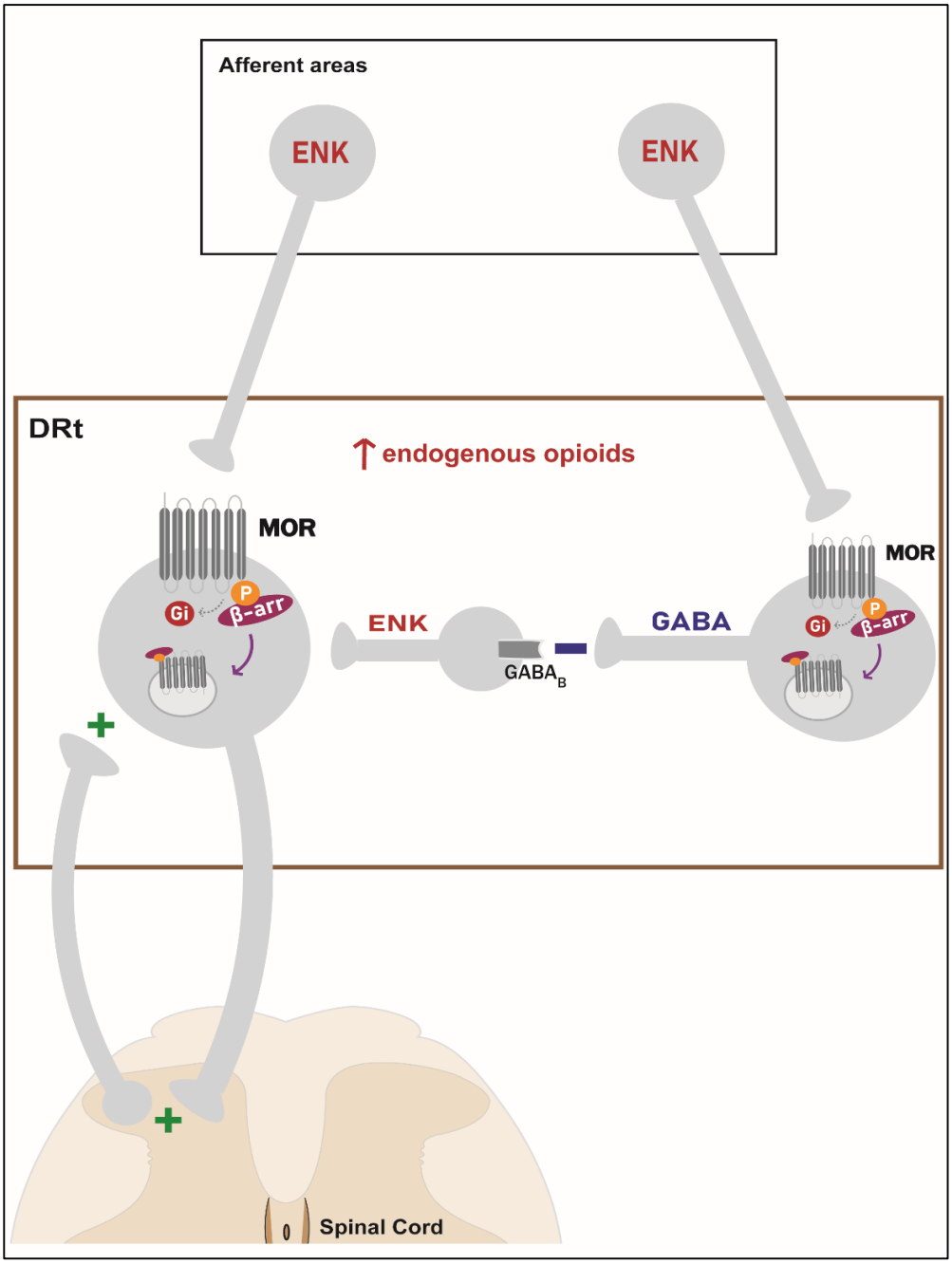

Figure 6- Proposed mechanisms of the opioidergic modulation of the DRt during neuropathic pain. Neuropathic pain induces the release of high levels of endogenous opioid peptides at the DRt, likely from local enkephalinergic interneurons and also from afferent areas. MOR activation promotes the dissociation of the inhibitory Gi-protein, followed by receptor phosphorylation and β-arrestin recruitment. Arrestins lead to MOR desensitization by preventing G-protein coupling and promoting receptor internalization. After internalization, the receptor can be delivered to lysosomes for degradation. Increased MOR phosphorylation, desensitization and down-regulation likely contribute to a loss of opioidergic inhibition of the DRt. MOR is expressed in spinally projecting neurons and also in GABAergic interneurons impinging upon enkephalinergic neurons. The loss of opioidergic inhibition of spinally-projecting neurons, which are engaged in a reverberative circuitry linking the DRt to the spinal dorsal horn (Lima and Almeida, 2002), probably contributes to enhance pain transmission. The loss of opioidergic inhibition in GABAergic interneurons should indirectly decrease the release of enkephalins at the DRt. However, overall this effect does not counterbalance the increase of endogenous enkephalins at the DRt, probably because neuropathic pain is accompanied by a reduced GABAergic inhibitory function (Li et al., 2019). DRt, dorsal reticular nucleus; ENK, enkephalinergic neurons; $GABA_B$, $GABA_B$ receptor; GABA, GABAergic neurons; MOR, μ-opioid receptor; β-arr, β-arrestin.

3. Opioidergic modulation of the DRt during Opioid Induced Hyperalgesia

Opioids have an important place in pharmacology. While their clinical use as analgesics is fundamental, their use is constrained by their side-effects. OIH is a state of enhanced pain sensitization in patients who are on opioid therapy. One of the mechanisms proposed to be involved in the development of OIH are alterations in the pain modulatory circuitry, which include the activation of descending pain facilitatory systems (Vanderah et al., 2001; Vera-Portocarrero et al., 2007). Descending facilitation may contribute to OIH through an enhancement of spinal dorsal horn excitability (Gardell et al., 2002; Heinl et al., 2011).

In the study presented in ***Publication II***, the pharmacological inactivation of the DRt completely reversed the mechanical and thermal hypersensitivity induced by chronic treatment with morphine. This indicates that the descending facilitation from the DRt is involved in OIH. Descending pain facilitation from another medullary area, the RVM, has been previously shown to be involved in the mediation of OIH induced by acute or prolonged administration of opioids (Rivat et al., 2009; Vanderah et al., 2001). The activation of descending pain facilitation from the RVM in OIH was shown to be mediated by enhanced endogenous CCK activity and activation of CCK2 receptors in the RVM (Xie et al., 2005). In our study, we show that one of the mechanisms underlying the involvement of the DRt in OIH is the activation of MOR. Interestingly, the CCK2 receptor is co-expressed with MOR in RVM neurons (Zhang et al., 2009a) functionally defined as the pronociceptive ON-cells, and CCK antagonists can block and reverse OIH (Xie et al., 2005). *In vitro* studies show that heteromerization of MOR and CCK receptors decreases MOR activity (Yang et al., 2018). It is therefore likely that enhanced CCK

activity by chronic morphine at the RVM, might be closely linked to MOR signaling at the RVM.

While MOR expressed in sensory neurons was shown to be critical to the initiation of OIH (Corder et al., 2017), the sustained activation of MOR in the DRt might trigger facilitatory influences to the spinal dorsal horn, contributing, alongside the peripheral mechanisms, to the maintenance of OIH. Nevertheless, at the DRt, MOR-independent mechanisms might also be involved in the mediation of OIH since pharmacological inactivation of the DRt fully blocks OIH while pharmacological and genetic manipulation of MOR only attenuated OIH. One of the mechanisms could involve the glutamatergic system and particularly the NMDA glutamate receptors, given its involvement in OIH (Roeckel et al., 2016) and in DRt-pronociceptive effects (Lima and Almeida, 2002). Several studies showed that the administration of a NMDA receptor antagonist diminishes or prevents OIH in animals (Arout et al., 2015; Li et al., 2001). At the DRt, glutamate administration induces a long-lasting increase in the responsiveness of spinal nociceptive neurons (Dugast et al., 2003). Glial cells might also be involved in the mediation of OIH at the DRt, possibly through TLR-4. Opioids activate TLR-4 that will trigger the production and the release of pro-inflammatory factors by glial cells. Once released, cytokines may induce abnormal spontaneous neuronal activity (Grace et al., 2015).

We found an increase in the number of cells expressing MOR at the DRt of morphine-treated animals but no difference in the MOR mRNA levels. The absence of effects of the chronic morphine treatment in MOR mRNA levels at the DRt is consistent with other studies showing the lack of effect of chronic morphine in the transcriptional regulation of MOR in the brain (Brodsky et al., 1995; Buzas et al., 1996; Castelli et al., 1997; Zhu et al., 2012). This suggest, that the high number of MOR-IR cells at the DRt

following morphine-infusion could be due to increased expression of MOR at the protein level and/or a change in MOR trafficking resulting in an accumulation of MOR at the plasma membrane. The MOR gene undergoes extensive alternative pre-mRNA splicing generating several classes of splice variants, including truncated single variants (Pasternak and Pan, 2013). It has been shown that 1TM splice variants, whose expression levels are increased in the brainstem after chronic morphine (Xu et al., 2015), increase the expression of the full-length MOR (Xu et al., 2013). This increased expression of the full-length protein induced by the 1TM variants was not associated with changes in mRNA levels. Instead, 1TM variants can dimerize with the full-length MOR in the endoplasmic reticulum, increasing its expression by a chaperone-like function that minimizes endoplasmic reticulum-associated degradation (Xu et al., 2013). The accumulation of the receptor at the plasma membrane is supported by the fact that morphine is a poor internalizing agonist and the internalization of the receptor is crucial for targeting the receptor to degradation (Williams et al., 2013). *In vivo*, chronic treatment with morphine was shown to induce different effects in the expression of MOR with either down-regulation, no change or up-regulation (Bernstein and Welch, 1998; Brady et al., 1989; Ray et al., 2004; Rothman et al., 1991; Stafford et al., 2001). Opioid tolerance has been associated, although not exclusively, with down-regulation of the receptor (Allouche et al., 2014; Williams et al., 2013).

Based in the cellular and behavioral results obtained in our studies we suggest that MOR signaling at the DRt switches from inhibitory to excitatory (Figure 7). At the cellular level, the shift to excitatory signaling is suggested by the increased expression of pCREB, a downstream marker of the excitatory signaling transduction pathway of MOR, at the DRt and decreased expression of pCREB after ultra-low dose naloxone treatment, which prevents MOR coupling to an excitatory Gs protein and restores MOR coupling to

a Gi/o inhibitory protein (Wang and Burns, 2009). The best well-established molecular adaptation to chronic opioid exposure following coupling of MOR to Gs is up-regulation of the cAMP/cAMP-dependent PKA signaling. Furthermore, CREB phosphorylation is predominantly made by PKA through binding of cAMP (Crain and Shen, 2000; Wang and Burns, 2009). However, the absence of effects of the PKA inhibitor in our work, discards the involvement of the PKA pathway at the DRt. Activation of PKC and ERKs pathways could be the signalling pathways involved since they can also phosphorylate CREB. ERKs are members of the mitogen-activated protein kinase family that transduce some extracellular stimuli into intracellular responses by altering gene expression or transcription. When activated, ERKs go from the cytosol to the nucleus and in turn phosphorylate CREB (Williams et al., 2001). It was also demonstrated that morphine induces ERKs phosphorylation (pERKs) via the PKC pathway (Sanna et al., 2014), and pharmacological inhibitors of PKC and ERK activity, specifically in descending pain modulatory areas, reduces morphine hyperalgesia (Bie and Pan, 2005). Additionally, some studies show that morphine leads to an increase in pERKs levels, which suggests that ERKs contribute to morphine-induced hyperalgesia (Sanna et al., 2015). Ongoing studies in our group show that sustained morphine administration increased the number of pERKs immunoreactive cells at the DRt (Sousa et al., 2019). Therefore, the PKC pathway may be involved in the increase of pCREB at the DRt of morphine infused animals.

The administration of the opioid agonist DAMGO at the DRt enhanced mechanical sensitivity in morphine-infused animals, whereas the opposite occurs in control animals. We did not detect an enhancement of thermal hypersensitivity probably due to methodological features of the test. In the hot plate test, the heat intensity is usually set up to observe responses within 5 to 10 s (Barrot, 2012), and morphine-infused animals

show withdrawal latencies on average near to 5 s, hence shorter latencies are likely not detected. The pronociceptive effects of DAMGO at the DRt of morphine-infused animals are likely mediated through MOR excitatory effects on DRt descending facilitation. This is supported by the attenuation of OIH development observed after MOR knockdown at the DRt. Finally, the behavioral data is consistent with the with MOR being expressed on DRt-spinally projecting neurons (Pinto et al., 2008b), which are excitatory and engaged in the reverberative loop responsible for pain amplification (Lima and Almeida, 2002). The switch of MOR signaling from inhibitory to excitatory at the DRt is further suggested by the pharmacological experiments performed with the ultra-low dose naloxone. The ultra-low dose of naloxone behaviorally attenuates OIH and restores the analgesic effects of DAMGO. Ultra-low doses of the opioid antagonist's naloxone or naltrexone prevent MOR coupling to Gs and restore MOR coupling to a Gi/o inhibitory protein (Crain and Shen, 2000). At ultra-low doses, the antagonists disrupt MOR coupling to Gs via their interaction with the MOR-interacting scaffolding protein filamin A, rather than a direct interaction with MOR, which occurs at higher doses (Wang et al., 2008). Blockade of MOR coupling to Gs by ultra-low doses of opioid antagonists was shown to enhance opioid analgesia, attenuate tolerance, dependence and also OIH (Crain and Shen, 1995, 2000, 2001; Largent-Milnes et al., 2008; Wang et al., 2005). The enhancement of opioid analgesia, attenuation of tolerance and OIH by ultra-low doses of an opioid antagonists co-treatment with opioids in preclinical and clinical studies further demonstrates the relevance of MOR coupling to Gs in the development of analgesic tolerance and OIH (Aguado et al., 2013; Chindalore et al., 2005; Firouzian et al., 2018; Gan et al., 1997). The effects of ultra-low dose of naloxone injections placed outside the DRt did not differ significantly from the injections located in the DRt. This means that upon injection into the DRt, naloxone diffuses to adjacent areas and therefore its site of action is not limited

to the DRt. Nevertheless, since increased MOR-Gs coupling increases pCREB levels and ultra-low dose of naloxone prevents the increase of pCREB levels (Wang and Burns, 2009), the decreased pCREB levels at the DRt, and not in the adjacent areas, after the microinjection of the ultra-low dose of naloxone, suggests that there is a reduction of MOR-Gs coupling only at the DRt. At the behavioral level, the reduction of MOR-Gs coupling in the DRt, and not in adjacent areas, is likely to contribute the most to the attenuation of OIH and to restore the analgesic effects of DAMGO at the DRt. This is supported by the fact that DAMGO in the DRt, but not in adjacent areas, produces an enhancement of morphine-induced mechanical hypersensitivity.

Based on the distribution of MOR expression in DRt neurons (Figure 7), the switch of MOR function to excitatory in spinally-projecting neurons likely contributes to increased activation of spino-DRt-spinal reverberative excitatory loop (Lima and Almeida, 2002), leading to the amplification of pain transmission. In the GABAergic interneurons, MOR excitatory signaling likely enhances GABA inhibition of enkephalinergic interneurons, consequently decreasing the levels of endogenous enkephalins at the DRt.

It has been shown that coupling of MOR to Gs protein can be mediated by different MOR isoforms. One of them is a truncated six transmembrane splice variant of MOR, the MOR-1K. The activation of MOR-1K induces excitatory cellular effects mediated through Gs activation (Gris et al., 2010), while activation of the 7TM canonical variant inhibits neurons through Gi activation. Furthermore, it has been shown that chronic morphine increased the expression of MOR-1K and that decreasing its expression attenuated OIH development and increased morphine analgesia (Oladosu et al., 2015). Chronic treatment with opioids also up-regulates the 7TM isoforms MOR-1B2 and MOR-1C1 (Chakrabarti et al., 2016; Verzillo et al., 2014). Recent *in vitro* studies show that

chronic morphine phosphorylates MOR-1B2 and MOR-1C1 carboxyl terminal, resulting in augmented association of these isoforms with Gs-proteins (Chakrabarti et al., 2019). These observations suggest that different MOR variants could be important in the excitatory effects of opioids. It would be interesting to evaluate, in future studies, whether chronic morphine at the DRt induces a differential expression of these isoforms and whether they are involved in the increased excitatory signaling of MOR at the DRt.

In summary, the study presented in ***Publication II*** shows that descending pain facilitation from the DRt is involved in OIH and that it entails the up regulation of MOR and altered MOR signaling pathways in DRt neurons, likely increasing descending pain facilitation (Figure 7). At the clinical practice, OIH has a relevant impact during opioid-based anesthesia and during postoperative analgesia. In fact, several clinical studies have reported the enhancement of postoperative pain after the administration of high doses of opioids (Fletcher and Martinez, 2014; Richebé et al., 2011; Schmidt et al., 2007). Studies suggest that enhanced descending facilitatory pathways are involved in the exacerbation of post-surgical pain after the administration of opioids. Descending facilitation of the RVM is involved in the enhanced sensory hypersensitivity in a model of post-surgical pain after fentanyl administration (Rivat et al., 2009). Ongoing studies in our group show that after morphine infusion cessation, behavioral hypersensitivity is completely abolished, but pCREB and pERKS levels at the DRt remain increased (Sousa et al., 2019). Furthermore, the pronociceptive effect of DAMGO at the DRt also remained after morphine treatment cessation (Sousa et al., 2019) and the injection of an ultra-low dose naloxone in the DRt enhanced the antinociceptive effects of a low dose of systemic morphine in animals with post-operative pain (*unpublished data*). These results are indicative of the maintenance of the switch of MOR signaling to excitatory in the DRt after opioid cessation. Therefore, the fact that intracellular cascades remain altered after

opioid cessation might impact negatively on the effects of opioids in future pain treatments. In conclusion, unveiling the molecular mechanisms of OIH will allow the development of new strategies to prevent or reverse the activation of pronociceptive neuronal circuits and to improve the management of different painful conditions where OIH has a clinical impact.

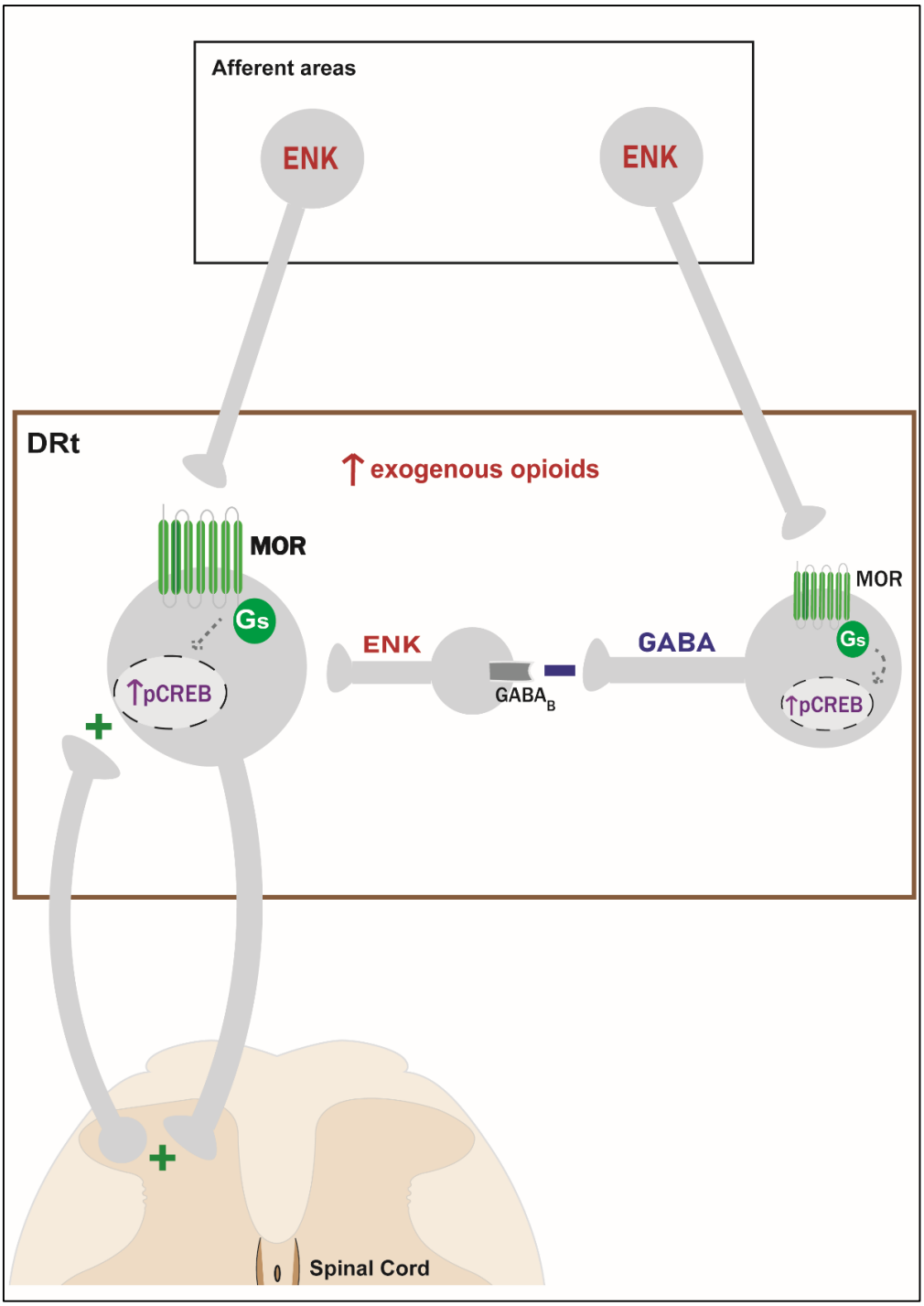

Figure 7- Proposed mechanism of the opioidergic modulation of the DRt during opioid induced hyperalgesia. Chronic morphine infusion promotes up-regulation of the receptor and switches MOR coupling to an excitatory G_s-protein. The latter event leads to the activation of intracellular transduction pathways resulting in increased expression of pCREB. MOR is expressed in spinally projecting neurons and also in GABAergic interneurons impinging upon enkephalinergic neurons. The switch of MOR signaling to excitatory in spinally-projecting neurons likely contributes to amplifying the reciprocal excitatory circuitry linking the DRt to the spinal dorsal horn (Lima and Almeida, 2002). The excitatory signaling in GABAergic interneurons likely enhances GABA inhibition of enkephalinergic interneurons therefore decreasing endogenous levels of enkephalins at the DRt. DRt, dorsal reticular nucleus; ENK, enkephalinergic neurons; $GABA_B$, $GABA_B$ receptor; GABA, GABAergic neurons; MOR, μ-opioid receptor; pCREB, phosphorylated cAMP response element binding protein.

4. Conclusions

The study presented in the first part of this dissertation (***Publication I***) evaluated the opioidergic modulation of the DRt during neuropathic pain and gave insights on how the impairment of the MOR function impacts the descending modulation of the DRt. The main results of this study suggest that the increased release of enkephalins at the DRt during persistent pain of neuropathic animals, likely leads to the down-regulation and desensitization of MOR (Figure 6). Additionally, we show that MOR plays a key role in the analgesic effects of systemic opioids, which becomes impaired following SNI. These results suggest that lack of MOR inhibitory function at the DRt in SNI animals might be one of the reasons behind decreased effectiveness of opioids in neuropathic pain (Martínez-Navarro et al., 2019). We studied the effects of the chronicity of neuropathic pain in the opioidergic system, however, future studies should address changes at earlier time-points. This would increase our knowledge about the mechanisms underlying the development of chronic pain taking place at descending pain modulatory areas.

The studies presented in the second part of this dissertation (***Publication II***) evaluated the opioidergic modulation of the DRt during OIH. The results of this study showed, for the first time, the involvement of the descending pain facilitation from the DRt during OIH, which is related with dysfunctions of MOR inhibitory function. The molecular and functional studies suggest that chronic treatment with morphine switch MOR signaling from inhibitory to excitatory at the DRt, which might represent one of the underlying mechanisms of increased descending facilitation during OIH (Figure 7). Since our results discard the involvement of the classical PKA pathway at the DRt, it would be interesting to address in future studies the involvement of the PKC pathway. It would also be interesting to study other mechanisms involved in OIH at the DRt, like the involvement

of the glutamatergic system and neuron-glial interactions. Additionally, it would be important to study for how long the alterations in the opioidergic system, induced by chronic treatment, remain after treatment cessation. It is known that paradoxical OIH is reversible and that it vanishes after cessation of opioids, as observed in humans (Younger et al., 2011) and in rodents (Celerier et al., 2001; Nation et al., 2018). Follow-up scans in humans conducted on an average of 4.7 months after cessation of opioids showed that several morphine-induced changes persisted (Younger et al., 2011), which suggests that despite the paradoxical pain sensation disappears, the alterations induced by the chronic treatment remain. Nonetheless, there are no studies evaluating whether cellular and molecular alterations are maintained after cessation of chronic opioid treatments and its consequences on future treatments. Therefore, it would be interesting to study the involvement of the DRt descending facilitation during OIH and after opioid treatment cessation in models of post-surgical pain, where OIH has a relevant clinical impact (Colvin et al., 2019).

In conclusion, the data gathered in this dissertation reveal that increased levels of opioid peptides, both endogenous (induced by neuropathic pain) and exogenous (in the OIH model), induced an impairment of the MOR inhibitory function at the DRt. Since MOR plays an important inhibitory function, these alterations likely contribute to enhancing descending facilitation from the DRt. The impairment of MOR inhibitory function at the DRt, observed in both models, results from different alterations in the receptor. MOR seems to adapt to different types of pain by diverse mechanisms that are nucleus specific. Our studies add another degree of complexity by showing different types of adaptations of MOR, in the same supraspinal area, in response to different types of pain. This reinforces the importance of studying the alterations in the receptor in different pain types and in different supraspinal sites involved in pain modulation. By improving

our knowledge about the different adaptations induced on MOR by distinct pathologies, it might be possible to develop specific strategies to better manage each disorder, improve the efficacy and minimize the long-term harms of the use of opioids.

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