Universidade do Minho
Escola de Ciências da Saúde

Diana Alexandra da Silva Amorim

Pronociception in experimental monoarthritis: the role of galanin in descending pathways

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Trabalho realizado sob a orientação do
Professor Doutor Armando Almeida
e co-orientação da
Professora Doutora Isaura Tavares

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DECLARAÇÃO

Nome: Diana Alexandra da Silva Amorim

Endereço electrónico: dianaamorim@ecsaude.uminho.pt

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Orientadores:

Professor Doutor Armando Almeida e Professora Doutora Isaura Tavares

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Aos meus pais

"Believe nothing no matter where you read it, or who said it ...

...unless it agrees with your own reason and common sense"

Buddha

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Pronociception in experimental monoarthritis: the role of galanin in descending pathways

Abstract

Acute pain is a protective mechanism that triggers escape behaviours in order to avoid a source of injury. However, when pain becomes constant and prolonged, such as in arthritis, it highly affects the quality of life. Moreover, arthritic pain is often accompanied by other disorders, such as mood alterations like anxiety and depression, which are usually overlooked when treating arthritic patients. Although the mechanisms underlying the comorbid development of anxiety and depression during chronic pain are not yet understood, it is clear that brain regions and neurotransmitter pathways involved in pain processing and modulation are also implicated in the modulation of mood and emotions. Among other neurotransmitters, galanin (GAL) appears to be an important player in both pain and emotions, with GAL infusion into the brain ventricles eliciting depressive-like behaviours and activation of the amygdala, a central area of emotional and pain processing, and the dorsomedial nucleus of the hypothalamus (DMH), a region that recently emerged as a hypothalamic region involved in anxiety response and capable of mediate behavioural hyperalgesia (increased nociception). The DMH pronociceptive action was correlated with alterations in the activity of rostral ventromedial medulla (RVM) On- and Off- pain modulatory cells. The RVM is part of the main pain inhibitory system although its facilitatory role has also been demonstrated. In addition to the RVM, the medullary dorsal reticular nucleus (DRt) is also a brainstem area associated to pronociception, with its neurones responding almost exclusively to noxious stimulation.

The kaolin/carrageenan (K/C) model of monoarthritis was used to assess the effect of prolonged arthritic-like pain in the knee upon nociception as well as upon the comorbid development of emotional-like disorders. Four weeks after saline (SHAM animals) or K/C (ARTH animals) injection into the right knee, animals were evaluated by the pressure application test directly in the injected knee to confirm the arthritic pain state. The open field and elevated plus maze tests were used to assess locomotion and anxiety-like behaviour; and the sucrose preference and forced swimming tests to evaluate depression-like behaviours. In order to study a potential therapeutic effect of amitriptyline, a tricyclic antidepressant, in the reversion of pain- and emotional-related behaviours in ARTH animals, a three-week period of amitriptyline treatment was performed. The contribution of GAL receptors for the DMH-mediated behavioural hyperalgesia

and associated pronociception, in SHAM and ARTH animals, was evaluated using heat noxious stimulation and electrophysiological recordings of RVM and spinal dorsal horn neuronal activities when pharmacologically manipulating the DMH with agonist and antagonists of GAL receptors. The role of the DRt as a potential brainstem mediator and the serotonin subtype-3 receptors (5HT3R) as potential spinal cord mediators of the descending GAL/DMH pronociceptive effect was assessed by administering GAL in the DMH with simultaneous blockage of the DRt and 5HT3R in the spinal cord, respectively, and measuring the behavioural response of SHAM and ARTH animals in the Hargreaves test.

Our results demonstrated that prolonged arthritis in the knee induces pain and comorbid development of anxiety- and depressive-like symptoms, which can be totally/partially reversed by the treatment with amitriptyline. In addition, the general activation of the DMH by glutamate resulted in behavioural hyperalgesia only in SHAM animals with concomitant alteration in the activities of RVM On- and Off-like cells. Interestingly, a GAL-dependent DMH pronociceptive pathway, independent of RVM On- and Off-like cells, is not only functional in SHAM but also in ARTH animals, as the administration of GAL in the DMH decreased the paw-withdrawal latency of the animals, an effect that was mimicked by the infusion of a GAL receptor 1 agonist. The reversion of the pronociceptive effect of GAL in the DMH was observed when inhibiting the DRt with lidocaine or by blocking spinal 5HT3R, although the behavioural hyperalgesia elicited by DRt activation was not eliminated by blocking these spinal receptors. Importantly, not only the DRt-dependent but also the spinal 5HT3R-dependent pathways are tonically activated in ARTH animals. Finally, preliminary results showed that RVM Neutral-cells could be the brainstem mediators of the GAL-DMH nociceptive facilitatory pathway that acts through the activation of spinal 5HT3R.

In conclusion, GAL receptors in the DMH appear to play a key role in nociceptive facilitation during chronic arthritis, with these receptors having a potential role in the integration of emotional and nociceptive information. Further studies should evaluate the role of GAL in the DMH upon the development of anxiety and depression-like pathologies; and to clarify upstream nuclei that could be activating this pathway, such as the amygdala and the bed nucleus of the stria terminalis, for a better understanding of the mechanisms underlying chronic pain and associated emotional disorders.

Pronociceção em monoartrite experimental: o papel da galanina nas vias descendentes

Resumo

A dor aguda é um mecanismo protetor que desencadeia comportamento de escape no sentido de evitar danos tecidulares. No entanto, quando esta se torna constante e prolongada, como no caso da artrite, tem um enorme impacto na qualidade de vida. Além disso, a dor artrítica é geralmente acompanhada por outras patologias, como alterações emocinais (ansiedade e depressão), que são normalmente sub-avaliadas quando se trata pacientes com artrite. Embora os mecanismos responsáveis pelo desenvolvimento de ansiedade e depressão durante dor crónica não estejam clarificados, a existência de regiões encefálicas e neurotransmissores envolvidos no processamento e modulação da dor que estão também implicados na modulação do humor e das emoções é amplamente conhecida. Entre outros neurotransmissores, a galanina (GAL) parece ser um importante interveniente tanto na dor como nas emoções, sendo que a injecção de GAL nos ventrículos cerebrais promove um comportamentos do tipo depressivo e ativa a amigdala, uma area importante no processamento das emoções e da dor, e o núcleo dorsomedial do hipotálamo (DMH), uma região que está envolvida na resposta à ansiedade e na mediação de hiperalgesia comportamental (aumentar a nociceção). A ação pronocicetiva do DMH foi correlacionada com alterações na atividade das células-"On" e –"Off" do bolbo rostral ventromedial (RVM). O RVM é parte do principal sistema de inibição descendente da dor, embora já tenha sido demonstrado o seu papel na facilitação da nociceção. Para além do RVM, também o núcleo dorsal reticular (DRt), no tronco cerebral, está associado a pronociceção, contendo neurónios que respondem preferencialmente a estímulos nóxicos.

O modelo de monoartrite induzido por caolina e carragenina (K/C) foi usado para estudar o efeito de dor artrítica prolongada no joelho na nociceção, assim como no desenvolvimento de patologias do tipo emocional. Quatro semanas após a injeção de soro fisiológico (animais SHAM) ou K/C (animais ARTH) no joelho direito, os animais foram avaliados no teste *pressure application measurement* diretamente sobre o joelho injetado para confirmar o estado de dor artrítica. Os testes *open field* e *elevated plus maze* foram usados para avaliar a locomoção e o comportamentos do tipo ansioso; e os testes *sucrose preference* e *forced swimming* para avaliar o comportamento do tipo depressivo. No sentido de estudar um potencial efeito terapêutico da amitriptilina, um antidepressivo triciclico, na reversão dos comportamentos dolorosos e emocionais em animais ARTH, foi realizado um tratamento durante três semanas. A contribuição

dos recetores de GAL na hiperalgesia e pronociceção mediadas pelo DMH, em animais SHAM e ARTH, foi avaliada usando estimulação nóxica quente e gravações eletrofisiológicas da atividade neuronal do RVM e do corno dorsal da medula espinhal, enquanto se manipulou farmacologicamente o DMH com agonistas e antagonistas dos recetores de GAL. O papel do DRt como um potencial mediador encefálico e dos recetores de serotonina do tipo-3 (5HT3R) como potenciais mediadores espinhais do efeito pronocicetivo descendente da GAL no DMH foi avaliado através da administração de GAL no DMH com o bloqueio simultâneo do DRt e dos 5HT3R na medula espinhal, respetivamente, e medindo a resposta comportamental dos animas SHAM e ARTH no teste de *Hargreaves*.

Os resultados demonstram que a artrite prolongada no joelho induz dor e o desenvolvimento de sintomas de ansiedade e depressão que podem ser totalmente/parcialmente revertidos pelo tratamento com amitriptilina. Além disso, uma ativação geral do DMH com glutamato resulta em hiperalgesia comportamental apenas em animais SHAM com alterações concomitantes na atividade das células do tipo On e Off do RVM. Interessante foi o facto da via pronociceptiva do DMH dependente de GAL, que é independente das células do tipo On e Off do RVM, se encontrar funcional não só em animais SHAM mas também em animais ARTH, uma vez que a administração de GAL no DMH diminuiu a latência de flexão da pata dos animais, um efeito que foi mimetizado pela injecção de um agonista dos recetores de GAL do tipo-1. A reversão do efeito pronocicetivo da GAL no DMH foi observada quando se inibiu o DRt com lidocaina ou bloqueou os 5HT3R espinhais, embora a hiperalgesia comportamental induzida pela ativação do DRt não tenha sido eliminada pelo bloqueio destes recetores espinhais. Importante é o facto de, tanto a via dependente do DRt como a via dependente dos 5HT3R espinais se encontrarem tonicamente ativas nos animais ARTH. Finalmente, resultados preliminares mostram que as células Neutras do RVM podem ser os mediadores supraspinhais da via facilitadora induzida pela GAL no DMH que atua pela ativação dos 5HT3R na medula espinal.

Em conclusão, os recetores de GAL no DMH parecem ser importantes na facilitação da nociceção durante artrite crónica, com estes recetores a terem um potencial papel na integração da informação emocional e nocicetiva. Estudos adicionais devem avaliar o papel da GAL no DMH sobre o desenvolvimento de patologias do tipo ansioso e depressivo; e clarificar quais os núcleus a montante que podem estar a ativar esta via, como por exemplo a amygdala e o núcleo da estria terminal, para uma melhor compreensão dos mecanismos subjacentes à dor crónica e patologias emocionais associadas.

Abbreviation list

5HT	-	5-hydroxytryptamine/serotonin
5HT3R	-	serotonin receptor subtype-3
ACC	-	anterior cingulate cortex
AMY	-	amygdala
ARTH	-	arthritic group
CGRP	-	calcitonin gene-related peptide
DMH	-	dorsomedial nucleus of the hypothalamus
DNIC	-	diffuse noxious inhibitory controls
DRt	-	medullary dorsal reticular nucleus
EPM	-	elevated plus maze
FST	-	forced swimming test
GABA	-	gamma-aminobutyric acid
GAL	-	galanin
GALR	-	galanin receptor
GLU	-	glutamate
i.c.v	-	intracerebroventricular
IASP	-	International Association for the Study of Pain
K/C	-	kaolin/carrageenan
LC	-	locus coeruleus
LIDO	-	lidocaine
M40	-	non-selective galanin receptor antagonist
M617	-	galanin receptor subtype-1 agonist
MIA	-	monoiodoacetate
NA	-	noradrenaline
NK-1	-	neurokinin subtype-1 receptor
NMDA	-	N-methyl-D-aspartate
NRM	-	nucleus raphe magnus
NS	-	nociceptive-specific
OA	-	osteoarthritis

OF - open field

OND - ondansetron (serotonin receptor subtype-3 antagonist)

PAG - periaqueductal grey matter

PAM - pressure application measurement

PF - paw-flick

PNC - partial nociceptive convergence

PWL - paw-withdrawal latency

RVM - rostral ventromedial medulla

SHAM - sham-operated group

SNRIs - serotonin–norepinephrine reuptake inhibitors

SP - substance P

SPT - sucrose preference test

SSRIs - selective-serotonin reuptake inhibitors

TCAs - tricyclic antidepressants

TF - tail-flick

TNC - total nociceptive convergence

WDR - wide-dynamic-range

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Chapter 1

Introduction

1. INTRODUCTION

1.1. Evolution of the concept of pain

The notion that pain could not be categorized as a sensation equivalent to touch was postulated by Aristotle (384-322 BC) when defining the five senses (vision, hearing, smell, taste and touch). Pain, unlike the traditional senses, could not be associated to a specific organ, thus, it was categorized as an emotion and considered a "passion of the soul", the antithesis of pleasure that was experienced by the heart (Perl, 2011). However, Herophilus (335-280 BC) defended another theory and proposed the brain as the organ of consciousness and sensation and that peripheral nerves and the spinal cord were the route to transmit a sensation from the periphery to the brain (Perl, 2011).

Only several centuries later, Avicenna (980-1038 AD) recognized the nervous system as a source of consciousness and sensation and was able to expressly show that pain differed from touch arguing that it is possible to lose the sense of touch while keeping pain, and vice-versa (Perl, 2011). In the beginning of the 20th century, Sherrington (1857-1952) established that pain should not be considered a sense as it can be elicited by widely different stimuli (e.g. mechanical, chemical, thermal stimuli) in contrast to the specific stimulus activating each of the five senses (Sherrington, 1906; Perl, 2011). He coined the term *"noxious"* to describe a stimulus capable of eliciting pain and the terms *"nociception"* and *"nociceptor"* to describe the distinctive activity of specific afferents carrying it (Sherrington, 1906; Perl, 2011).

Early theories on pain were the "Specificity" and the "Sumation" or "Intensity" theories, reporting that distinctive afferents were responsible for pain and touch and that pain was the result of a growing amount of stimulation of the same fibres, respectively (Moayedi and Davis, 2013). Later, Melzack and Wall (Melzack and Wall, 1965) proposed the "Gate control" theory, suggesting a dynamic role of the central nervous system in pain processing, being able to inhibit, excite and modulate the transmission of nociceptive information (Moayedi and Davis, 2013). More recently the "Neuromatrix" or "Pain matrix" theory focus on the role of brain regions upon pain perception (Melzack, 1999; Tracey and Mantyh, 2007).

1.2. The current concept of pain processing

Pain is defined, by the International Association for the Study of Pain (IASP), as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Loeser and Treede, 2008), a definition that highlights the fact that pain perception is not necessarily proportional to the intensity of a stimulus or similar amongst different individuals.

Pain is the result of the supraspinal interpretation of the nociceptive inputs taking into account the emotional state, memories and expectations of each person. Thus, it is now accepted that a painful experience is multidimensional and comprises three distinct but complementary dimensions: the sensory-discriminative (intensity, location, quality, and duration), the motivational-affective (unpleasantness) and the cognitive-evaluative (appraisal, cultural values, context, and cognitive state) dimensions (Tracey and Mantyh, 2007; Moayedi and Davis, 2013).

1.2.1. Nociceptors

Nowadays it is well established that, when a noxious stimulus, either from mechanical, thermal or chemical nature, is applied at the periphery this information is transduced into an action potential and conveyed to spinal dorsal neurones by primary afferent fibres (nociceptors) (Millan, 1999; D'Mello and Dickenson, 2008; Basbaum et al., 2009) (**Fig.1**). Typically, a nociceptor has peripheral free nerve endings and central axonal branches that innervate both the target organ and the spinal cord (Millan, 1999; D'Mello and Dickenson, 2008; Basbaum et al., 2009), respectively.

There are two types of nociceptors, Aδ- and C-fibres. Aδ-fibres are medium-diameter cells and are thinly myelinated while C-fibres are characterised by their small-diameter cell body with unmyelinated axons. As nociceptive signals are transmitted faster by Aδ-fibres when compared with C-fibres, they have been associated with the "first" pain, a sharp, pricking and well localized sensation, while C-fibres are responsible for the "second" pain, which is delayed and more diffuse. Aδ-fibres transmit mainly high-threshold mechanical and thermal (heat) information and C-fibres are polymodal afferents, activated by high-threshold mechanical, thermal and chemical stimuli (Millan, 1999; Julius and Basbaum, 2001; Basbaum et al., 2009).

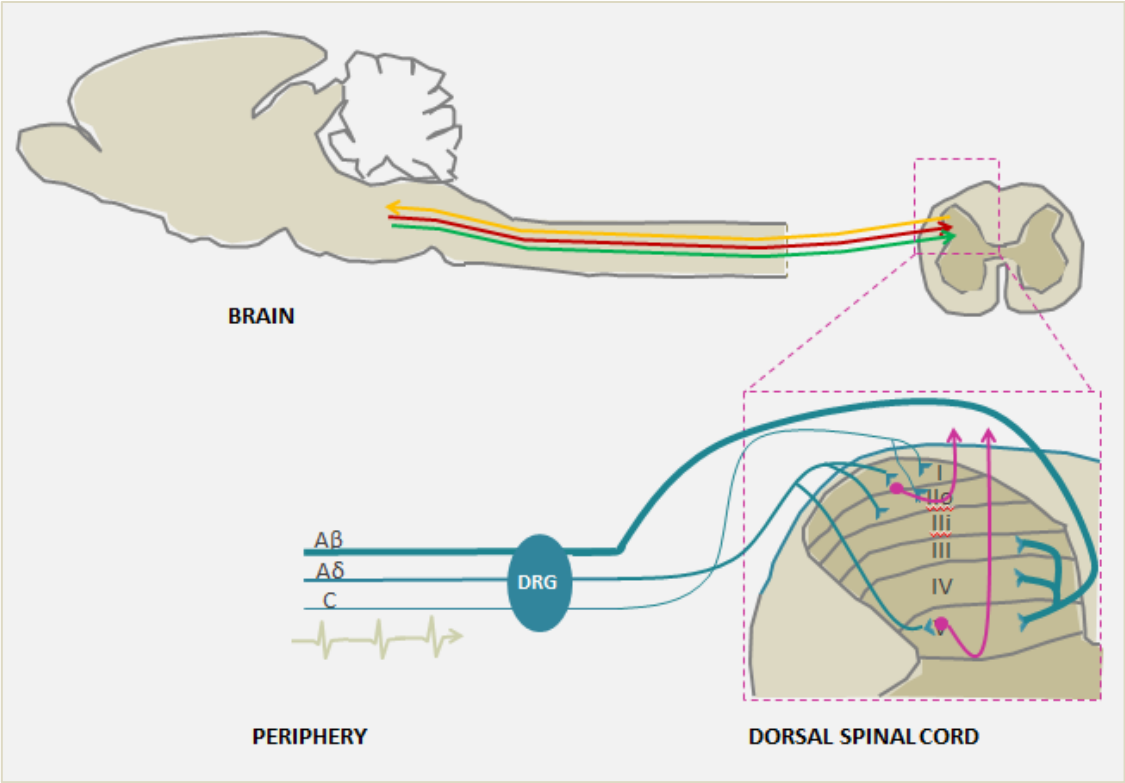


Figure 1. Schematic representation of pain pathways. A peripheral noxious stimulus (thermal, mechanical or chemical) applied to the body is converted into action potentials (transduction) via specialized primary afferent fibers (*blue lines*), called nociceptors. This nociceptive information is then conducted to the dorsal horn of the spinal cord via two types of primary afferent neurones, thinly myelinated, faster conducting Aδ and unmyelinated, slowly conducting C fibres, that synapse with second order neurones (*purple circles and lines*)(transmission to the central nervous system). The nociceptive information is then transmitted via ascending nociceptive pathways (*yellow line*) to different supraspinal regions, where it is processed taking into account the sensory-discriminative, motivational-affective and cognitive-evaluative dimensions of pain (perception). Descending pathways from supraspinal areas to the spinal dorsal horn modulate the nociceptive transmission at this level, acting either by promoting (*green line*) or supressing (*red line*) nociception (descending modulation).

In addition there is a third type of fibres, Aβ-fibres that are large diameter cells with thick myelinated axons allowing fast conduction of impulses. These fibres are involved in proprioception and tactile processing and do not contribute to the transmission of nociceptive information in normal conditions (Millan, 1999; Julius and Basbaum, 2001; Basbaum et al., 2009). However, after peripheral nerve injury or inflammation these fibres can transmit nociceptive inputs (Millan, 1999).

In the spinal cord and medulla oblongata, the nociceptive information is transmitted from primary afferents to neurones in the grey matter (second-order neurones) (Millan, 1999; D'Mello and

Dickenson, 2008; Basbaum et al., 2009; Todd, 2010) in an organized manner, where superficial laminae receive information from fine calibre C-fibres while deep laminae receive inputs mainly from larger diameter neurones. Aδ- and C-fibres synapse with neurones located mainly in the superficial dorsal horn [C-fibres: laminae I and II; Aδ-fibres: laminae I and V] (Millan, 1999; D'Mello and Dickenson, 2008; Basbaum et al., 2009; Todd, 2010).

1.2.2. Ascending pathways

Once the noxious information arrive to the dorsal spinal cord, these inputs are forwarded to the brain through three major pathways: (i) the spinothalamic tract, carries nociceptive information to the thalamus, which is subsequently distributed to several cortical structures involved in the sensory-discriminative components (lateral spinotalamic tract) and in the motivational-affective component (medial spinotalamic tract) of the pain experience; (ii) the spinoreticular tract, in which reticular regions project to the medial thalamus, which is then connected with brain areas relevant for the motivational and cognitive dimensions of pain, and; (iii) the spinomesencephalic tract that targets the periaqueductal grey matter (PAG), a major antinociceptive area (Basbaum et al., 2009). An additional pathway that seems specifically devoted to the emotional component of pain is the spino-parabrachial-amygdaloid pathway (Neugebauer et al., 2009).

The major outputs of the dorsal spinal cord originate in laminae I and V (Millan, 1999; D'Mello and Dickenson, 2008; Basbaum et al., 2009). Dorsal horn neurones that project to the brain can be classified as (i) wide-dynamic-range (WDR) neurones if they respond to innocuous and noxious stimuli and the response is proportional to the intensity of the stimuli; (ii) nociceptive-specific (NS) neurones if they respond exclusively to noxious stimuli and (iii) non-nociceptive neurones that respond only to tactile/innocuous stimulation (D'Mello and Dickenson, 2008; Loeser and Treede, 2008).

1.2.3. Descending modulation

After supraspinal processing, in light of several factors such as attention, context, emotions and mood, spinal transmission of nociceptive inputs are modulated by descending (from the brain to the spinal cord) pathways that either facilitate (pronociception) or inhibit (antinociception) the nociceptive transmission in the spinal dorsal horn (Millan, 1999; D'Mello and Dickenson, 2008).

Classically, the endogenous pain control system was considered to be mediated by reciprocal projections between the PAG, the rostral ventromedial medulla (RVM) and the spinal cord (Millan, 2002; D'Mello and Dickenson, 2008), although a large number of brain structures have been associated to descending modulation (Millan, 2002; Almeida et al., 2006).

In fact, PAG activation results in deep and intense analgesia, demonstrating that this midbrain area is able to inhibit nociceptive transmission (Mayer et al., 1971; Gebhart, 2004). The analgesic effect of the PAG is opioid-dependent (Basbaum and Fields, 1984; Gebhart, 2004; Mohammad-Zadeh et al., 2008) and spinal inhibition occurs through RVM neurones that project directly to dorsal horn neurones blocking nociceptive transmission (Heinricher et al., 2009). This endogenous pain control system can be activated by neurotransmitters that are released during specific environmental or physiological conditions in which it is essential to decrease pain.

Although the majority of studies on pain mechanisms are focused on pain inhibition there are some brain regions that were shown to facilitate pain (Porreca et al., 2002; Ren and Dubner, 2002) such as the anterior cingulate cortex (ACC) (Zhang et al., 2005a), amygdala (AMY) (Neugebauer et al., 2004), dorsomedial nucleus of the hypothalamus (DMH) (Martenson et al., 2009), RVM (Heinricher et al., 2009) and medullary dorsal reticular nucleus (DRt) (Lima and Almeida, 2002). In addition, some emotions such as anxiety and pain anticipation are also known to exacerbate pain (Hsieh et al., 1999; Ploghaus et al., 1999, 2000, 2001; Porro et al., 2002; Porro, 2003).

1.2.4. Acute pain versus chronic pain

Acute pain is part of our main defence/alert mechanism allowing us to perceive potentially dangerous (noxious) stimuli. In normal conditions, there is a dynamic balance between descending inhibitory and facilitatory influences (Kovelowski et al., 2000; Porreca et al., 2002; Bee and Dickenson, 2007). A protective imbalance towards anti- or pronociception usually occurs in order to cope with changes in the environmental and/or physiological conditions. For example, while antinociception allows a better performance in life threatening situations (enhancing the fight-or-flight response) during an illness pronociception will evoke protective behaviours towards the organism or an area affected (McNally, 1999; Heinricher et al., 2009).

When persistent nociceptive signalling imbalances the pain modulatory system, pronociceptive influences prevail over antinociceptive leading to the development of chronic pain syndromes. In

these conditions, the perception of a noxious stimulus can be enhanced (hyperalgesia) and, more importantly, innocuous stimuli can be perceived as painful (allodynia) or pain can be felt in the absence of any stimulation (resting/spontaneous pain) (Loeser and Treede, 2008). Thus, while acute pain is easily managed, chronic pain remains a challenge as classic analgesics are mostly inefficient in the treatment of this pathology.

1.3. Chronic arthritis

1.3.1. Epidemiology

Arthritis, or inflammation of the joints, causes a dramatic decline in the quality of life of patients and has a tremendous impact on the economy not only due to the substantial costs associated to its treatment that burden national health systems, but also due to indirect costs associated to disability leaves. About 20% of adults worldwide suffer from chronic arthritis (Helmick et al., 2008) and pain is the main cause for people to seek medical care.

Osteoarthritis (OA), the most common type of arthritis, affects 27 million people in the US, with the knee, hip and hand joints being the most affected (Johnson and Hunter, 2014). Moreover, the prevalence of OA in developing countries is expected to double by 2020, as it is highly associated to increased obesity and aging (Johnson and Hunter, 2014).

1.3.2. Peripheral sensitization

By definition, sensitization is the *"increased responsiveness of neurones to their normal input or recruitment of a response to normally subthreshold inputs"* (Loeser and Treede, 2008). Joints are innervated mainly by peptidergic neurones expressing substance P (SP) and calcitonin-gene related peptide (CGRP) (O'Brien et al., 1989; Iwasaki et al., 1995; Tamura et al., 1998). During inflammation of the joint the expression of SP increases (Buma et al., 2000), an event that has been associated to peripheral sensitization of mechanoreceptors (Heppelmann and Pawlak, 1997).

In inflammatory conditions, proinflammatory and proalgesic agents are released from the damage tissues, immune cells and also from the nociceptors at the periphery (Basbaum et al., 2009; Lee et al., 2013). These nociceptors express membrane receptors that recognize the

above mentioned agents and activate specific signalling pathways that, when persistently activated, induce plastic changes altering their response properties (Basbaum et al., 2009).

In the case of arthritis, peripheral sensitization results from increased responsiveness and decreased activation threshold of primary afferent neurones. Moreover, in arthritis, *"silent"* or *"sleeping"* nociceptors that are normally mechanoinsensitive, become responsive to movements of the joint (Schaible et al., 2011). Taken together, these changes lead to the development of mechanical allodynia that is often reported by patents while performing a normal joint movement.

1.3.3. Central sensitization

In pathological conditions, in addition to nociceptors, spinal neurones also increase their receptive fields, responsiveness to suprathreshold stimuli and decrease their response threshold. Persistent nociceptive signalling alters the neurochemistry of the spinal cord enhancing peripheral inputs to the dorsal horn, which in turn alter the normal function of local interneurones and descending modulatory pathways (Millan, 1999, 2002; D'Mello and Dickenson, 2008). Central sensitization is highly correlated with increased release of excitatory neurotransmitters, mainly SP, glutamate (GLU) and CGRP, in the spinal cord (Millan, 1999; D'Mello and Dickenson, 2008; Basbaum et al., 2009).

During joint inflammation, CGRP is released in the spinal cord by primary afferents and promotes the development as well as the maintenance of spinal hypersensitivity (Neugebauer et al., 1996). Similarly, GLU receptors are important players in both the initiation and maintenance of spinal sensitization (Dougherty and Willis, 1992). Some studies showed that spinal administration of N-methyl-D-aspartate (NMDA) receptor agonists induced thermal hyperalgesia and mechanical allodynia (Coderre and Melzack, 1992; Malmberg and Yaksh, 1993), two hallmarks of chronic pain.

In addition, SP is co-released with CGRP and GLU by primary afferent spinal endings in response to noxious stimulation, playing an important role in nociceptive transmission in normal and prolonged pain conditions where the activation of its NK-1 receptor promotes central sensitization (De Biasi and Rustioni, 1988; Tuchscherer and Seybold, 1989; Schaible et al., 1990; Ma and Woolf, 1995; Allen et al., 1997; Mantyh, 1997; Afrah et al., 2002; Khasabov et al., 2002).

Dorsal root neurones co-expressing galanin (GAL), SP and CGRP that synapse in dorsal horn neurones were also proposed to play a role in nociceptive transmission (Ch'ng et al., 1985; Bedecs et al., 1992; O'Donnell et al., 1999). Several studies have confirmed this hypothesis although GAL's effect varies, either promoting anti- or pronociception, depending on the dosage and the experimental condition (Kuraishi et al., 1991; Wiesenfeld-Hallin et al., 1993; Reeve et al., 2000). Low doses of spinal GAL were associated with nociceptive facilitation, enhancing the release of excitatory neurotransmitters from nerve terminals (Yue et al., 2011), through the activation of GALR2/R3, whereas high doses of GAL promote antinociception, an effect mediated by GALR1 in the spinal cord (Liu et al., 2001; Hua et al., 2004).

In addition, descending projections can also enhance nociceptive transmission through the release of SP and serotonin (5HT) in the spinal cord (Oyama et al., 1996; Millan, 2002; Porreca et al., 2002; Lu and Perl, 2007). Of special interest is the 5HT3 receptor (5HT3R) that has been shown to play a role in descending nociceptive facilitation (Ali et al., 1996; Millan, 2002; Rahman et al., 2009; Yanarates et al., 2010; Gu et al., 2011). These receptors are predominantly expressed in the spinal terminals of small diameter afferent fibres (Guo et al., 2014) where they act as a pre-synaptic modulatory mechanism of nociceptive transmission.

5HT3R contribution to nociceptive facilitation during chronic pain was demonstrated by the efficacy of ondansetron (OND), a 5HT3R antagonist, in inhibiting nociceptive-associated responses in animals and humans with chronic pain (McCleane et al., 2003), suggesting that a descending serotonergic facilitatory drive occurs in these circumstances. Thus, plastic changes during chronic pain states are not restricted the spinal cord but also result from changes in the brain.

1.4. Descending pronociception

Descending nociceptive facilitation is essential for both the development and maintenance of chronic pain. Many supraspinal regions have been demonstrated to facilitate nociception in normal and/or pathological conditions, namely caudal areas of the rat brain, such as the medullary DRt (Lima and Almeida, 2002) and RVM (Urban and Gebhart, 1999; Urban et al., 1999) and more frontal areas, such as the DMH (Martenson et al., 2009), ACC (Zhang et al., 2005a) and AMY (Li and Neugebauer, 2004) (**Fig. 2**).

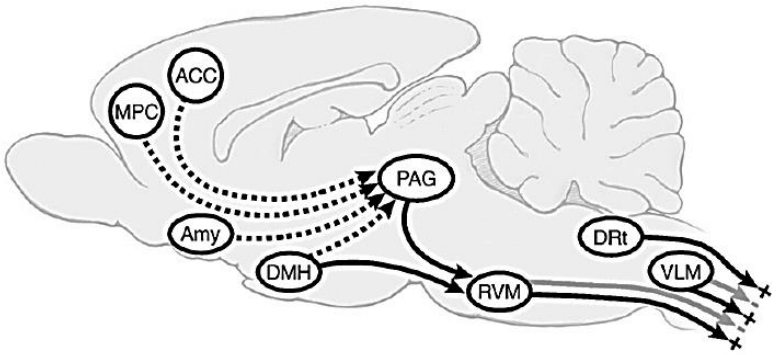


Figure 2. Schematic illustration of descending pain modulatory pathways in the rat brain. The rostral ventromedial medulla (RVM) receives inputs either directly or indirectly, through the midline periaqueductal grey matter (PAG), from limbic forebrain areas that participate in nociceptive processing, such as the medial prefrontal cortex (MPC), anterior cingulate cortex (ACC), amygdala (Amy) and dorsomedial nucleus of the hypothalamus (DMH), exerting its modulatory action over the dorsal horn of the spinal cord by facilitating or inhibiting nociceptive transmission. Other brainstem regions may also directly modulate nociceptive transmission in the dorsal spinal cord, namely the ventromedial medulla (VLM), an area that, as the RVM, exerts both inhibitory (*grey lines*) and facilitatory (*black lines*) influences, and the medullary dorsal reticular nucleus (DRt) that promotes nociception. (Heinricher et al., 2009).

1.4.1. Descending facilitation from caudal areas

1.4.1.1. Medullary dorsal reticular nucleus (DRt)

The DRt is located laterally in the caudal brainstem and is reciprocally connected with the spinal dorsal horn, where it modulates nociceptive transmission from the periphery to the central nervous system (Lima and Almeida, 2002). In the brain, the DRt projects to several areas implicated in pain modulation, such as the PAG, RVM, caudal ventrolateral medulla, nucleus tractus solitaries, AMY and medial thalamus, namely to the parafascicular and ventromedial thalamic nuclei (Leite-Almeida et al., 2006). Its projections to several areas of the medial thalamus, AMY and bed nucleus of the stria terminalis implicate the DRt in the processing of the motivational-affective component of pain.

Studies focused on the role of the DRt in pain modulation, revealed that neurones in this region respond exclusively or mostly to peripheral noxious stimulation transmitted by Aδ- and C-fibres (Villanueva et al., 1988, 1989; Bing et al., 1989). Moreover, electrophysiological studies showed the existence of two types of cells in the DRt in accordance with their receptive field: (i) neurones with total nociceptive convergence (TNC) and (ii) neurones with partial nociceptive convergence (PNC). TNC are present in larger numbers (84% of DRt cells) and do not exhibit spontaneous

activity or respond to innocuous peripheral stimulation. By contrast, PNC neurones do not show spontaneous activity but respond to noxious and innocuous stimulation (Villanueva et al., 1989).

In terms of nociceptive behaviour, DRt stimulation induces hyperalgesia in acute pain (Almeida et al., 1996), while its lesion has an analgesic effect in both acute (Almeida et al., 1996) and inflammatory pain (Almeida et al., 1999). Similarly, the number of c-Fof spinal cord cells activated by peripheral noxious stimulation decreased after lesioning the DRt (Almeida et al., 1999). Additionally, DRt stimulation by GLU increases post-discharge activity of spinal WDR neurones to noxious electrical stimulation (Dugast et al., 2003).

The spino-DRt-spinal loop seems to be an excitatory descending drive (Almeida et al., 1993, 2000) acting upon dorsal horn WDR neurons by enhancing their capacity to respond to peripheral stimulation through prolongation of their discharge (Dugast et al., 2003). The DRt pronociceptive effect is controlled by incoming opoidergic and gamma-aminobutyric acid (GABA) inhibition as μ-opioid and GABAB receptors are co-expressed in DRt neurones (Pinto et al., 2008). Martins and colleagues (Martins et al., 2008) demonstrated that opioidergic inhibition of the DRt can supress its pronociceptive effect and that the activation of α1-adrenoreceptors in the DRt leads to an increase of neuropathic pain-related behaviours (Martins et al., 2010).

Besides spinal WDR wind-up [or prolonged increase in neuronal excitability induced by low-frequency repetitive stimulation of unmyelinated afferents (C-fibres)], DRt neurones also show wind-up following electrical stimulation of the spinal cord or the nucleus reticularis gigantocellularis, an effect partially dependent on persistent sodium currents and under a tonic GABAA-mediated inhibitory control (Soto et al., 2008; Soto and Canedo, 2011). Additionally, an integrative role of the DRt in the mediation of the descending nociceptive facilitation from the ACC was suggested, since the electrolytic lesion of the DRt block the ACC-induced facilitation of spinal nociception (Zhang et al., 2005a).

Up-to-now, only a few studies were performed to clarify the role of the DRt in chronic pain conditions. However, it has been shown that during both chronic neuropathic (Mao et al., 1993) and inflammatory pain (Neto et al., 1999), the DRt is strongly activated. More recently, Sotgiu and co-workers (Sotgiu et al., 2008) demonstrated that the blockage of the DRt of neuropathic animals resulted in decreased spontaneous and evoked activities (induced by mechanical stimulation of the skin supplied by the sciatic nerve) of spinal WDR neurones, suggesting a facilitatory role of the DRt in the maintenance of neuronal sensitization in this pathology.

In parallel to its role in descending facilitation of nociception triggered from noxious stimulation applied to an area of the body (homotopic stimulation), the DRt is also able to inhibit nociception but from heterotopic sites (outside the receptive field of neurones processing homotopic stimulation), a phenomenon called Diffuse Noxious Inhibitory Controls (DNIC) (Bouhassira et al., 1992; Lima and Almeida, 2002). Indeed, DNIC is not only an inhibitory phenomenon, since the depression of background activity results in further reinforcement (facilitation) of the nociceptive message being applied to a specific part of the body.

1.4.1.2. Rostral ventromedial medulla (RVM)

The RVM is a brainstem structure that includes the nucleus raphe magnus (NRM), nucleus raphe pallidus, nucleus raphe obscurus, and adjacent nuclei of the reticular formation, such as the nucleus reticularis gigantocellularis and the nucleus reticularis gigantocellularis pars α. The RVM modulates spinal nociceptive transmission through projections to the dorsal horn of the spinal cord (Fields et al., 1995).

Ascending spinal tracts that target brainstem neurones are essential for integration of the nociceptive information and the activation of autonomic and homeostatic processes (Thurston-Stanfield et al., 1999; Lima et al., 2002). Moreover, the RVM connects with areas involved in pain processing, namely the PAG, DRt, parabrachial nucleus, locus coeruleus (LC), hypothalamus, AMY, prefrontal cortex (Murphy et al., 1999; Odeh and Antal, 2001; Leite-Almeida et al., 2006; Verner et al., 2008).

The RVM was initially associated only to descending inhibition (Basbaum and Fields, 1984), however, behavioural and electrophysiological studies showed that RVM also facilitated behavioural nociception (Zhuo and Gebhart, 1990a, 1990b, 1991, 1997, 2002a, 2002b; Cervero et al., 1991; Urban and Gebhart, 1999). RVM-mediated descending pain modulation is mediated by three electrophysiologically different classes of cells (i) On- (pronociceptive), (ii) Off- (antinociceptive) and (iii) Neutral-cells, which have been widely studied in pain research in the last three decades (Fields, 1983; Heinricher et al., 1989, 2009). Electrophysiological recordings of RVM cells demonstrated that when a noxious stimulus is applied at the periphery On-cells increase their activity immediately before a behavioural withdrawal response is observed, while RVM Off-cells decrease their activity at the same time. Initially, the third type of RVM cells was designated as Neutral-cells as they do not alter their activity during peripheral noxious stimulation

(Fields et al, 1983). Later works showed that these cells respond to nociceptive stimulation during prolonged inflammatory disorders (Heinricher et al., 1989).

Interestingly, although pathways descending from the raphe nuclei are serotonergic, 5HT expression was found only in RVM Neutral-cells and not in On- or Off-cells (Potrebic et al., 1994). Although the neurotransmitters released by On- and Off-cells remain unknown, several neurotransmitters are co-expressed in 5HT-expressing RVM neurones including the GABA, glycine, acetylcholine, somatostatin, SP, enkephalin, dynorphin, GAL, thyrotropin-releasing hormone, and cholecystokinin (Millan, 2002).

The NRM, a RVM region mainly expressing 5HT, seems to be a key player in this RVM facilitatory role, since NRM activation by GLU results in nociceptive facilitation (Porreca et al., 2002) and the blockage of GLU receptors in this region reverses hyperalgesia observed during inflammation (Urban and Gebhart, 1999). Moreover, increased NMDA receptors expression has been reported in the NRM after inflammation (Miki et al., 2002).

More recently, it was shown that the RVM antinociceptive or pronociceptive effect in chronic conditions depended on the source of the input activating this nucleus (Urban et al., 1999; Vanegas and Schaible, 2004). When the RVM was activated as a result of primary hyperalgesia (increased sensitivity within the injured area) antinociceptive pathways were evoked while secondary hyperalgesia hyperalgesia (increased sensitivity outside/surrounding the injured area) evoked RVM-mediated facilitation (Urban et al., 1999; Vanegas and Schaible, 2004).

1.4.2. Descending facilitation from frontal and medial brain areas

While brainstem projections directly mediate nociceptive transmission in the spinal cord, its activity is in turn modulated by frontal and medial brain areas, such as the DMH, ACC and AMY, also play an important role in pain modulation, namely in its affective an cognitive dimentions. In fact, several areas of the limbic system, such as the AMY and the prefrontal cortex that mediate the affective component of pain, or the hypothalamus (such as the paraventricular, arcuate and dorsomedial nuclei) that mediate the autonomic response to pain, exert a top-down modulation of brainstem activity and constitute the more frontal part of the pain modulatory system (Weisenberg et al., 1996; Rainville et al., 1997; Willoch et al., 2000; Longe et al., 2001; Bantick et al., 2002; Petrovic and Ingvar, 2002).

1.4.2.1. Dorsomedial hypothalamic nucleus (DMH)

The DMH is located in the medial hypothalamus, caudally to the paraventricular and dorsal to the ventromedial nuclei of the hypothalamus (Paxinos and Watson, 2007). The DMH shares extensive reciprocal projections within the hypothalamus, with the paraventricular nucleus and nuclei of the preoptic and periventricular regions. In addition, the DMH also presents reciprocal connections with several telencephalic areas, such as the septal region, hippocampus, and AMY; and brainstem regions, such as the PAG, parabrachial nucleus, ventrolateral medulla and DRt (Thompson et al., 1996; Martins et al., 2008).

The DMH mediates autonomic, endocrine, and behavioural changes in response to stress and anxiety in both anesthetized (DiMicco and Monroe, 1998) and wake behaving animals (Stotz-Potter et al., 1996). Although the DMH is strongly connected with nuclei involved in pain modulation, such as the raphe magnus nucleus (Samuels et al., 2004; Sarkar et al., 2007) in the RVM (Heinricher et al., 2009; Ossipov et al., 2010), its role in descending pain modulation is still poorly understood.

Like the RVM, the DMH has been reported to either inhibit (innate fear-induced antinociception; (Freitas et al., 2009, 2013; Biagioni et al., 2012, 2013) or facilitate pain (Martenson et al., 2009) pain; in the latter, DMH disinhibition with the GABAA receptor antagonist bicuculline induced behavioural hyperalgesia. This effect was mediated by the RVM, where increased activity of pronociceptive RVM On-cells and suppression of the activity of antinociceptive RVM Off-cells, was observed in control animals (Martenson et al., 2009). More recently, the same group showed that DMH neurones projecting to the RVM expressed the pronociceptive peptide cholecystokinin, confirming the DMH as a mediator of behavioural hyperalgesia (Wagner et al., 2013).

1.4.2.2. Anterior cingulate cortex (ACC)

The ACC is part of the cingulate cortex, a prefrontal region that surrounds the frontal part of the corpus callosum. Based on functional evidences, the ACC was divided in the cognitive component (in the dorsal part), mainly connected to other regions of the prefrontal cortex and parietal cortex, and the emotional component (ventral) connected with the AMY, nucleus accumbens, hypothalamus and anterior insula.

The implication of the ACC in pain modulation has been reported in both humans and rodents (Casey et al., 1994; Pastoriza et al., 1996; Rainville et al., 1997; Petrovic et al., 2000; Bantick et al., 2002; Zhang et al., 2005a). Human studies revealed that during moderate to intense noxious peripheral stimulation, the activity of the ACC is enhanced (Casey et al., 1994) highlighting a possible role of this area in pain modulation. Additionally, ACC neuronal activity was associated with the perception of the unpleasantness of a pain stimulus (Rainville et al., 1997).

ACC activity is modulated by attention as cognitive distractions attenuated pain and pain-evoked activity of this area amongst others (Petrovic et al., 2000; Bantick et al., 2002). Animal studies are in line with human studies showing that ACC lesions reduce behavioural nociceptive responses in rats (Pastoriza et al., 1996) and the electrical stimulation or activation of ACC metabotropic GLU receptors facilitates nociception (Calejesan et al., 2000). This indicates that the ACC enhances pain perception, an effect exerted through descending pathways targeting the RVM (Calejesan et al., 2000) and the DRt (Zhang et al., 2005a).

1.4.2.3. Amygdala (AMY)

The AMY is a group of several nuclei located deep in the temporal lobes of the brain and closely connected with the hypothalamus, cingulate cortex and hippocampus. It is a key area within the limbic system and plays a critical role in the modulation of pain-related negative emotional effects (Neugebauer et al., 2009).

The AMY receives nociceptive signals from the spino-parabrachial-amygdaloid pathway targeting the latero-capsular part of the central AMY (Jasmin et al., 1997; Neugebauer et al., 2009), while in parallel sensory information (including nociceptive input) is conveyed through the basolateral part of the AMY from thalamic and cortical areas (Phelps and LeDoux, 2005; Neugebauer et al., 2009).

Human studies showed that the AMY becomes increasingly active during peripheral noxious stimulation (Bornhövd et al., 2002), suggesting that it is involved in the forebrain pain modulatory system. In animals models of peripheral inflammation, ERK expression analysis confirmed increased AMY activity in these disorders (Carrasquillo and Gereau, 2007).

Interestingly, although a role of the AMY on pain is well accepted, a dual effect has been reported, with some studies showing antinociception (Manning and Mayer, 1995; Helmstetter et

al., 1998; McGaraughty and Heinricher, 2002; Nandigama and Borszcz, 2003) while others showing pronociception (Greenwood-Van Meerveld et al., 2001; Qin et al., 2003; Viisanen and Pertovaara, 2007; Ansah et al., 2009). The pronociceptive effect of the AMY was correlated with the activation of metabotropic GLU receptors subtype-1/5, since the administration of an agonist or GLU, increased the discharge rate of presumably pronociceptive On-cells in the RVM and suppressed presumably antinociceptive neurones in the LC, respectively, of nerve-injured but not healthy animals (Viisanen and Pertovaara, 2007; Ansah et al., 2009). In an animal model of arthritic pain, the activation of both NMDA and non-NMDA receptors in the AMY contributed to pain-related sensitization of AMY neurones (Li and Neugebauer, 2004) and the activation of the extracellular signal-regulated kinase in the AMY was suggested to contribute to inflammatory hypersensitivity (Carrasquillo and Gereau, 2007).

1.5. Chronic pain and comorbid emotional disorders

Chronic pain, including arthritis, is often accompanied by the development of anxiety and/or depression that are usually under-evaluated and are not taken into account when programming pain control therapies for chronic pain patients (Bair et al., 2003). A study using an USA cohort of arthritic patients showed that 30.5% of the patients report comorbid anxiety, 17.5% comorbid depression and 14.7% both (Murphy et al., 2012).

Moreover, a recent study demonstrated that patients with pain in multiple locations, particularly involving the joints, have increased risk to develop depression or anxiety in the future (Gerrits et al., 2014). Hence, when treating arthritic pain patients, clinicians should also evaluate the existence of emotional comorbidities and provide treatment.

Although the mechanisms underlying the comorbid development of anxiety and depression during chronic pain are not yet understood, it is clear that brain regions and neurotransmitter pathways involved in pain processing and modulation are also involved in the modulation of mood and emotions (Robinson et al., 2009). In fact, the development of chronic pain and mood disorders interact with each other reciprocally (Liu and Chen, 2014).

Osteoarthritic pain patients show a decrease in grey matter in the ACC, right insular cortex and operculum, dorsolateral prefrontal cortex, AMY and brainstem, an effect that was partially reversed after pain was managed (Rodriguez-Raecke et al., 2009). In an animal model of

arthritis, mechanical stimulation of the inflamed knee joint increased firing rate of AMY neurones (Neugebauer and Li, 2003).

1.5.1. Supraspinal neurotransmitters involved in pain and emotions

Depression, anxiety and pain are complex diseases involving not only changes in the neuroplasticity of modulatory circuits but also alterations in neurotransmitter content, namely 5HT, NA and GAL (Bao et al., 2012; Kormos and Gaszner, 2013) (**Fig. 3**).

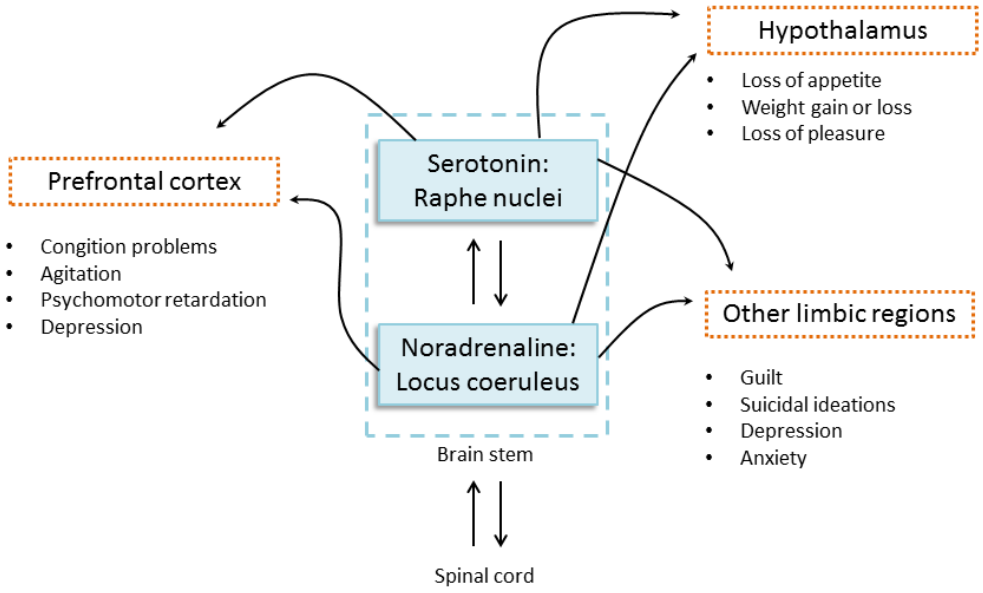


Figure 3. Serotonin and noradrenaline pathways associated with emotional disorders and pain. Interactions between the brainstem and other higher brain areas and their clinical manifestations. [Modified from (Jann and Slade, 2007)].

1.5.1.1. Serotonin (5-hydroxytryptamine; 5HT)

Serotonin or 5-hydroxytryptamine, first described in 1948 (Rapport et al., 1948) is synthetized from the conversion of tryptophan by two enzymes, the tryptophan hydroxylase and amino acid decarboxylase (Mohammad-Zadeh et al., 2008). 5HT is present in the gastrointestinal tract (Mohammad-Zadeh et al., 2008) and, since it cannot cross the blood-brain barrier, all brain 5HT is produced *in situ*.

In the brain, the 5HT is mainly expressed in raphe nuclei and changes in serotonergic transmission are widely associated to both chronic pain and emotional disorders (Robinson et al., 2009). In fact, alteration in 5HT content in certain brain regions, such as the dorsal raphe nucleus (DRN) and the NRM (in the RVM) are associated with the development of pain and emotional pathologies (Harro and Oreland, 2001).

A key role for 5HT in depression was shown by decreasing the ingestion of the 5HT precursor, tryptophan, causing the relapse of depressed individuals, an effect that was reversed after treatment with a specific-serotonin reuptake inhibitor (Delgado et al., 1999). Moreover, a single nucleotide polymorphism in the tryptophan hydroxylase-2 (tph2) gene, which results in about 80% loss of 5-HT synthesis (*in vitro*) was identified in a subset of depressed patients (Zhang et al., 2005b). Interestingly, the same genetic alteration in a mouse model induced depression-like behaviour in these animals (Jacobsen et al., 2012). Indirect evidences also confirmed the role of the 5HT in depression since blocking its reuptake through membrane transporters and thus increasing the extracellular levels of 5HT, decreased depressive symptoms in patients and animal models alike (Verdu et al., 2008; Saito et al., 2014).

Important, but not surprising, is the fact that antidepressants are used to treat chronic pain (Verdu et al., 2008), since alterations in 5HT-mediated pathways were reported during chronic pain and depression (Robinson et al., 2009).

1.5.1.2. Noradrenaline (NA)

NA is produced at the periphery by adrenal glands acting as a hormone of the autonomic nervous system. In the central nervous system, NA is synthetized mainly by neurones located in the brainstem, where it acts as a neurotransmitter. NA is synthesized from dopamine by the dopamine β-hydroxylase and exerts its action through adrenergic receptors.

A role for NA-mediated pathways in the modulation of pain and emotions is widely accepted with the LC being a key brain region of NA synthesis and descending modulation (Pertovaara, 2006; Itoi and Sugimoto, 2010). In parallel, the dysfunction of LC NA neurones has been postulated as a mechanism underlying depression (Ressler and Nemeroff, 1999; Harro and Oreland, 2001). In fact, most antidepressant drugs act by blocking the re-uptake of NA from the synaptic cleft (Hamon and Blier, 2013). Relapse symptoms in depressed individuals induced by blocking the

synthesis of catecholamines were reversed after treatment with a specific-noradrenaline reuptake inhibitor (Miller et al., 1996).

As for 5HT, NA is mainly associated with nociceptive inhibition (Jones, 1991; Millan, 2002; Pertovaara, 2006), however, it became clear that, during prolonged pain, NA may also facilitate nociceptive transmission (Jeong and Holden, 2009). Damage of LC NA neurones in animals with inflammatory pain prevented the development of hyperalgesia, a syndrome usually observed in these animals (Tsuruoka and Willis, 1996). Recently, using optogenetic tools Hickey and colleagues (Hickey et al., 2014) described two distinct population of NA neurones in the LC, one that supress and another that facilitates thermal nociception, suggesting a bidirectional influence of the LC upon nociceptive transmission.

1.5.1.3. Galanin (GAL)

GAL has also been studied in the context of both depression and pain (Mitsukawa et al., 2008; Kormos and Gaszner, 2013). This neurotransmitter is a 29 or 30 amino acid peptide whose name derives from the existence of a glycine in its N-terminal and an alanine in its C-terminal (Tatemoto et al., 1983; Webling et al., 2012). The biological activity of GAL is highly dependent on the N-terminal sequence, being the first 14 amino acids conserved in different species (Langel and Bartfai, 1998; Webling et al., 2012), allowing the establishment of the structural conformations needed to interact with its receptors.

To date, three GAL receptor (GALR) subtypes were described, GALR1, GALR2 and GALR3 (Fathi et al., 1997; Wang et al., 1997; Webling et al., 2012). All these receptors are G-protein coupled receptors, with GALR1 and GAL3 activating mainly inhibitory while GALR2 activating mainly excitatory intracellular transduction pathways (Wang et al., 1998; Lang et al., 2007).

Both GAL and GALRs display a broad distribution in the central and peripheral nervous system of the rat (Waters and Krause, 2000; Jacobowitz et al., 2004) suggesting a variety of biological functions of this peptide (Lang et al., 2007). In the brain, there are some differences in the expressing pattern of GALR1 and GALR2 subtypes. GALR1 are present within discrete regions of the hippocampus, AMY, thalamus, and LC while GALR2 are predominantly expressed in the dentate gyrus, substantia nigra, ventral tegmental area and dorsal raphe (Waters and Krause, 2000; Jacobowitz et al., 2004). In the hypothalamus the presence of all the three GALR subtypes has been reported, namely in the arcuate, paraventricular, ventromedial and dorsomedial

hypothalamic nuclei (Mitchell et al., 1997; Waters and Krause, 2000; Mennicken et al., 2002; Jacobowitz et al., 2004).

The role of supraspinal GAL in pain modulation is less explored than its action controlling nociceptive transmission at the spinal cord level. In the brain, the antinociceptive effect of GAL has been widely demonstrated as its direct infusion in the PAG, arcuate and tuberomamillar nuclei, nucleus accumbens and central AMY or its administration in the brain ventricles decreased nociception, in both acute and persistent pain conditions (Wang et al., 1999; Imbe et al., 2004; Sun et al., 2004; Gu et al., 2007; Jin et al., 2010; Li et al., 2012; Xu et al., 2012). Most of the reports correlate the supraspinal GAL antinociceptive action with the activation of GALR1 (Sun et al., 2004; Fu et al., 2011; Li et al., 2012; Kong and Yu, 2013).

The association of GAL in depression was established two decades ago when it was demonstrated its co-expression with NA in the LC, and 5HT in the dorsal raphe nucleus (DRN) and AMY (Skofitsch and Jacobowitz, 1985; Jacobowitz et al., 2004), three of the main brain regions known to promote depression in humans and rats (Lin et al., 2011; Hamon and Blier, 2013; Willner et al., 2013). In these regions, GAL exerts a tonic inhibitory effect decreasing the firing rate of NA and 5HT neurones (Seutin et al., 1989; Sevcik et al., 1993; Pieribone et al., 1995; Xu et al., 1998).

Behavioural data allowed the association of GAL to the development of depressive-like behaviour in rats since its administration in the ventral tegmental area, a region greatly involved in the dopaminergic system, resulted in increased immobility time in the forced swimming test (FST) (Weiss et al., 1998), an effect that was also observed when GAL was infused into the brain ventricles (Kuteeva et al., 2008). Moreover, using the same behavioural test, the intracerebroventricular (i.c.v) administration of a GALR1 agonist (M617) induced a pro-depressive effect, which contrasts with the anti-depressive action exerted by the GALR2 agonist (Kuteeva et al., 2008). Accordingly, an increase in GAL expression and GALR2 binding site density was induced by antidepressant drugs (Lu et al., 2005). More recently, polymorphisms in GAL gene were observed in the genome of depressed patients (Unschuld et al., 2010; Wray et al., 2012).

1.5.1.4. Antidepressants in the treatment of chronic pain

Conventional analgesics, such as the non-steroidal anti-inflammatory drugs, are not always effective in chronic pain with antidepressants being good therapeutic alternatives in the management of chronic pain (Saito et al., 2014). In fact, as described above, most of antidepressants target 5HT and/or NA pathways that are also involved in the pathophysiology of pain. However, the analgesic effect of antidepressants appears to have distinct mechanisms from the ones underlying their antidepressant action, since the dosages effective in the treatment of chronic pain are usually lower than the required to treat depression (Mika et al., 2013). Moreover, the time needed to achieve the analgesic effect is often shorter than for antidepressant therapy (Mika et al., 2013).

In general, antidepressants that target both 5HT and NA have a greater analgesic effect than antidepressants targeting each of the neurotransmitter system alone, being tricyclic antidepressants (TCAs) the first-line choice to treat chronic pain, with benefits in the treatment of comorbid mood or anxiety disorders (Katzman et al., 2014). TCAs, such as amitriptyline, imipramine and clomipramine, acts blocking the reuptake of 5HT and NA, with the 5HT reuptake inhibition being exerted by the compounds themselves, whereas the NA reuptake inhibition results from the action of their respective metabolites, nortriptyline, desipramine and desmethylclomipramine (Mika et al., 2013). Besides the effect of TCAs on 5HT and NA reuptake inhibition, they can also interact with adrenergic, cholinergic and sodium channels, which could explain their wide-ranging side-effects. In order to avoid some of these effects of the TCAs, selective-serotonin and noradrenaline reuptake inhibitors (SNRIs), such as duloxetine, venlafaxine and milnacipran, have been studied (Mika et al., 2013). Also selective-serotonin reuptake inhibitors (SSRIs), such as fluoxetine and sertraline, have been evaluated, showing advantages regarding side-effects, however, their efficacy on the treatment of chronic pain is considerably lower (33%) when compared to SNRIs and TCAs (80%) (Micó et al., 2006).

1.6. Aims

A reciprocal potentiation between pain and emotional pathologies has been proposed in the last decades, nonetheless the specific circuits and neurochemical mediators underlying this interaction are still mostly unclear. In this context, the DMH, a hypothalamic region that plays a role in the processing of emotions, has recently been proposed to enhance nociception although its potential contribution towards chronic pain as yet to be studied. Identically, although GAL plays an important role in counteracting nociceptive transmission in the spinal cord in acute and chronic inflammatory disorders, very little is known about its role on the supraspinal modulation of pain. In this thesis we focus on the DMH as a potential player in the nociceptive facilitation observed during prolonged arthritis as well as on its contribution to the development of comorbid emotional-like disorders in rats. More specifically, we aimed at:

(i) Evaluating the influence of monoarthritis of the knee upon the development of comorbid anxiety- and depression-like behaviours in rodents and the ability of amitriptyline, a tricyclic antidepressant, to reverse nociceptive and emotional-related impairments (**Chapter 2.1**);

(ii) Studying the role of the RVM as a brainstem mediator of the DMH descending pronociceptive effect (**Chapter 2.2**);

(iii) Assessing the effect of GAL and GALRs in the DMH upon nociceptive behaviour and the activity of pain modulatory RVM cells in healthy and monoarthritic animals (**Chapter 2.3**);

(iv) Analysing the role of the DRt as a brainstem mediator of the descending pronociceptive effect induced by GAL in the DMH in healthy and monoarthritic animals (**Chapter 2.4**);

(v) Analysing the role of 5HT3Rs as spinal targets of the descending pronociceptive effect induced by GAL in the DMH in healthy and monoarthritic animals (**Chapter 2.4**);

(vi) Evaluating the contribution of the RVM towards behaviour hyperalgesia induced by GAL in the DMH in healthy and monoarthritic animals (**Chapter 2.5**).

1.7. References

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Chapter 2

Experimental Work

Chapter 2.1

Amorim D, David-Pereira A, Pertovaara A, Almeida A, Pinto-Ribeiro F

Amitriptyline reverses hyperalgesia and improves associated mood-like disorders in a model of experimental monoarthritis

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Research report

Amitriptyline reverses hyperalgesia and improves associated mood-like disorders in a model of experimental monoarthritis

D. Amorim [a,b], A. David-Pereira [a,b], A. Pertovaara [c], A. Almeida [a,b], F. Pinto-Ribeiro [a,b,*]

[a] Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), Campus of Gualtar, University of Minho, 4710-057 Braga, Portugal
[b] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[c] Institute of Biomedicine/Physiology, University of Helsinki, Helsinki, Finland

HIGHLIGHTS

- Mood disorders in monoarthritic rats mimic those in osteoarthritic patients.
- Monoarthritic rodents display mechanical hyperalgesia and mood-like disorders.
- Amitriptyline reverses mechanical hyperalgesia in monoarthritic animals.
- Amitriptyline partly reverses depressive-like behaviour in monoarthritic animals.

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ABSTRACT

Affective disorders are common comorbidities of chronic inflammatory pain that are often overlooked in primary care. As the impact of inflammatory pain upon mood-like disorders in animal models is not well known, our objective was to assess whether prolonged experimental monoarthritis (ARTH) induced the development of anxiety and depressive-like behaviours in rodents and if amitriptyline, an antidepressant commonly used in the treatment of chronic pain, could reverse both nociceptive and mood-like impairments. Experimental ARTH was induced through an injection of kaolin/carrageenan into the right knee joint with control (SHAM) animals injected with saline. Four weeks after induction, ARTH animals displayed mechanical hyperalgesia and a depressive-like phenotype as they showed a significant increase in immobility and a decrease in the latency to immobility in the forced-swimming test at the expense of the time spent climbing/swimming. ARTH animals also displayed a decreased sucrose preference, an index of anhedonia and anxiety-like behaviour as time spent exploring the open arms of the elevated-plus-maze was decreased when compared to controls. The anxiety-like phenotype was also supported by an increase in the number of fecal boli left in the open field. In ARTH animals, the administration of amitriptyline decreased mechanical hyperalgesia and increased sucrose preference and the time spent climbing, although it had a deleterious effect in the performance of control animals. Our data show that this model of ARTH can be useful for the study of chronic pain-mood disorders comorbidities and that amitriptyline is able to partly reverse the associated nociceptive and emotional impairments.

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1. Introduction

Joint pain is a condition that affects millions of people, especially the elderly, representing a huge burden on National Health Programs [1]. Pain is the most common complaint in arthritis but often patients exhibit clinical signs of comorbid depression and anxiety [2] that frequently go untreated [3].

Curiously, although many animal models of chronic inflammatory pain are available and extensively used, only a few studies addressed inflammatory pain and mood disorders comorbidity [4–7]. However, the animal models and time points chosen to perform the behavioural analysis are different among studies. For example, the time frame of osteoarthritis (OA) settlement is one feature of the behavioural experimental designs that could by itself limit the full development of neuropsychological impairments

Abbreviations: AMI, amitriptyline hydrocloride; ARTH, K/C induced monoarthritis; EPM, elevated plus maze; FC, femoral condyle; FP, femoral plateaux; FST, forced swimming test; gf, gram force; K/C, kaolin/carrageenan; LWT, limb withdrawal threshold; M, meniscus; OA, osteoarthritis; OF, open field test; PAM, pressure application measurement; PL, patellar ligament; SAL, saline; SCB, subchondral bone; SHAM, control animals; SPT, sucrose preference test; TCA, tricyclic antidepressant; TP, tibial plateau.

* Corresponding author at: Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal. Tel.: +351 253604852; fax: +351 253604809.
E-mail addresses: dianaamorim@ecsaude.uminho.pt (D. Amorim), anapereira@ecsaude.uminho.pt (A. David-Pereira), antti.pertovaara@helsinki.fi (A. Pertovaara), aalmeida@ecsaude.uminho.pt (A. Almeida), filiparibeiro@ecsaude.uminho.pt (F. Pinto-Ribeiro).

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(anxiety- and depressive-like behaviour) in a slow developing disease such as OA. Accordingly, recent data obtained from studies on chronic neuropathy [8] demonstrated that the experimental time frame adopted is critical to model the affective pathologies associated to chronic pain. These authors showed that anxiety and depressive-like behaviours developed in a time-dependent manner, with the former present at three weeks of neuropathy while the latter was expressed only after 6 weeks of neuropathy.

In spite of all the evidence demonstrating the existence of pain-depression comorbidity, the actual mechanisms underlying this interaction are still mostly unknown [3]. While chronic pain patients are up to four times more prone to develop major depressive disorders than the general population [9], more than half of depressed patients present comorbid chronic pain [8], with the effects of having both chronic pain and depression being worse than suffering from chronic pain or depression alone [10]. Therefore, antidepressants, including tricyclic antidepressants (TCAs), are extensively used in the management of both chronic pain and depression [11]. TCAs are the first-line drugs for the treatment of chronic pain and amitriptyline (AMI), the most commonly used TCA to treat depression in chronic pain patients [12], has also been associated with significant analgesia in different animal models of chronic neuropathic pain [13].

Accordingly, in the work herein, we used the kaolin/carrageenan model of experimental knee monoarthritis (MA) to establish a direct link between prolonged MA and the development of mood-like disorders in rodents. After four weeks of MA, we treated the animals with AMI to evaluate whether this antidepressant could reverse the nociceptive and mood-like changes induced by the model.

2. Materials and methods

2.1. Animals and ethical questions

The experiments were performed in adult male Wistar rats (n = 44, Charles Rivers, Barcelona, Spain) weighting 225–250 g at the beginning of the experiment. The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive 86/609/EEC and 2010/63/EU concerning the use of animals for scientific purposes. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Animals were housed in pairs under a 12 h light cycle (starting at 8:00 a.m.) with food and water available *ad libitum*. General health parameters were surveyed twice per week by the resident veterinary and animal weight was recorded every week throughout the experimental period. Before the beginning of the experiment, all animals were handled daily by the experimenter for a week and on the day of the experimental sessions animals were left in the experimental room for an hour in order to habituate to the surroundings.

2.2. Induction of monoarthritis

The induction of arthritis (ARTH) was performed as described in detail elsewhere [14]. Briefly, 3% kaolin and 3% carrageenan (Sigma–Aldrich, St. Louis, MO, USA) were dissolved in sterile saline solution (0.9% NaCl) and injected into the synovial cavity of the right knee joint at a volume of 0.1 mL. This model produces mechanical hyperalgesia, which begins just in a few hours after surgery and lasts for several weeks [15]. Control animals (SHAM) were injected with 0.1 mL saline in the synovial cavity of the right knee joint.

2.3. Behavioural assessments

2.3.1. Nociceptive behaviour

In each animal, the development of arthritis was verified 1–2 h prior to each experiment. Only those rats that vocalized every time after five flexion–extension movements of the knee joint were considered to have developed osteoarthritis, and were included in the ARTH group. SHAM animals did not vocalize to any of the five consecutive flexion–extension movements of the knee joint.

2.3.1.1. Pressure application measurement. The application of noxious pressure to the primary site of injury is a classical approach to measure mechanical hyperalgesia [16], both in humans and animals [17]. Here, a new pressure application measurement (PAM) method was used. It allows an accurate behavioural measurement of primary mechanical hypersensitivity in rodents with chronic inflammatory joint pain [18] by the application of a force range of 0–1500 g. To perform the test and with the animal securely held, the force transducer unit (fitted to the experimenter's thumb) is placed on one side of the animal's knee joint and the forefinger on the other and an increasingly force is gradually applied across the joint until a behavioural response is observed (paw-withdrawal, freezing of whisker movement, wriggling or vocalization) with a cut-off of 5 s. The peak force (in grams force (gf)) applied immediately prior to the behavioural response is recorded as the limb withdrawal threshold (LWT). LWT was measured twice in both the ipsilateral and contralateral limbs at 1 min intervals. The mean LWTs were calculated per animal. At the end of the session animals were returned to their home cage.

2.3.2. Locomotor activity and anxious-like behaviour

2.3.2.1. Open-field test. The open field (OF) test was used to evaluate differences in the locomotor ability between SHAM and ARTH animals and followed a protocol previously implemented in our lab [19]. In summary, locomotor behaviour was assessed by measuring the total distance travelled by the animal inside the arena and anxiety-like behaviour was evaluated through the analysis of the time spent in the centre of the arena and by counting the number of faeces (*fecal boli*) remaining in the arena at the end of each trial [20]. The OF test was performed in a square arena (43.2 cm wide with the central area of the arena corresponding to a square 21.6 cm wide and equidistant from the borders) with transparent acrylic walls (Med Associates Inc., St. Albans, Vermont, USA) in a brightly illuminated room (240 lx in the centre of the arena). The test started when the animal was placed at the centre of the arena and its exploratory activity was automatically registered during 5 min. The arena was cleaned with 10% alcohol between each trial.

2.3.2.2. Elevated-plus maze. The elevated-plus maze (EPM) was used to assess anxiety-like behaviour and followed a protocol previously implemented in our lab [21]. The EPM apparatus (ENV-560; MedAssociates, St. Albans, Vermont, USA) consisted of two opposite open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm), which were elevated 72.4 cm above the floor. At the beginning of the test the animal was placed in the centre of the maze and allowed 5 min to freely explore the maze. During the duration of the trial, behaviour was recorded through the use of an infra-red photobeam system connected to a computer with software (MedPCIV, MedAssociates) that allows for the quantification of the time spent in each arm. Between trials, the maze was cleaned with 10% ethanol. The percentage of time spent in the open arms was used as an index of anxiety-like behaviour.

2.3.3. Depressive-like behaviour

2.3.3.1. Sucrose preference test. Anhedonia, measured as a reduction in sucrose preference (SPT), was assessed through the

application of a protocol adapted from Bessa et al. [21]. This protocol includes an initial exposure of the animals to the sucrose solution a week before the induction of ARTH. During this session, performed during their active period (8:00 p.m.), animals (without any previous water and food restriction) had free access to both water and the sucrose solution for 2 h. In addition, the behaviour of the animals was monitored by the experimenter in order to certify that all animals tasted the sucrose solution. On the test day and after 24 h of food/water deprivation, each animal was presented with two pre-weighted bottles [water and sucrose solution (2%)] for one hour (8:30–9:30 p.m). Afterwards, the bottles were re-weighted and sucrose preference was calculated using the equation:

$$\text{Sucrose_preference} = \left[\frac{\text{sucrose_intake}}{(\text{sucrose} + \text{water})\text{_intake}}\right] \times 100.$$

For experiment 1, the results are presented as the difference in sucrose preference between the data obtained at four weeks post-ARTH induction and the basal assessment that preceded the induction of monoarthritis. In experiment 2, the results are presented as the difference in sucrose preference between the data obtained during the fourth week of pharmacological treatment and the assessment performed at four weeks post-ARTH induction.

2.3.3.2. Forced swimming test. Learned helplessness was evaluated in the modified forced-swimming test (FST) [22] and followed a protocol previously implemented in our lab [21]. Animals were submitted to a pre-test session (10 min) in which they were individually placed in cylinders filled with water (25 °C; depth 30 cm). Twenty-four hours later animals were again placed in the cylinders for a period of 5 min and the testing session was recorded with a video camera. The quantification of (i) latency to immobility, (ii) time spent immobile, (iii) time spent swimming and (iv) time spent climbing was performed by a blind observer that rated the videos using the Etholog software [23]. Note that the time spent swimming and climbing was discriminated as opposed to floating as previously described by Rénéric et al. [24]. Learned helplessness behaviour was defined as an increase in time of immobility at the expense of the time spent swimming/climbing and a decrease in the latency to immobility.

2.4. Drug treatment

Four weeks after arthritis induction, amitriptyline hydrochloride (AMI; Sigma–Aldrich, St. Louis, MO, USA) was dissolved in saline (SAL) and administered intraperitoneally (i.p.) at a dose of 15 mg/kg daily for three weeks, until the beginning of the behavioural tests. The pharmacological treatment with AMI or SAL was maintained during the duration of the behavioural evaluation with injections being administered daily at the end of the behavioural session.

2.5. Experimental design

The present study was divided in two main experiments (Fig. 1A), (i) the first one involving the assessment of the comorbid development of mood-like disorders in animals with experimental ARTH and (ii) the second determining the potential of amitriptyline in reversing both the nociceptive and the emotional-like components of chronic experimental ARTH.

In experiment 1, one week preceding the intrasynovial injection animals were presented to a sucrose solution and in the day before knee injection animals were submitted to a baseline evaluation in the sucrose preference test (SPT). After four weeks of the knee injection animals were tested in the SPT, PAM, OF, EPM and FST. At the end of the behavioural sessions animals were sacrificed with a lethal dose of pentobarbital and the knee joints were removed for further histological processing and analysis.

In experiment 2, one week preceding the intrasynovial injection animals were presented to a sucrose solution and in the day before the beginning of the treatment animals were submitted to a baseline evaluation in the sucrose preference test (SPT). After four weeks of the intrasynovial injection SHAM and ARTH animals were administered either saline (SHAM_SAL and ARTH_SAL) or amitriptyline (SHAM_AMY and ARTH_AMY). Three weeks after the beginning of the treatment, animals were behaviourally tested following the same experimental design as in experiment 1 (Fig. 1A).

2.6. Histology

Right knees from SHAM and ARTH animals were excised and fixed in paraformaldehyde solution (4%). Knees were subsequently submerged in a decalcifying solution (BiodecR, Bio-Optica, Milan, Italy) for 48 h, paraffin embedded, cut into 4-μm sections and stained with hematoxylin and eosin (Fig. 1B).

2.7. Statistical analysis

The GraphPad Prism® 6 software (GraphPad Software Inc, La Jolla, CA, USA) was used to perform the statistical analysis. Differences in weight gain between ARTH and SHAM groups were evaluated by applying a mixed-design two-way analysis of variance (ANOVA) followed by t-test with a Bonferroni correction for multiple comparisons. The comparisons of differences between groups in experiment 1 were performed using a Student's *t*-test for unpaired data. A two-way ANOVA followed by *t*-test with a Bonferroni correction for multiple comparisons was used to compare results from the behavioural assessment at the end of experiment 2, except for the PAM test where a repeated measures two-way analysis of variance followed by t-test with a Bonferroni correction for multiple comparisons was performed. Statistical significance was accepted for $P < 0.05$. Data in the results section are expressed as mean ± standard deviation (SD).

3. Results

3.1. Body weight

In experiment 1, body weight of all animals increased significantly (main effect of elapsed time: $F_{4,40} = 136.00$, $P < 0.001$) but ARTH animals gained less body weight during the experimental period than SHAM animals (interaction between elapsed time and the experimental group: $F_{4,40} = 3.16$, $P = 0.024$). In experiment 2, the administration of AMI induced an overall 10% decrease in the body weight of SHAM and ARTH animals when compared with SAL-treated animals (interaction between weight gain and experimental group during AMI treatment: $F_{9,112} = 20.68$, $P < 0.001$).

3.2. Nociceptive behaviour

All animals in the ARTH group developed a clear swelling of the treated knee joint and all gave a vocalization response during a minor extension and flexion of the affected limb. SHAM animals displayed no obvious swelling of the knee joint and did not vocalize when the limb was flexed.

3.3. Histological analysis

As shown in Fig. 1B1, SHAM animals presented no surface abnormalities in the joint. In ARTH animals an overall tissue degeneration is evident with the medial femoral plateaux (FP) being more affected than the tibial plateaux (Fig. 1B2). In the FP it is possible to

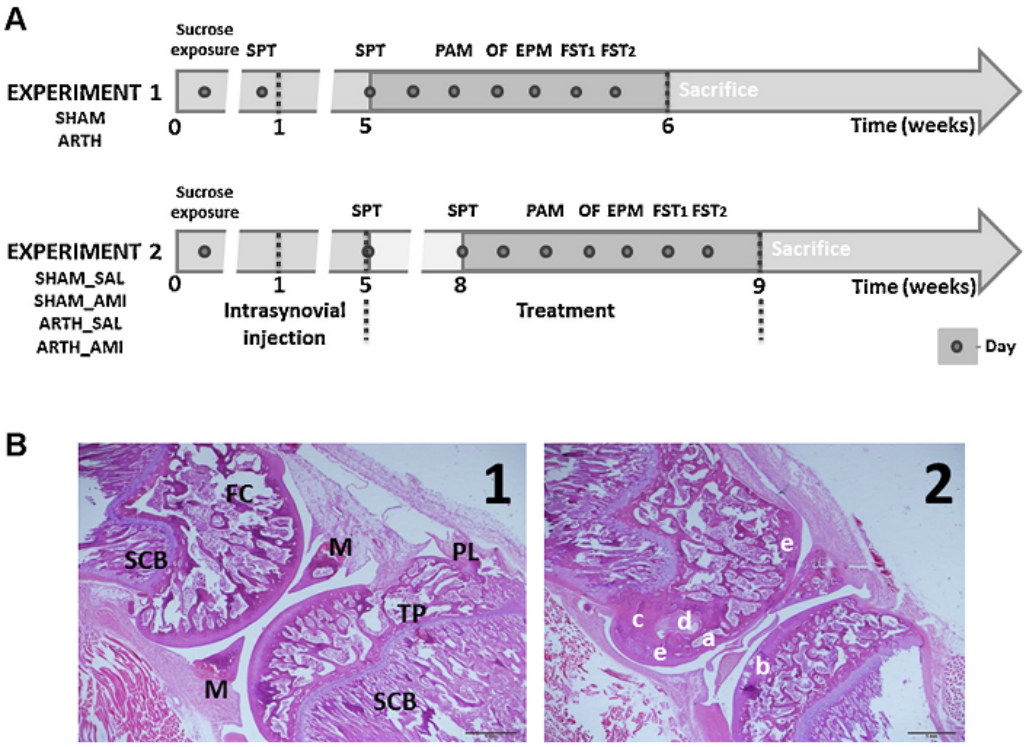


Fig. 1. (A) Time course of the two main experiments, (i) the first one involving the assessment of the comorbid development of mood-like disorders in animals with experimental ARTH and (ii) the second that evaluated the potential of amitriptyline in reversing both the nociceptive and the emotional-like components of chronic experimental ARTH. One week preceding the intrasynovial injection animals were presented to a sucrose solution and in the day before the knee injection animals were submitted to a baseline evaluation in the sucrose preference test (SPT). Four weeks after the intrasynovial injection the first test performed was the SPT followed by 24 h of rest to compensate for the fasting period. The next evaluation was the pressure application measurement (PAM), on day 3 followed by the open field test (OF) and elevated plus maze (EPM) on days 4 and 5, respectively. Finally, we opted to perform the forced swimming test (FST) in the last two days so that the physical effort associated to it would not bias the results of the other tests. In experiment 2, animals were divided into four experimental groups: SHAM animals that received saline (SHAM_SAL) or amitriptyline (SHAM_AMI) and ARTH animals that received saline (ARTH_SAL) or amitriptyline (ARTH_AMI). Four weeks after the intrasynovial injection animals were submitted to a baseline evaluation in the SPT followed by three weeks of daily injections of SAL or AMI. In the fourth week of the treatment, animals were behaviourally tested following the same experimental design as in experiment 1. (B) Sections of the femoro-tibial joint space stained with eosin-haematoxylin of SHAM and ARTH animals four weeks after the induction of arthritis. In SHAM animals (B1) the structure of the femoro-tibial joint is preserved (M: meniscus; PL: patellar ligament; FC: femoral condyle; TP: tibial plateau and SCB: subcondral bone). In ARTH animals (B2), cartilage damage (a) can be observed in addition to fibrillation and marked thinning of the central part of the articular surface. In the FC, subchondral sclerosis (c), cyst formation (d) and inflammation (infiltration of immune cells) (e) are present while in the TP only cyst formation (b) is observed at this time point.

observe focal damage of cartilage surface associated to a decrease in cartilage thickness in the central area, reduplication of the tidemark and some surface fibrillation. Additionally, there is an increase in subchondral bone volume concomitant with subchondral sclerosis, decreased bone marrow area and subchondral cyst formation.

3.4. Experiment 1

3.4.1. Mechanical hyperalgesia

LWT was significantly decreased in ARTH animals compared to SHAM ($t_{10} = 4.885$, $P < 0.001$) confirming their arthritic state (Fig. 2A).

3.4.2. Locomotor activity

In the OF test, total distance travelled by the animals was not different between SHAM and ARTH animals ($t_{10} = 0.790$, $P = 0.448$) (Fig. 2B).

3.4.3. Anxiety-like behaviour

In the OF, ARTH animals showed a strong tendency to spend less time in the centre of the arena ($t_{10} = 2.122$, $P = 0.060$) (Fig. 2C) and left a significantly higher number of *fecal boli* in the OF arena ($t_{10} = 3.078$, $P = 0.012$) (Fig. 2D) when compared to SHAM animals, indicating they developed an anxious-like phenotype. This was confirmed in the EPM test, as ARTH animals also spent less time in the open arms ($t_{10} = 2.665$, $P = 0.024$) (Fig. 2E).

3.4.4. Depressive-like behaviour

ARTH animals displayed a depressive-like phenotype as these animals showed a significantly decreased preference for sucrose solution (*i.e.*, anhedonia-like behaviour) when compared with the SHAM group ($t_{10} = 2.319$, $P = 0.043$) (Fig. 2F). No differences in liquid consumption per body weight unit were found between the experimental groups ($t_{10} = 1.755$, $P = 0.110$). SPT results are further substantiated in the FST where ARTH animals showed a significant decrease in the latency to immobility ($t_{10} = 2.845$, $P = 0.017$) (Fig. 3A). This finding was accompanied by an increase in the immobility time ($t_{10} = 3.630$, $P = 0.005$) (Fig. 3B) and a corresponding decrease of the time spent swimming ($t_{10} = 2.955$, $P = 0.014$) (Fig. 3C) and climbing ($t_{10} = 3.267$, $P = 0.009$) (Fig. 3D) by ARTH animals.

3.5. Experiment 2

3.5.1. Mechanical hyperalgesia after treatment

LWT was significantly different between the experimental groups (main effect of the experimental group: $F_{1,28} = 12.310$, $P = 0.002$) with ARTH animals displaying a lower LWT when compared to SHAM animals (data not shown). Additionally, LWT also

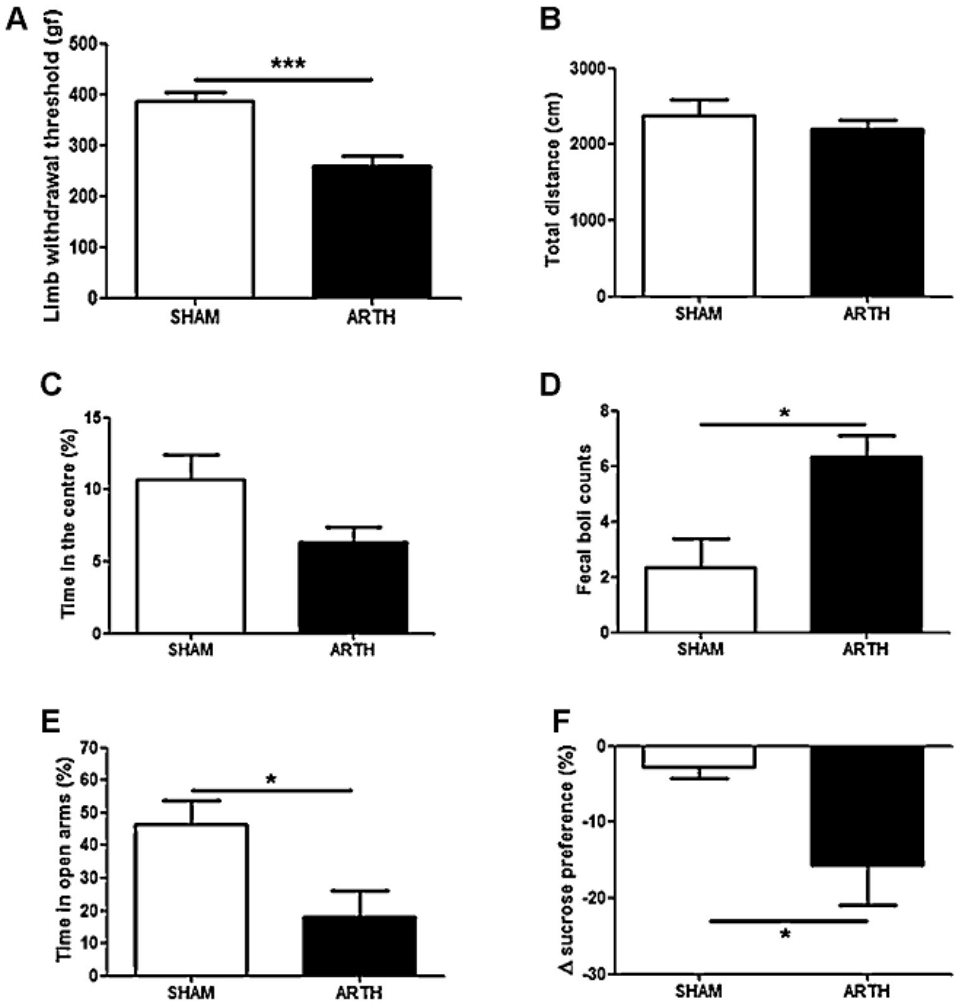


Fig. 2. Behavioural assessment four weeks after arthritis induction, in experiment 1. Evaluation of the right (ipsilateral) limb withdrawal latency (LWT) in the pressure application measurement (PAM). The LWT of arthritic (ARTH) group was significantly decreased when compared to results in control (SHAM) animals (mechanical hyperalgesia) (A). No differences were found in locomotor behaviour between arthritic (ARTH) and control (SHAM) groups, as indicated by the total distance travelled in the open field (OF) test (B). ARTH animals showed increased anxiety-like behaviour as indicated by: the tendency to avoid the centre of the OF arena (C); the significantly higher number of *fecal boli* left in the arena at the end of the trial (D); and the reduction in time spent in the open arms of the EPM test, when compared to SHAM animals (E). ARTH animals displayed an anhedonic-like behaviour as showed by the significant decrease in the preference for sucrose solution in the sucrose-preference test (SPT) (F) (Δsucrose preference is the variation in sucrose preference between the data obtained four weeks after and one day before monoarthritis induction). Results are expressed as mean + SEM ($n = 6$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$.

varied with the drug treatment (interaction between the experimental group and drug treatment: $F_{1,28} = 7.565$, $P = 0.010$) since ARTH animals treated with AMI displayed a higher LWT when compared to ARTH treated with SAL. *Post hoc* tests showed that AMI was able to reverse primary hyperalgesia as shown by the increase in LWT of ARTH_AMI animals to the level of SHAM animals (Fig. 4A).

3.5.2. Locomotor activity

In the OF test, total distance travelled by the animals was not different between experimental groups (main effect of test group: $F_{1,28} = 0.618$, $P = 0.438$) (Fig. 4B).

3.5.3. Anxiety-like behaviour after treatment

In the OF test, there were no differences in the time spent in the centre of the arena (main effect of test group: $F_{1,28} = 2.405$, $P = 0.1322$) (Fig. 4C). The number of *fecal boli* that remain in the OF arena varied with the drug treatment (interaction between the experimental group and drug treatment: $F_{1,28} = 7.488$, $P = 0.011$) (Fig. 4D). In the EPM there were no significant differences between the groups in the time spent in the open arms (main effect of test group: $F_{1,28} = 1.348$, $P = 0.256$) (Fig. 4E). Note that SHAM_AMI animals had behavioural performances similar to ARTH_SAL animals (Fig. 4).

3.5.4. Depressive-like behaviour after treatment

Sucrose preference was significantly different between the experimental groups (main effect of test group: $F_{1,28} = 5.536$, $P = 0.026$) with SHAM animals displaying more preference for the sugary beverage when compared to ARTH animals. Additionally, sucrose preference also varied with the drug treatment (interaction between group and drug: $F_{1,28} = 6.044$, $P = 0.020$) since ARTH animals treated with AMI consumed considerably more sucrose than ARTH animals treated with SAL. *Post hoc* tests confirm that ARTH_AMI animals increased sucrose preference when compared with ARTH_SAL animals (Fig. 4F). In the FST, the latency to immobility between the groups varied with the drug treatment (interaction between the group and drug: $F_{1,28} = 6.710$, $P = 0.015$) as SHAM and ARTH animals treated with AMI display a lower latency to immobility. *Post hoc* tests indicated that the latency to immobility was significantly shorter in the SAL-treated ARTH

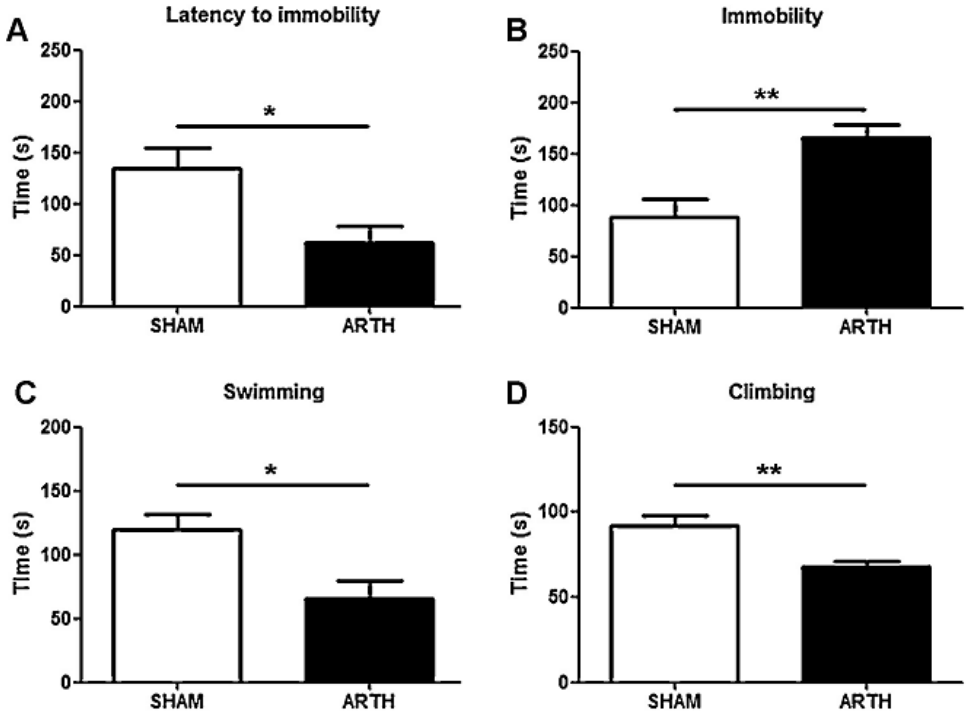


Fig. 3. Behavioural assessment in the forced swimming test (FST), in experiment 1. Four weeks after the induction of experimental arthritis, ARTH animals become immobile significantly earlier than SHAM animals in the forced swimming test (FST) (A). ARTH animals spent significantly more time immobile than SHAM animals (B), at the expense of the time spent swimming (C) and climbing (D) in the FST test. Results are expressed as mean + SEM ($n = 6$ per group). *$P < 0.05$, **$P < 0.01$.

than SHAM group and also between SAL-treated SHAM and AMI-treated SHAM (Fig. 5A). The duration of the immobility time was significantly different between treatments (main effect of test drug: $F_{1,28} = 5.303$, $P = 0.029$) as SHAM animals treated with AMI increased the amount of immobility time. In parallel, the difference in the immobility time between the groups varied with the drug treatment (interaction between group and drug: $F_{1,28} = 6.801$, $P = 0.015$) as ARTH animals treated with AMI showed no difference in immobility time while SHAM treated with AMI significant increased the time spent immobile. *Post hoc* tests confirm that the duration of immobility time was significantly longer in the SAL-treated ARTH than SHAM group (Fig. 5B). Concerning the duration of the active period, the time spent swimming was significantly different between the experimental groups (main effect of the group: $F_{1,28} = 5.658$, $P = 0.024$) since ARTH animals spent less time swimming when compared to SHAM. In addition this parameter also varied with the treatment (main effect of the drug: $F_{1,28} = 15.470$, $P < 0.001$) since the time spent swimming was decreased in SHAM animals after the treatment with AMI when compared with SAL-treated SHAM. Moreover, the difference in swimming time between the groups was dependent on the drug treatment (interaction between the group and drug: $F_{1,28} = 5.198$, $P = 0.030$) since the effects of AMI administration were restricted to SHAM animals. *Post hoc* tests confirm that the swimming time was shorter in the SAL-treated ARTH than SHAM group (Fig. 5C).

The difference in time spent climbing varied with the drug treatment and the experimental group (interaction between the group and drug: $F_{1,28} = 14.800$, $P < 0.001$) since ARTH animals treated with AMI increased the time spent climbing while AMI-treated SHAM animals decreased it. *Post hoc* tests confirm that the duration of the climbing time was significantly longer in the AMI-treated ARTH than SHAM group (Fig. 5D). Overall, SHAM animals treated with AMI had behavioural performances similar to ARTH animals treated with SAL (Fig. 5).

4. Discussion

In this study, we demonstrate that the K/C animal model can be used as a valid method to study the comorbidity between arthritis and mood disorders in the rat. In addition to sustained hyperalgesia (decreased withdrawal threshold to noxious mechanical stimulation of the inflamed knee), animals with four weeks of K/C-induced arthritis exhibited anxiety-like behaviour (low exploratory activity in the EPM and increased defecation in the OF arena), anhedonic behaviour (reduced preference for sucrose) and learned helplessness (decreased mobility in the FST). We also demonstrated that after three weeks of antidepressant treatment, AMI is able to decrease mechanical hyperalgesia and partly reverse depressive-like behaviour, as the preference for sucrose is increased in ARTH animals and they spend more time climbing in the forced swimming test. Due to the importance of using animal models that mimic the greatest number of features of the human disorders, we anticipate that the concomitant study of pain and emotional-like behaviours in the experimental model of arthritis will significantly increase our understanding of the mechanisms underlying comorbidity of chronic pain and mood disorders.

In general K/C-induced arthritis has been established as model of acute monoarthritis that partly mimics the initial inflammatory stages of OA [25]. The advantage of also administering kaolin is the mechanical damage to intra-joint structures [26] triggered by this clay, which is absent in inflammatory models using carrageenan alone. Taking into account that inflammatory disorders are slowly progressing degenerative diseases it is most surprising that the majority of studies using this model use a restricted time frame (*e.g.* 4 h [27], 5–6 h [28] and 1 week [29]). Our data contrast with most studies, reporting marked cellular infiltration, as we observe a clear degeneration of the femoral plateaux (thinning of the cartilage, fibrillation and subchondral sclerosis). This degree of joint degeneration partly mimics features of later stages of chronic inflammatory diseases, in particular of osteoarthritis. Indeed and

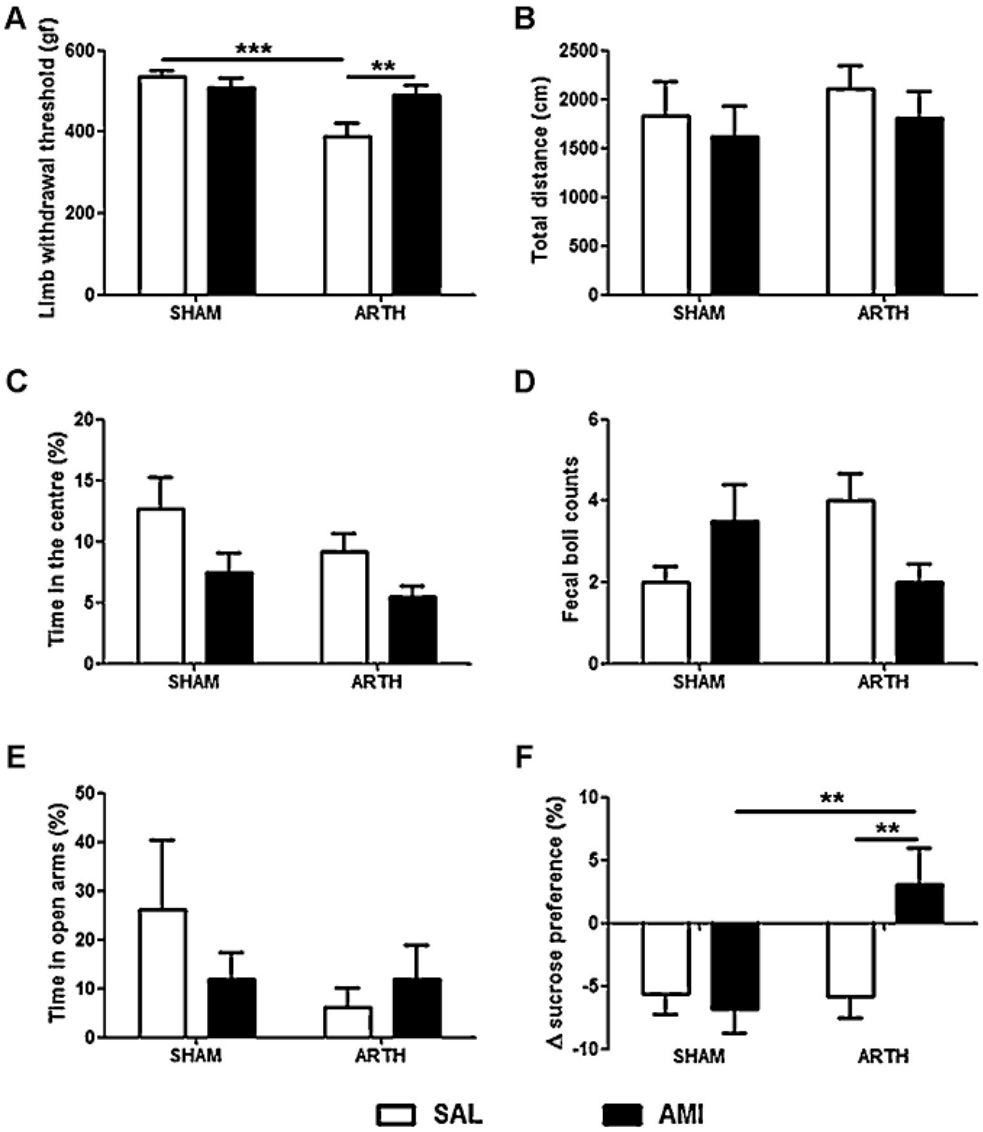


Fig. 4. Behavioural assessment after treatment with amitriptyline (AMI), in experiment 2. Evaluation of the right (ipsilateral) limb withdrawal latency (LWT) in the pressure application measurement (PAM). The LWT of arthritic (ARTH) animals treated with AMI increased when compared to ARTH animals treated with saline (SAL), reaching control (SHAM) levels (abolished mechanical hyperalgesia) (A). No differences were found in locomotor activity between arthritic (ARTH) and control (SHAM) groups, as indicated by the total distance travelled in the open-field (OF) test (B). ARTH animal treated with AMI showed no difference in anxiety-like behaviour when compared to ARTH animals treated with SAL in both the time spend in the centre of the OF arena (C) and the number of *fecal boli* (D). In the elevated plus maze (EPM) test no differences were observed in the time spent in open arms (E). In the sucrose preference test (SPT), ARTH animals treated with AMI increased sucrose preference when compared with ARTH animals treated with SAL (F) (Δsucrose preference is the variation in sucrose preference between the data obtained three weeks after and one day before the beginning of the treatment). Results are expressed as mean + SEM ($n = 8$ per group). $^{**}P < 0.01$, $^{***}P < 0.001$.

according to Pearson's [30] report, our animals display a knee joint degeneration level concomitant with stage three of human osteoarthritis that comprises the presence of deep fissures, loss of cartilage, remodelling of the bone plate and reduplication of the tidemark. Nonetheless, further studies involving a time dependent evaluation of the succession of events affecting knee joint structure are needed for this K/C-induced arthritis to be considered a valid osteoarthritis model.

Anxiety and depression [31] are important comorbidities that have been positively correlated with pain severity in arthritic patients. These mood disorders are 2 to 3 times more prevalent in OA patients than in patients with other chronic pathologies [3]. However, to date and to the best of our knowledge, only one experimental animal study [32] has addressed the comorbidity of pain and depression in OA, in spite of the availability of many animal models mimicking specific aspects of the OA pathology [33]. It should be emphasized that in our study anxiety- and depression-like behaviours were assessed after a sustained hyperalgesic state that had lasted 28 days, whereas the majority of the studies from other authors used more restricted time-frames (see above).

Our data shows that ARTH animals with 28 days of the K/C-induced arthritis display a profound mechanical hyperalgesia in the ipsilateral knee (PAM test), which parallels the hyperalgesic response of OA patients to noxious stimulation of the knee [34]. Interestingly, some reports have positively associated motor disability with the development of depression in OA patients [31]. However, although our ARTH rats present mechanical hyperalgesia, they did not display any locomotor impairments as the total distance travelled in the OF was not different from SHAM animals. Hence, in this work the development of mood-like disorders could not be associated to locomotor disability of rodents.

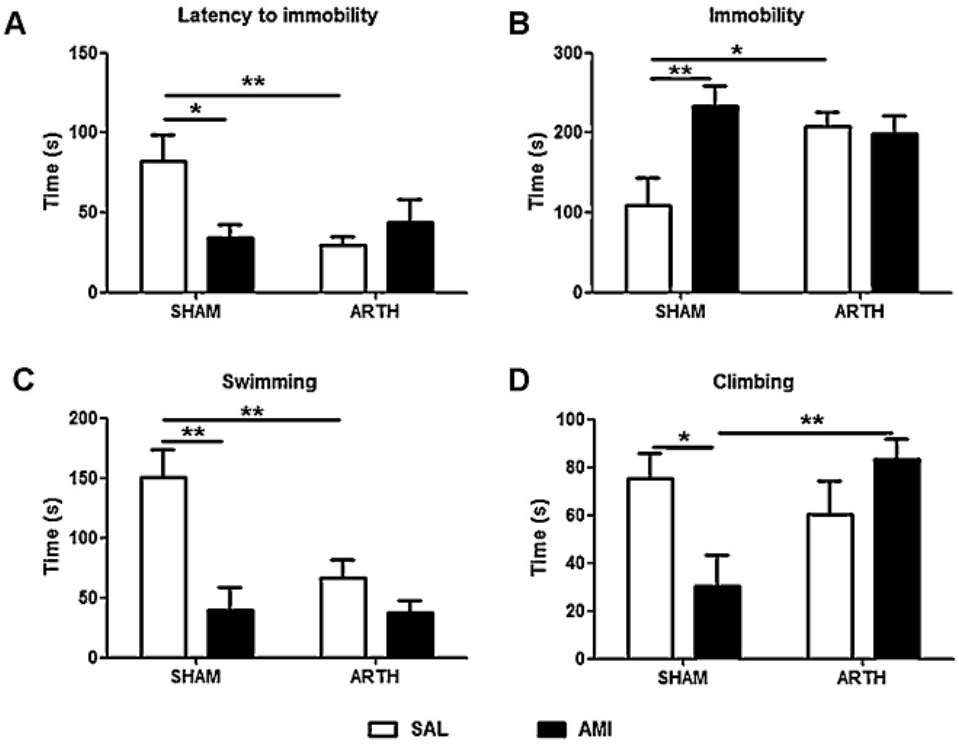


Fig. 5. Behavioural assessment in the forced swimming test (FST) after treatment with amitriptyline (AMI), in experiment 2. AMI did not reverse the effect of arthritis on the latency to immobility (A), the time spent immobile (B) or the time spent in swimming (C). Interestingly, ARTH animals injected with AMI increased the time spent climbing (D) but only when compared with SHAM treated animals. Additionally, note that following AMI treatment of ARTH animals, no differences were observed with the SHAM group treated with SAL in the latency to immobility (A), total immobility (B) and time spent climbing (D). Results are expressed as mean + SEM ($n = 8$ per group). *$P < 0.05$, **$P < 0.01$.

Clinical treatment of joint disorders focuses mainly on ameliorating pain and functional limitations [35], while less attention is paid to mood comorbidities [2]. However, several studies have shown that the prevalence of anxiety and depression *e.g.* in OA patients is very high [2,31], around 41% and 62%, respectively, although the recognition of both depression and anxiety in primary care settings is typically lower, less than 50% [2]. Similarly, in preclinical OA studies most authors have focused on acute changes in behaviour or molecular and cellular responses [4–7,27–29,36,37]. In experimental animal studies on neuropathic pain, comorbid anxiety- and depression-like behaviour has been studied more extensively. For example, Yalcin et al. [8] assessing anxiety and depression-related behaviours in a mouse model of neuropathic pain demonstrated a time-dependent development of mood-like disorders. Their findings indicate that anxiety-related behaviour was developed 4 weeks after induction of the neuropathy, whereas depression-related behaviours were observed 2-4 weeks later.

In the currently used K/C model, the development of anxiety-like behaviour had already been shown by Ji et al. [28] as early as 24 h after its induction. Using a similar set-up, we demonstrated that ARTH animals still displayed anxiety-like behaviour at four weeks post-induction as shown by the significant decrease in the time spent in the open arms of the EPM. In addition, ARTH animals also displayed an anxious-like phenotype in the open field test as this group left a significantly higher number of *fecal boli* in the OF arena when compared to SHAM animals. Despite its simplicity, defecation is considered an important indicator of emotional responses, particularly in anxiety-like behaviour [20].

While the results by Yalcin et al. [8] showed that the development of depression-like behaviour in neuropathic animals took 6–8 weeks, our results indicate that in the K/C model the depressive-like phenotype appeared earlier, at four weeks. This was shown by a decrease in the latency to immobility, an increase in the duration of immobility and a decrease in active behaviours (climbing and swimming) in the FST. This type of behavioural change in experimental animals is considered an index of learned helplessness that presumably represents human "resignation" [38]. Yet, based on studies on neuropathic pain by Wang et al. [39], it could be argued that the differences obtained between SHAM and ARTH animals in the FST could be explained by motor deficits of ARTH animals. However, ARTH animals of the present study failed to display any motor impairments in the OF. Nor could we detect any obvious difference in the use of the left and right hind paws during the FST. Moreover, the depressive-like phenotype obtained in the FST is further supported by the SPT data, a test that does not implicate/require motor coordination.

Although the analysis of anhedonia (lack of interest in pleasure) in rodents has to be approached with caution, a reduced preference for a sweet solution is considered to share some analogy with anhedonia and depressive behaviour in humans [40] and therefore, the SPT is widely used in stress studies [41]. Our ARTH animals showed a significant decrease in the preference for a sweet solution when compared to SHAM, hence displaying a clear hedonic deficit. It has been argued that sucrose intake can also be influenced by body weight [41], but comparing the ratio between animal body weight and liquid consumption between groups revealed no significant differences between ARTH and SHAM animals. This finding gives further support to the presence of an anhedonic state in ARTH animals.

In what concerns the performance of AMI-treated SHAM animals, there appears to be an anxiogenic and pro-depressive effect of AMI on these animals as shown in the OF and the FST tests. In clinical practice, antidepressants are known to have deleterious effects when administered to non-depressed patients

[42] comprising, amongst others, sedation, lower reaction time in the choice reaction time task, sensory impairments, disruption of discrimination ability, dry mouth, dizziness and constipation [42,43]. In what concerns the effects of prolonged AMI administration to animals only a few works reported frequency-related depression of ventricular conduction in dogs [44,45]. Nonetheless, in a preliminary study (unpublished data) where we compared the motor performance of AMI- and SAL-treated SHAM animals in the Rotarod test (increasingly faster rotations) no differences could be found between experimental groups. These results are in accordance with a report from Bomholt et al. [46] also showing that the acute administration of AMI did not alter the motor performance of SHAM animals in the Rotarod test. Based on these results we considered that the detrimental effects of AMI administration to SHAM animals were not motor related.

Recurrent episodes of inflammation at the damaged joints in arthritic patients lead to the release of inflammatory mediators that activate voltage-gated sodium channels causing peripheral/central sensitization and ultimately, persistent inflammatory pain [47]. Descending monoaminergic pathways play an important role in inhibiting pain transmission at the spinal cord level [48]. Tricyclic antidepressants (TCAs), which inhibit the reuptake of serotonin and noradrenalin, are essential components of the current pain management therapeutic strategies in chronic pain disorders, including rheumatoid arthritis [49]. Yet, Richards et al. [49] while studying the effect of AMI in controlling pain in a data base of controlled trials in adults with rheumatoid arthritis or ankylosing spondylitis could not confirm the efficacy of this TCA as an analgesic. No available data could be found concerning the use of AMI for pain management in monoarthritic/osteoarthritic patients. Nonetheless, our data shows that the administration of AMI to ARTH animals significantly decreased mechanical hyperalgesia after three weeks of treatment. Interestingly, this effect might result not only from increased availability of brain serotonin and noradrenaline and subsequent activation of descending pathways as demonstrated in other works [48,50], but also from a peripheral effect as this drug decreases the activity of sodium and calcium voltage dependent channels [51]. This hypothesis is supported by demonstration that AMI selectively inhibits ectopic discharge of low frequency and bursting discharge in animal models of neuropathic pain [52], and that the antidepressants' ability to block sodium channels could effectively suppress persistent peripheral signaling [53].

Although AMI had no effect upon anxiety-like behaviour, it efficiently reversed the hedonic-like behaviour as measured in the SPT. These results are consistent with data from other studies showing that the decrease in depressive-like behaviour results from the modulation of the activity of serotonin transporters [54] and of several receptors [54,55]. However taking into account our results from the FST it is unlikely that the effect of AMI reported herein is related to the modulation of serotonin receptors, as no differences were found for the swimming component of the test, but rather to an effect of this drug on noradrenergic receptors. In fact, similarly to what was observed by Detke et al. [56], in rats injected with specific noradrenaline uptake inhibitors, the administration of AMI to ARTH animals selectively increased climbing behaviour without altering the time spent swimming.

5. Conclusions

So far, it is not clear which components of chronic inflammation are directly involved in the development of mood disorders in human patients with an inflammatory joint disorder such as OA [57]. The causes of pain-depression comorbidity in patients with arthritis are complex (involving sensory, affective and cognitive processes) and the abnormal peripheral and central mechanisms of pain processing involved in this comorbidity are far from being understood. Our present results support the proposal that the K/C model in the rat provides a good pre-clinical tool for studying the link between chronic inflammatory joint pain and mood disorders, as well as a model for testing novel drugs that may modulate pain intensity, mood disorders, or both. Importantly, AMI was able to reverse the nociceptive and hedonic components of chronic inflammatory pain with its influence likely due to a simultaneous combination of central and peripheral effects.

Competing interest

The authors declare that they have no competing interests.

Author contributions

DA and ADP carried out the experimental work and scored the tests. DA analysed the data. DA and FPR designed the experiment, coordinated the tasks and wrote the manuscript. AP and AA revised the manuscript. All authors read and approved the final manuscript.

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Chapter 2.2

Pinto-Ribeiro F*, Amorim D*, David-Pereira A, Monteiro AM, Costa P, Pertovaara A, Almeida A

Pronociception from the dorsomedial nucleus of the hypothalamus is mediated by the rostral ventromedial medulla in healthy controls but is absent in arthritic animals

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Research report

Pronociception from the dorsomedial nucleus of the hypothalamus is mediated by the rostral ventromedial medulla in healthy controls but is absent in arthritic animals

Filipa Pinto-Ribeiro[a,b,1], Diana Amorim[a,b,1], Ana David-Pereira[a], Ana Maria Monteiro[a,c], Patricio Costa[a,b], Antti Pertovaara[d], Armando Almeida[a,b,*]

[a] Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), Campus of Gualtar, University of Minho, 4750-057 Braga, Portugal
[b] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[c] Department of Neurology, Hospital S. João, Porto, Portugal
[d] Biomedicum Helsinki, Institute of Biomedicine/Physiology, University of Helsinki, POB 63, Helsinki, Finland

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ABSTRACT

The dorsomedial nucleus of the hypothalamus (DMH) has been proposed to participate in stress-induced hyperalgesia through facilitation of pronociceptive cells in the rostroventromedial medulla (RVM). We hypothesized that the DMH participates in hyperalgesia induced by arthritis. The DMH was pharmacologically manipulated while assessing heat-evoked nociceptive behavior or the discharge rates of pronociceptive RVM ON- and antinociceptive RVM OFF-like cells in NAIVE, SHAM and monoarthritic (ARTH) animals. In NAIVE and SHAM animals, the changes in nociceptive behavior induced by activation of the DMH by glutamate and inhibition by lidocaine were in line with earlier evidence indicating that the DMH has a nociceptive facilitating role. However, in ARTH animals, neither activation nor inhibition of the DMH influenced pain-like behavior evoked by stimulation of an uninflamed skin region (paw and tail). In accordance with these behavioral results, activation or inhibition of the DMH induced pronociceptive changes in the discharge rates of RVM cells in NAIVE and SHAM animals, which suggests that the DMH has a pronociceptive role mediated by the RVM in normal animals. However, in ARTH animals, both glutamate and lidocaine in the DMH failed to influence either pain-like behavior or noxious stimulation-evoked responses of RVM cells, while blocking the DMH increased spontaneous activity in the pronociceptive RVM ON cells. Our data indicate that the DMH participates in descending facilitation of cutaneous nociception in healthy controls, but it is not engaged in the regulation of cutaneous nociception in monoarthritic animals, while a minor role in tonic suppression of nociception in arthritis cannot be discarded.

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1. Introduction

It is widely accepted that central sensitization contributes to the development and maintenance of chronic pain (Schaible et al., 2002). Alterations of the descending pain control systems exert an important role in central sensitization (Latremoliere and Woolf, 2009; Sandkühler, 2009). In chronic pain conditions, functional changes have been described both in descending facilitatory and inhibitory circuits (Herrero and Cervero, 1996; Gozariu et al., 1998; Burgess et al., 2002) that influence nociception at the spinal cord level (Ren and Dubner, 1996; Urban and Gebhart, 1999). Several studies have already demonstrated that pathways descending from the rostral ventromedial medulla (RVM) facilitate pain-related responses in some chronic pain conditions (Burgess et al., 2002; Ossipov et al., 2010). More specifically, it has been reported that changes in the activity of RVM cells are essential to the development of hyperalgesia (Ossipov et al., 2005). Nociception is enhanced when the basal and stimulus-evoked activities of pronociceptive RVM ON-cells are increased and those of antinociceptive OFF-like cells are decreased (Miki et al., 2002). In chronic pain models, the net effect by the RVM may be pronociception, since blockade

Abbreviations: ARTH, arthritic animals/arthritis; CTRL, control animals/controls; DMH, dorsomedial nucleus of the hypothalamus; GLU, glutamate; H, peripheral noxious heat stimulation; HPA, hypothalamus–pituitary–adrenal axis; LIDO, lidocaine; MS, mechanical stimulation; NPS, noxious peripheral stimulation; P, peripheral noxious mechanical stimulation; PAG, periaqueductal gray matter; PW, paw-withdrawal; RVM, rostral ventromedial medulla; SAL, saline solution; TF, tail-flick; TS, thermal stimulation.

* Corresponding author at: Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), Campus of Gualtar, University of Minho, 4710-057 Braga, Portugal. Tel.: +351 253604808; fax: +351 253604809.
E-mail address: aalmeida@ecsaude.uminho.pt (A. Almeida).
[1] These authors contributed equally for this paper.

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of the RVM has reduced hyperalgesia (see for review, Urban and Gebhart, 1999). Interestingly, Urban and Gebhart (1999) reported that blocking the RVM reversed secondary hyperalgesia (*i.e.*, hyperalgesia adjacent to the injured region) to heat in monoarthritic or mustard oil-treated animals but failed to reduce primary hyperalgesia (*i.e.*, hyperalgesia in the injured region) in animals with paw inflammation. This result is in line with the hypothesis that the RVM-mediated descending modulation of spinal nociception may vary from inhibition to facilitation depending on whether the spinal neuron receives inputs from the inflamed/injured area or from adjacent areas, respectively (Vanegas, 2004; Vanegas and Schaible, 2004).

The dorsomedial nucleus of the hypothalamus (DMH) is among the supramedullary structures that have been shown to contribute to descending pain control through a relay in the RVM (Heinricher et al., 2009). In healthy rats, the DMH has a top-down pronociceptive action mediated by the RVM. This was shown by the finding that disinhibition of the DMH with the $GABA_A$ receptor antagonist bicuculline induced behavioral hyperalgesia that was accompanied by increased activation of pronociceptive RVM ON-cells and decreased activation of antinociceptive RVM OFF-cells (Martenson et al., 2009). However, it is still not known whether a chronic pain condition, such as arthritis, induces changes in the top-down pronociceptive action by the DMH. Therefore, we assessed whether the top-down effect by the DMH on the discharge rates of RVM cells and pain behavior differs between monoarthritic and healthy control animals. Since the DMH has been shown to promote nociception through a relay in the RVM in healthy controls (Martenson et al., 2009) and since the descending pain facilitation by the RVM in pathophysiological conditions has been most prominent outside of the injured region (Urban et al., 1999), pain behavior in the monoarthritic animals was assessed by stimulating the intact skin outside the dermatome corresponding to the inflamed joint (paw and tail).

We hypothesized that arthritis increases the descending pronociceptive action of the DMH and thereby contributes to chronic pain and hypersensitivity in arthritis. Results of the present study, however, were opposite to that predicted by our working hypothesis: assessment of pain behavior and the discharge rates of pro- and antinociceptive RVM cells indicated that the top-down pronociceptive action by the DMH was reduced in arthritis.

2. Materials and methods

2.1. Animals, ethical issues and anesthesia

The experiments were performed in adult male *Wistar* rats with 175–250 g (Charles River, Barcelona, Spain). The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive 86/609/EEC and 2010/63/EU concerning the use of animals for scientific purposes. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

During surgery and electrophysiological sessions, the anesthesia was induced (50 mg/kg, i.p.) and maintained by administering pentobarbitone (15–20 mg/kg/h, i.p.). After the initial anesthesia for the placement of the animal in the stereotaxic frame and for the placement of the injection cannula and the recording electrode, the animals received three additional reinforcements (20 mg/kg) at 60 min intervals or more frequently when needed (see below). The level of anesthesia was monitored by verifying dilation of the pupils, the general muscle tone and behavioral responses to noxious pinching every 15 min. If the pinch-evoked response was stronger than a brief muscle twitch restricted to the stimulated limb, the animal was given an additional dose of pentobarbitone (20 mg/kg i.p.) and the anesthesia level was re-assessed within 5 min. After the completion of the behavioral tasks or the electrophysiological sessions, animals received a lethal dose of pentobarbitone and the brains were removed for histological confirmation of cannula placement (Fig. 1B–K) or electrode recording site.

2.2. Induction of arthritis

The induction of arthritis (ARTH) was performed 21 days before the actual experiments, as described in detail elsewhere (Ansah and Pertovaara, 2007). Briefly, a solution of 3% kaolin and 3% carrageenan (Sigma–Aldrich, St. Louis, MO, USA) was injected into the synovial cavity of the right knee joint at a volume of 0.1 mL. This model produces mechanical hyperalgesia, which begins just in a few hours after surgery and extends up to 8 weeks (Radhakrishnan et al., 2003). In each animal, development of arthritis was verified 1–2 h prior to each experiment. Only those rats that vocalized every time after five flexion-extension movements of the knee joint were considered to have arthritis and were included in the arthritis group. SHAM animals were injected with 0.1 mL of saline in the synovial cavity of the left knee joint. NAIVE animals were not submitted to any type of treatments of the synovial cavity.

2.3. Procedures for intracerebral microinjections

For intracerebral drug administration, one (electrophysiology) or two (behavioral assessments) stainless steel guide cannulae (26 gauge; PlasticsOne, Roanoke, VA, USA) were implanted in the DMH and the RVM (behavioral assessments). Animals were allowed to recover from the surgery for at least one week before any tests were performed.

Test drugs were administered in either the DMH or the RVM through a 33-gauge injection cannula (PlasticsOne) with an injection volume of 0.5 μL. The spread of the injected drugs within the brain was expected to have a diameter of at least 1 mm (Myers, 1966). The efficacy of injections was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 20 s and the injection cannula was left in place for additional 30 s to minimize the return of drug solution back to the injection cannula.

Additionally, for physiological confirmation of cannula placement in the DMH (disinhibiton of the DMH and nociceptive facilitation), an injection of bicuculline methiodide, a $GABA_A$ receptor antagonist (0.4 mM; Tocris Bioscience, Bristol, UK), was performed in the DMH in each animal before the beginning of the behavioral testing (Fig. 1A) as well as at the end of each electrophysiological session, in order to reproduce the earlier results by Martenson et al. (2009). Only the injections involving the DMH (Fig. 1B–G) resulted in decreased paw-withdrawal latency. No alterations were observed after injections outside the DMH area, or an antinociceptive effect was observed (*i.e.*, ventromedial hypothalamic nucleus) (Fig. 1H–K).

2.4. Behavioral assessment of nociception

The rats were habituated to the experimental conditions by allowing them to spend 1–2 h daily in the laboratory during two to three days preceding any testing. For assessing nociception in unanaesthetized animals, radiant heat-induced latencies for withdrawing the tail and hindpaw (The Plantar Test Instrument Model 37370, Ugo Basile, Varese, Italy) were determined. Paw-withdrawal (PW) and tail-flick (TF) latencies were determined in separate sessions. In each drug treatment session, the withdrawal latency was assessed prior to drug administration and at various intervals

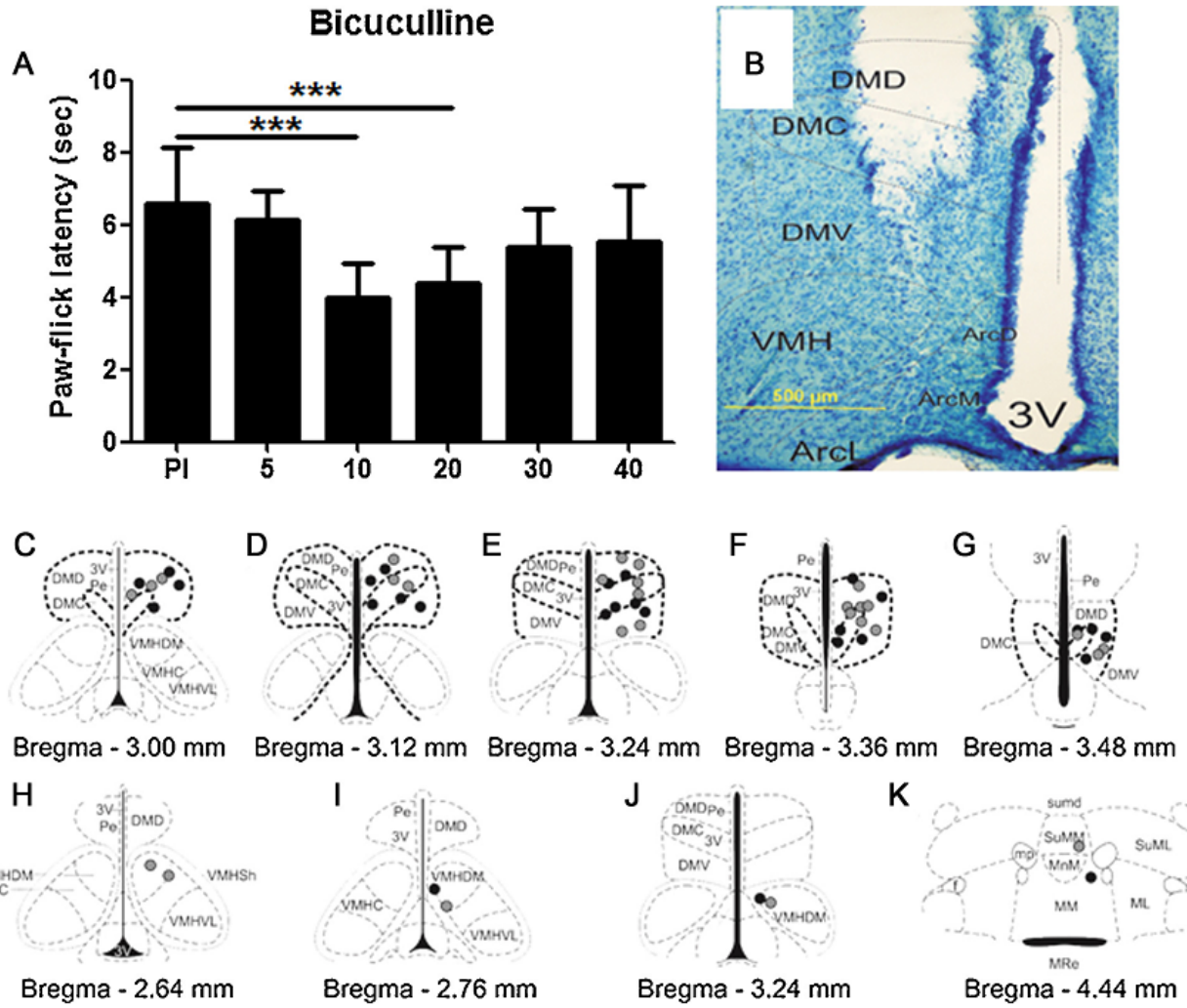


Fig. 1. Pharmacological and anatomical confirmation of drug injection sites in the dorsomedial nucleus of the hypothalamus (DMH). (A) Evolution of radiant heat-evoked paw-withdrawal responses after the administration of the $GABA_A$-receptor antagonist, bicuculline, into the DMH. PI represents the response latency before DMH injection, and the numbers in the *X*-axis represent minutes after the injection. (B) Photomicrograph of an example of the drug injection site in the DMH of the rat brain (AP: −3.24 mm from bregma) superimposed with the appropriate legend of Paxinos and Watson (2007) stereotaxic atlas. (C–G) Schematic representation of other injection sites in the DMH (C: −3.00 mm, D: −3.12 mm, E: −3.24 mm, and F: −3.36 mm; G: −3.48 mm). (H–K) Schematic representation of injection sites outside the DMH (H: −2.64 mm, I: −2.76 mm, J: −3.24 mm, and K: −4.44 mm; in all graphs, the AP distance is from bregma). DMD, DMC, DMV – dorsal, compact and ventral portions of the DMH; ARC – arcuate nucleus; 3V – third ventricle. In A, error bars represent S.D. ($n = 8$). ***$P < 0.001$ (*t*-test with a Bonferroni correction for multiple comparisons; reference: PI, latency before injection).

following the intracerebral injections. At each time point, the measurement was repeated (bilaterally for PW) twice at an interval of 1 min (except for GLU due to its fast effect) and the mean of these values was used in further calculations. Cut-off time was 15 s.

2.5. Course of the behavioral study

The behavioral study started three weeks after the induction of arthritis and at least one week following insertion of the guide cannula in the DMH. Saline (SAL) was used in control injections of drugs, while NAIVE and SHAM animals were used as controls for ARTH animals. Paw-withdrawal latencies were measured 30 s after hypothalamic microinjection of GLU or 10 and 20 min after hypothalamic microinjection of LIDO, since the maximum effects by hypothalamic injections of these compounds have been obtained at these time points (Pinto-Ribeiro et al., 2008, 2011). The involvement of the RVM in mediating the descending pronociceptive drive from the DMH was assessed by pretreating the RVM with LIDO 15 min before administering GLU into the DMH. The interval between behavioral assessments of different drug treatment conditions in the same animal was at least two days. The order of testing different treatment conditions varied between the animals.

2.6. Recording of neuronal responses in nociceptive RVM cells

The electrophysiological recordings of the activity of RVM neurones followed a protocol described detail in Pinto-Ribeiro et al. (2008). Briefly, in anaesthetized animals a recording electrode was placed in the RVM (AP: −1.92 mm from the interaural line, LM: 0.1–0.6 mm, and DV: 10.3 mm) (Paxinos and Watson, 2007) using a standard stereotaxic frame. Single neurone activity was recorded extracellularly with a tungsten electrode (tip impedance 5–10 MΩ at 1 kHz) and data sampling was performed using a CED Micro 1401 interface and Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

For the characterization of RVM cells, we adapted the classification scheme developed by Fields et al., 1983 and Fields and Heinricher (1985). RVM neurones were classified as ON-like cells if their activity increased during peripheral noxious stimulation of the tail, OFF-like cells if their activity decreased during the noxious stimulation period and NEUTRAL-cells if no changes or only a

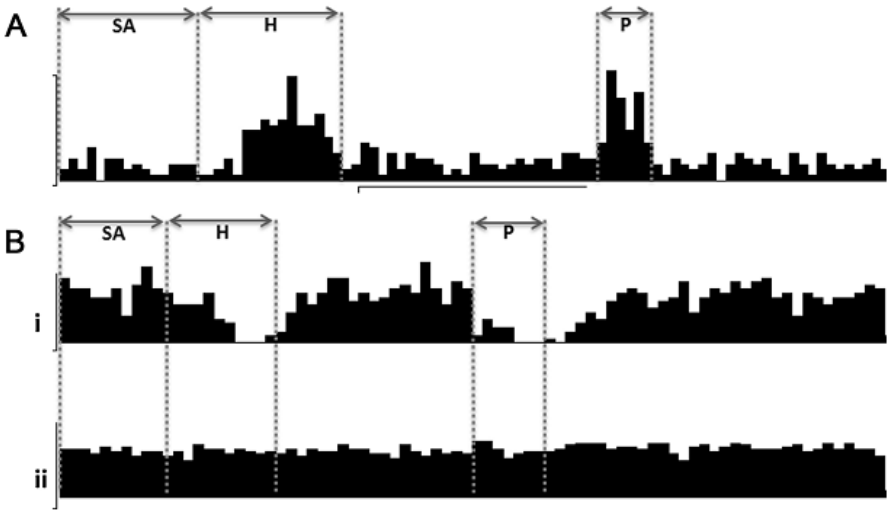


Fig. 2. Examples of original recordings of the activity of pain-modulatory cells in the rostroventromedial medulla (RVM). (A) Neurone that increased activity (ON-like cell) during noxious heating (H) and mechanical stimulation (P) of the tail when compared to basal activity (SA). (B) Simultaneous recording of two cells in the RVM. The first neurone (i) decreased its activity during both noxious heat and mechanical stimulation (OFF-like cell), whereas the second neurone (ii) failed to respond to either type of noxious peripheral stimulation (NEUTRAL-like cell). The vertical calibration bars for neuronal response represent 20 Hz in A, 25 Hz for trace (i) in B, and 35 Hz for trace (ii) in B. The horizontal calibration bar represents 25 s for graph A and 20 s for graph B.

negligible (<10%) alteration in the discharge rates was observed (Fig. 2). It should be pointed out that in the present study the changes in the activity of RVM neurones were not directly correlated with withdrawal responses, unlike in the original classification scheme of Fields et al., 1983, but cell classification was solely based on the response to peripheral noxious stimulation of the tail, as in several earlier studies (Pertovaara et al., 2001; Miki et al., 2002; Pinto-Ribeiro et al., 2008; Sanoja et al., 2010; Sugino et al., 2012). As a result of this, RVM cells studied in the present study were designated as ON-like and OFF-like cells rather than ON- or OFF-cells. NEUTRAL-cells were not considered further in the present study.

The evaluation of the response of RVM cells to peripheral noxious stimulation consisted of the following assessments performed successively: (i) spontaneous activity (first 20 s of recording without any stimulation); (ii) response to heating of the tail (10 s or until a withdrawal response was observed), and (iii) response to pinching of the tail for 5 s with a surgical clamp that produced painful sensation when applied to the hand of the experimenter.

2.7. Course of the electrophysiological study

Electrophysiological recordings of RVM cells were performed three weeks following the induction of arthritis. Pharmacological manipulations started after the cell was classified as ON-like or OFF-like one and its spontaneous and noxious stimulation-evoked activity had been recorded. In a single session, one to four cells could be recorded simultaneously; the same cells were recorded throughout the whole session unless (1) the neurone stopped responding for more than an hour, in which case we would search for another recording site, or (2) the activity of the cell would not return to the same basal level as at the start of the recording in which case no more recordings were performed. Each session comprised the microinjection of SAL either GLU or LIDO, followed by a final injection of bicuculline. At the end of each recording session, an electrolytic lesion was made in the last recording site.

2.8. Drugs

Solutions for intracerebral drug injection in the DMH were prepared with sterilized saline 0.9% (Unither, Amiens, France; pH 7.2) except for LIDO, which was acquired as a solution. Each injection had a volume of 0.5 μL and contained either 50 nmol of GLU (Merck, Darmstadt, Germany) or LIDO 2% (B. Braun Medical, Barcarena, Portugal). These doses were chosen according to previous studies (Pinto-Ribeiro et al., 2008; Ansah et al., 2009).

2.9. Statistical analyses

Behavioral data analysis was performed using an independent-samples *t*-test and one-way analysis of variance (ANOVA). The potential effect of drugs upon the spontaneous and noxious-evoked activity of pain modulatory-like cells of the RVM at specific timepoints was examined by using two-way analysis of variance (ANOVA). *Post hoc* tests were performed using *t*-test with a Bonferroni correction for multiple comparisons. $P < 0.05$ was considered to represent a significant difference. Data in the results section is presented as mean ± S.D.

3. Results

3.1. Induction of monoarthritis failed to alter pain-like behavior induced by noxious stimulation of non-inflamed skin areas

All animals in the ARTH group had monoarthritis of the knee as indicated by vocalization every time after flexion-extension movement of the affected but not the contralateral knee joint. In NAIVE or SHAM animals, flexion-extension movement of the knee joint failed to produce vocalizations. The baseline TF was not significantly different among the experimental groups ($F_{2,25} = 3.340$, $P = 0.5682$; one-way-ANOVA) (Fig. 3A). Baseline PW latencies were not significantly different between the left and right hindpaws in the NAIVE ($t_{(0,636)} = 0.53$; paired *t*-test), SHAM ($t_{(0,491)} = 0.64$) or ARTH ($t_{(2,107)} = 0.07$) groups. Therefore, the mean PW data for left and right hindpaws in each experimental group were used in further calculations. The baseline PW was significantly different among the experimental groups ($F_{2,28} = 4.279$, $P < 0.0247$; one-way-ANOVA). The ARTH group had the longest PW latency (7.03 ± 0.13 s) in the baseline condition, while the PW latencies were in the same range in NAIVE (6.14 ± 0.26 s) and SHAM (5.94 ± 0.08 s) groups (Fig. 3B). One NAIVE animal and one SHAM animal were excluded from the experiment for severe weight loss during the first week of

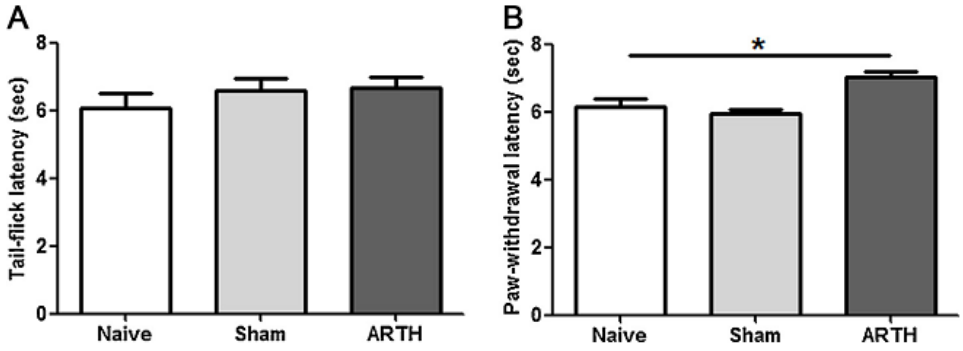


Fig. 3. Baseline evaluation of tail-flick (A) and paw-withdrawal (B) latencies in NAIVE, SHAM and arthritic (ARTH) animals. The graphs show the mean response latency + S.D. *$P < 0.05$ (reference in A and B: the NAIVE group).

behavioral assessment. We were unable to ascertain a probable cause for the weight loss, although we performed necropsis.

3.2. Effect of DMH manipulation upon nociceptive behavior

3.2.1. DMH activation increased nociceptive behavior in control but not arthritic animals

TF and PW latencies were assessed following GLU administration in the DMH to study phasic modulation of pain behavior by activation of the DMH. GLU in the DMH produced a significant decrease of the TF latency (main effect of GLU: $F_{1,56} = 33.37$, $P < 0.0001$). The TF latency was significantly different among the experimental groups (main effect of the experimental group: $F_{2,56} = 4.915$, $P = 0.01$) and the difference in the TF latency among experimental groups varied with the drug treatment (interaction between experimental group and drug treatment: $F_{2,56} = 4.928$, $P = 0.0107$). *Post hoc* testing indicated that the TF latency was decreased by GLU in the DMH only in the NAIVE and SHAM groups but not in the ARTH group (Fig. 4A).

Identically, GLU in the DMH induced a significant decrease of the PW latency (main effect of GLU: $F_{1,59} = 15.59$, $P = 0.0002$). PW latency was significantly different among the experimental groups (main effect of the experimental group: $F_{2,59} = 5.555$, $P = 0.0062$). Again, *post hoc* testing showed that the PW latency was decreased by GLU in the DMH only in the NAIVE and SHAM groups but not in the ARTH group (Fig. 4B).

3.2.2. DMH inhibition decreased nociceptive behavior in control but not arthritic animals

Lido in the DMH significantly increased TF latency (main effect of LIDO: $F_{1,65} = 24.94$, $P < 0.0001$) although this effect was not significantly different among experimental groups (main effect of the

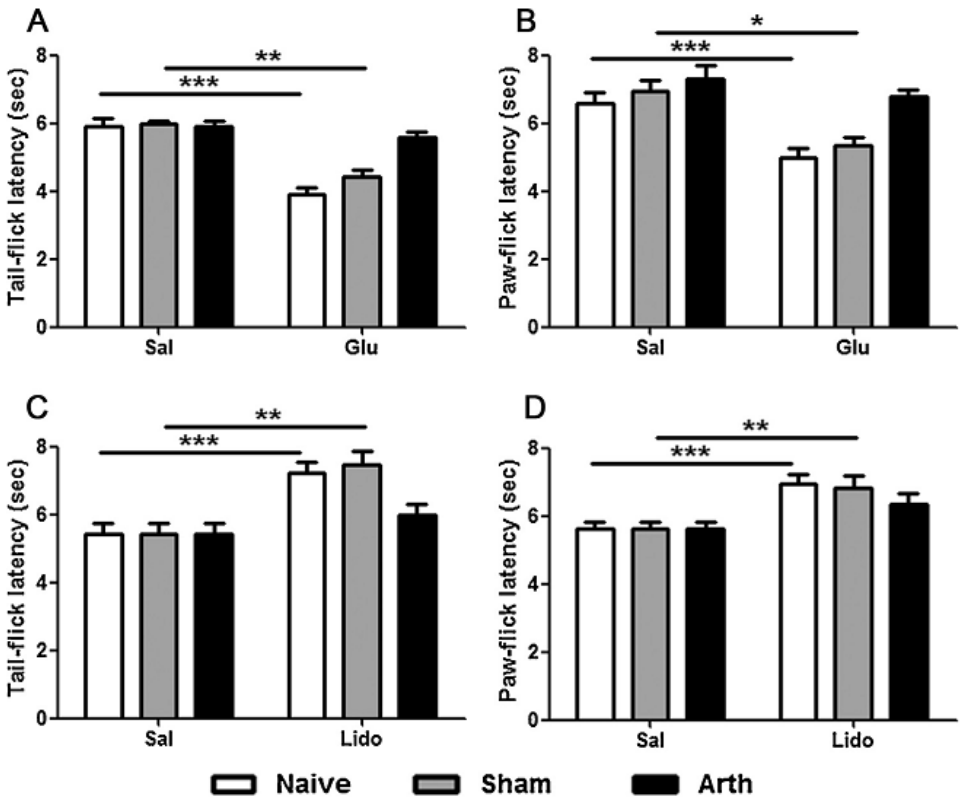


Fig. 4. Effect of DMH pharmacological manipulation of NAIVE, SHAM and arthritic (ARTH) animals on pain behavior assessed with the radiant heat-induced tail-flick (A, C) (TF) and paw-withdrawal (PW) (B, D) tests. (SAL – saline control; GLU – glutamate; LIDO – lidocaine). The graphs show the mean response latency + S.D. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (reference in A and B: the corresponding SAL group; reference in C and D: the corresponding PI group).

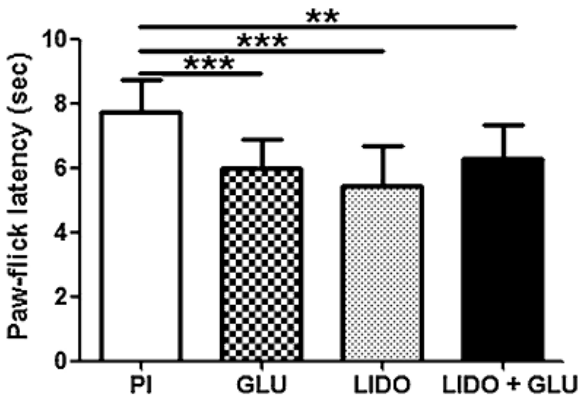


Fig. 5. Comparison of paw-withdrawal (PW) latencies in NAIVE animals after (i) microinjection of glutamate (GLU) into the DMH, (ii) lidocaine (LIDO) into the rostroventromedial medulla (RVM) and (iii) lidocaine into the RVM followed by glutamate into the DMH (20 min later). Note that all the pharmacological treatments significantly decreased PW latencies when compared to preinjection (PI) baseline responses, but no differences were observed between treatments. Graphs show mean latency + S.D. ($n = 14$). $^{**}P < 0.01$, $^{***}P < 0.001$ (t-test with a Bonferroni correction for multiple comparisons; reference: the PI group).

experimental group: $F_{2,56} = 2.451$, $P = 0.0941$). *Post hoc* testing indicated that the TF latency was increased by LIDO in the DMH only in the NAIVE and SHAM groups but not in the ARTH group (Fig. 4C).

Identically, LIDO in the DMH induced a significant increase in PW latency (main effect of LIDO: $F_{2,64} = 27.55$, $P < 0.0001$) although this effect was not significantly different among experimental groups (main effect of the experimental group: $F_{1,64} = 0.8238$, $P = 0.4434$). Again, *post hoc* testing showed that the PW latency was increased by LIDO in the DMH only in the NAIVE and SHAM groups but not in the ARTH group (Fig. 4D).

3.3. Increased nociceptive behavior by the DMH is mediated by the RVM

Next, we assessed whether the RVM is a possible downstream medullary relay for the DMH-induced pronociceptive action in normal animals. Therefore, we determined PW latency following administration of GLU into the DMH of SHAM animals with a LIDO-induced block of the RVM. For comparison, PW latency was also measured following administration of GLU into the DMH without a concomitant block of the RVM, or following a LIDO induced block of the RVM without a concomitant administration of GLU into the DMH. All three treatments (the activation of the DMH by GLU, the inhibition of the RVM by LIDO, or the concomitant inhibition of the RVM with LIDO and activation of the DMH by GLU) resulted in a decrease in PW latency that was significantly different from the pre-treatment baseline ($F_{3,55} = 13.51$, $P < 0.0001$). *Post hoc* tests indicated that there were no differences in the post-injection PW latencies among the three treatment groups (Fig. 5).

3.4. Effect of DMH manipulation upon the activity of RVM cells

3.4.1. The sample of RVM cells studied

A total of 102 RVM cells of SHAM animals were analyzed [69 ON-like cells: 39 ON-like cells tested with mechanical stimuli (MS; noxious pinch of the tail) and 30 ON-like cells tested with thermal stimuli (TS; noxious heating of the tail); 33 OFF-like cells: 19 OFF-like cells tested with MS and 14 OFF-like cells tested with TS], while the number of RVM cells analyzed in ARTH animals was 253 [137 ON-like cells: 71 ON-like cells tested with MS and 66 ON-like cells tested with TS; 116 OFF-like cells: 65 OFF-like cells tested with MS and 51 OFF-like cells tested with TS].

3.4.2. DMH manipulation altered the spontaneous activity of RVM cells in a pronociceptive fashion, an effect that was absent in arthritic animals

Microinjection of drugs into the DMH altered significantly the spontaneous activity of ON-like RVM cells ($F_{2,159} = 5.307$, $P = 0.0059$). This effect varied with the experimental group ($F_{2,159} = 11.18$, $P < 0.0001$). *Post hoc* testing indicated that GLU significantly increased ON-like cell spontaneous activity in the SHAM group while LIDO increased ON-like cell spontaneous activity in the ARTH group (Fig. 6A).

Similarly, spontaneous activity of OFF-like RVM cells was altered by drug treatments of the DMH ($F_{2,90} = 10.38$, $P < 0.0001$), an effect that also varied with the experimental group ($F_{2,90} = 12.37$, $P < 0.0001$). *Post hoc* testing showed that GLU significantly decreased OFF-like cell spontaneous activity only in the SHAM group whereas LIDO had no effect on spontaneous activity of OFF-like cells, independent of the experimental group (Fig. 6B).

3.4.3. DMH manipulation altered the noxious stimulation-evoked responses of RVM cells, an effect that was absent in arthritic animals

Drug treatments of the DMH had a significant main effect on the magnitude of the heat stimulation-evoked and or mechanical stimulation-evoked response in RVM ON-like cells (HEAT: $F_{2,77} = 14.66$, $P < 0.0001$ and MECHANICAL: $F_{2,95} = 26.18$, $P < 0.0001$), which varied between experimental groups (HEAT: $F_{2,77} = 13.35$, $P < 0.0001$ and MECHANICAL: $F_{2,95} = 26.22$, $P < 0.0001$). *Post hoc* testing showed that GLU increased and LIDO decreased ON-like cell responses to heat in SHAM animals whereas no effect was observed in the ARTH group (Fig. 7A, C). Concomitantly, drug treatment of the DMH also significantly altered the noxious stimulation-evoked activity of OFF-like RVM cells (HEAT: $F_{2,47} = 22.34$, $P < 0.0001$ and MECHANICAL: $F_{2,66} = 43.52$, $P < 0.0001$). Again, the effect of drug treatment on the peripheral stimulation-evoked responses of OFF-like cells varied with experimental group (HEAT: $F_{2,47} = 18.13$, $P < 0.0001$ and MECHANICAL: $F_{2,66} = 47.03$, $P < 0.0001$). Although no effect upon ARTH animals was observed, in the SHAM group GLU decreased and LIDO increased RVM OFF-like cell responses during heat stimulation (Fig. 7B, D).

4. Discussion

In line with earlier results (Martenson et al., 2009), our study indicates that the DMH has a pronociceptive role, enhancing acute pain-like behavior of rodents in a phasic and tonic fashion. Moreover, the DMH-induced facilitation of pain behavior was accompanied by the induction of pronociceptive changes in the activity of pain-modulatory RVM ON-like and OFF-like cells of healthy control animals as in the study of Martenson et al. (2009). Importantly, in contrast to other supraspinal areas that enhance nociception in acute and chronic pain conditions (Almeida et al., 1996, 1999; Pinto et al., 2008; Sotgiu et al., 2008; Martins et al., 2010), the novel finding of the present study was that the DMH-induced facilitation of pain-like behavior was absent in arthritic animals. Concomitantly, the pronociceptive effect of the DMH upon pain-regulating RVM cells was also absent in arthritic animals. The present data suggests that the pronociceptive role of the DMH is restricted to acute pain but is lost or altered in prolonged pain conditions (like arthritis).

4.1. Role of the DMH in acute pain modulation

In accordance to a potential role in pain facilitation, the DMH activation by GLU and inhibition by LIDO clearly demonstrated a facilitatory role for this nucleus in acute pain behavior. These results are in line with earlier data showing that disinhibition of the DMH

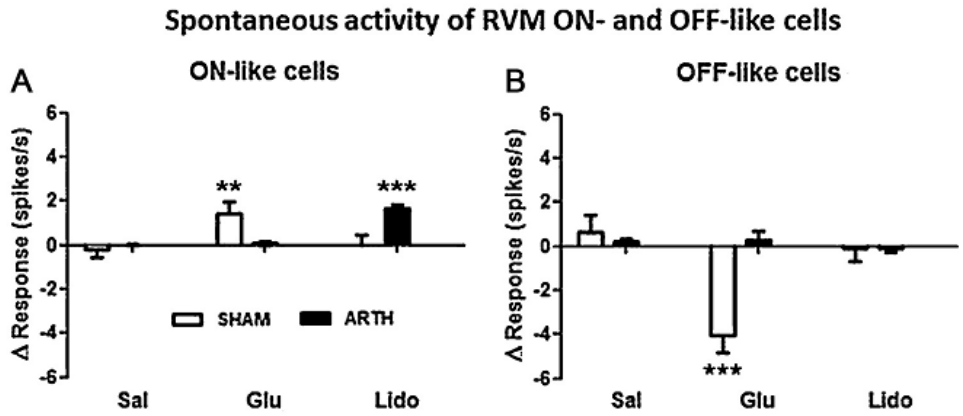


Fig. 6. Effect of DMH drug administration on the spontaneous activity of rostroventromedial medullary (RVM) ON-like (A) and OFF-like (B) cells in SHAM and arthritic (ARTH) animals. (Sal – saline; Glu – glutamate; Lido – lidocaine). Results are presented as mean + SEM (ON-like cells, n_{SHAM} = 16 and n_{ARTH} = 28; OFF-like cells, n_{SHAM} = 5 and n_{ARTH} = 21). ***P < 0.001.

by bicuculline methiodide, a $GABA_A$ receptor antagonist, induces behavioral hyperalgesia (Martenson et al., 2009), a finding that was replicated also in the present study.

In an attempt to integrate DMH and brainstem activity, Zaretskaia et al. (2008) evaluated the level of c-Fos expression in the rat brain after disinhibition of the DMH. Among central nervous system regions in which DMH disinhibition enhanced c-Fos expression was the RVM (Zaretskaia et al., 2008). This is noteworthy since the RVM, together with some other brainstem areas (Lima and Almeida, 2002; Kincaid et al., 2005; Waters and Lumb, 2008; Marques-Lopes et al., 2010), is able to facilitate nociception. Additionally, electrophysiological studies have already demonstrated a pronociceptive action of the DMH on the RVM (Heinricher et al., 2009). In the present study, GLU in the DMH of healthy control animals induced pronociceptive effects both on noxious stimulus-evoked responses and spontaneous activities of RVM ON-like and OFF-like cells. Thus, the GLU-induced phasic pronociceptive effect in control animals can be explained by a direct descending action

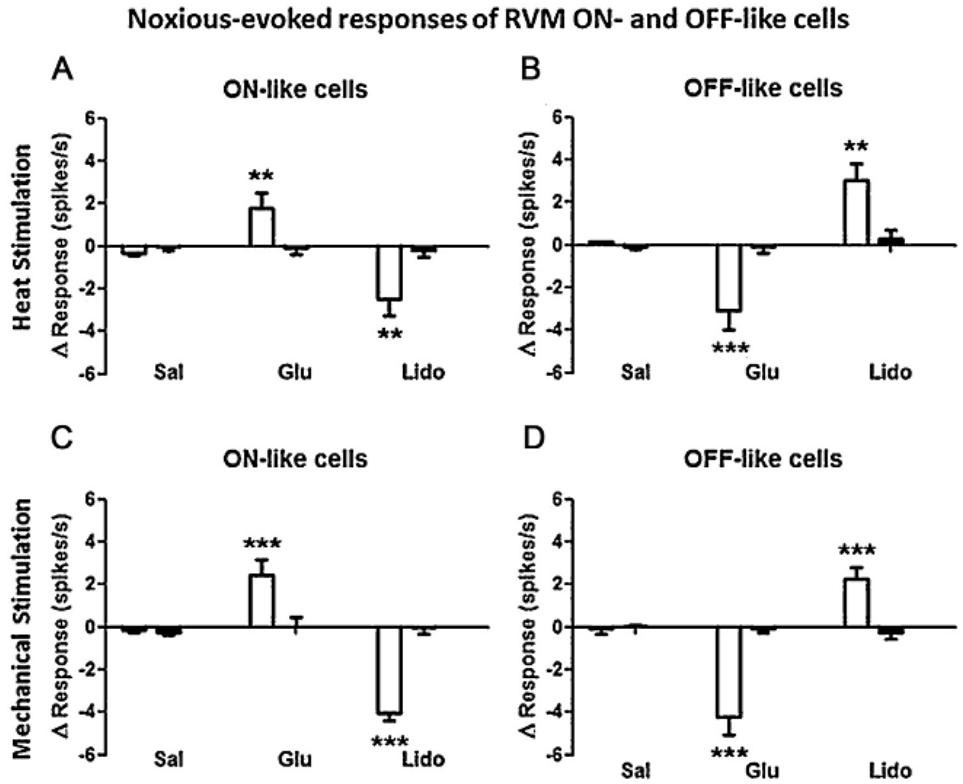


Fig. 7. Effect of DMH drug administration on the noxious heat- (A, B) and mechanically-evoked (C, D) responses of rostroventromedial medullary (RVM) ON-like (A, C) and OFF-like (B, D) cells in SHAM and arthritic (ARTH) animals. (Sal – saline; Glu – glutamate; Lido – lidocaine). *Y*-Axis shows the noxious stimulation-induced change in the discharge rate. 0 spikes/s represents the spontaneous discharge rate before GLU injection, values > 0 spikes/s represent stimulus-evoked increases and values < 0 spikes/s represent stimulus-induced decreases in the discharge rate. Results are presented as mean + SEM (SAL: ON-like cells, n_{SHAM} = 16 and n_{ARTH} = 28; OFF-like cells, n_{SHAM} = 5 and n_{ARTH} = 21; GLU: ON-like cells, thermal testing: n_{SHAM} = 11, n_{ARTH} = 18, mechanical testing: n_{SHAM} = 15, n_{ARTH} = 24; OFF-like cells, thermal testing: n_{SHAM} = 6, n_{ARTH} = 17, mechanical testing: n_{SHAM} = 6, n_{ARTH} = 22; LIDO: ON-like cells, thermal testing: n_{SHAM} = 8, n_{ARTH} = 17, mechanical testing: n_{SHAM} = 9, n_{ARTH} = 22; OFF-like cells, thermal testing: n_{SHAM} = 6, n_{ARTH} = 8, mechanical testing: n_{SHAM} = 11, n_{ARTH} = 11). *P < 0.05, **P < 0.01, ***P < 0.001.

of the DMH on RVM cells that was followed by enhanced spinal nociceptive transmission. The hypothesis that the phasic pronociceptive effect induced by activation of the DMH in control animals was mediated by the RVM rather than other areas of the brain is partly supported by the finding that GLU activation of the DMH after blocking the RVM failed to result in an additional increase in the descending pronociceptive effect (blocking the RVM is already pronociceptive by itself).

LIDO treatment was used to study tonic control of nociception by the DMH. Blocking the DMH with LIDO in control animals failed to change the spontaneous activity of RVM ON-like or OFF-like cells while it reduced the magnitude of the noxious stimulation-evoked responses of RVM ON- and OFF-like cells. The reduction in stimulus-evoked responses of RVM cells following LIDO treatment of the DMH was accompanied by a reduction in stimulus-evoked pain behavior. This finding suggests that, in control animals, the tonic descending effect of the DMH on RVM cells and pain behavior is presynaptic rather than postsynaptic to the RVM. This hypothesis is in line with the work of Yoshimura and Hidemasa (2006) that showed that evoked activity of spinal neurones was under pre-synaptic control of descending noradrenergic projections.

Several authors have reported that the RVM contributes to the descending modulation of nociception in chronic pain conditions (*e.g.*, Pertovaara et al., 1996; Vera-Portocarrero et al., 2011; Ambriz-Tututi et al., 2011; Mase et al., 2011). Spinally-projecting RVM neurones, known to be involved in this process (Urban et al., 1999; Porreca et al., 2002) include those expressing $GABA_A$ (Heinricher and Kaplan, 1991) receptors. Interestingly, the existence of a GABAergic output from the DMH (Acosta-Galvan et al., 2011) and of DMH neurons projecting to the RVM (Thompson et al., 1996) have already been demonstrated. Therefore, it is conceivable that the acute hyperalgesia observed in control animals following GLU administration into the DMH might result from activation of DMH GABAergic projecting neurones that target spinally-projecting RVM neurones. Further studies involving pharmacological manipulations of the spinal cord and RVM during DMH activation should clarify the neurotransmitter mechanisms of this hypothalamic–medullary interaction.

4.2. Role of the DMH in arthritis

The failure to influence pain behavior by activation or inhibition of the DMH in arthritic animals, which was paralleled by a similar failure in altering the activity of RVM nociceptive neurones was intriguing. An increase in the descending pronociceptive drive originating in the DMH would have been expected as chronic inflammatory disorders lead to changes toward pronociception in the balance of descending modulation (Miki et al., 2002; Dubner, 2005; Woolf and Salter, 2006; Lagraize et al., 2010). On the other hand, chronic inflammation also results in an increased descending antinociceptive drive (Cervero et al., 1991; Danziger et al., 2001) in an attempt to counteract increased pain. It is conceivable that the loss of descending pain-facilitatory effect by the DMH following development of arthritis might also result from this compensatory phenomenon.

An aspect to take into consideration is that we did not evaluate primary hyperalgesia (knee). While it has been demonstrated (Urban et al., 1999) that hyperalgesia resulting from an injury is not restricted to the lesioned region, some authors argue that descending pain modulatory pathways differently modulate peripheral inputs according to their location (Vanegas and Schaible, 2004). Indeed, Urban and Gebhart (1999) showed the occurrence of descending facilitation of nociceptive inputs originating from healthy areas while inputs arising from the injury site were inhibited. Interestingly, RVM lesions would prevent the descending facilitatory drive of inputs originating from healthy areas surrounding the lesion site but not from the lesion site (Urban and Gebhart, 1999). In this context, the loss of the DMH-induced pain facilitation in arthritic animals may result from the remodeling of the pathways signaling and modulating nociception in prolonged chronic conditions, both at the spinal and supraspinal levels. Whether this "shut-down" of the DMH is associated with changes in the neurochemistry of the hypothalamus or the brainstem remains to be determined.

At the hypothalamic level, we have previously demonstrated that after the induction of arthritis, the paraventricular nucleus of the hypothalamus (PVN) tonically enhanced the firing rate of antinociceptive RVM OFF-like cells, in parallel to an antinociceptive behavioral effect (Pinto-Ribeiro et al., 2008). Similarly, it is possible that in the present study arthritis enhanced the tonic inhibitory drive from the hypothalamus, either locally by inhibiting pronociceptive DMH activity, at the brainstem level by inhibiting pronociceptive activity of RVM cells, or/and spinally by inhibiting nociceptive transmission.

Interestingly, the increase in the spontaneous activity of pronociceptive RVM ON-like cells after blocking the DMH with LIDO in ARTH animals was an exception to the general data, suggesting the existence of a tonic inhibitory descending drive in prolonged arthritis. This result not only further supports the hypothesis of the existence of hypothalamic plasticity in chronic inflammation but also implicates that the DMH may contribute to a tonically-mediated inhibition of pronociceptive RVM cells after the development of arthritis. This tonic inhibition of the RVM ON-like cell activity by the DMH in arthritic animals, however, did not produce a net change in the balance of descending pain-facilitatory and -inhibitory controls as indicated by the failure to produce a change in pain behavior of arthritic animals after blocking the DMH. This is probably due to the development of a strong pronociceptive descending drive in the chronic phase of arthritis (Danziger et al., 2001).

5. Conclusions

The DMH plays a role in acute pain facilitation in normal animals, and this effect is associated with pronociceptive alterations in the activity of pain-regulating RVM cells. However, in arthritic animals, the DMH-induced pronociception is lost, probably due to remodeling of the hypothalamic circuits mediating nociception. Additionally, a tonic descending inhibitory drive originating in the DMH was present in arthritic but not control animals, further supporting the hypothesis of remodeling of hypothalamic circuits in chronic inflammatory conditions.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Chapter 2.3

Amorim D, David-Pereira A, Marques P, Puga S, Rebelo P, Costa, P, Pertovaara A, Almeida A, Pinto-Ribeiro F

A role of supraspinal galanin in behavioural hyperalgesia in the rat

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A role of supraspinal galanin in behavioural hyperalgesia in the rat

Diana Amorim[1,2], Ana David-Pereira[1,2], Patrícia Marques[1,2], Sónia Puga[1,2], Patrícia Rebelo[1,2], Patrício Costa[1,2], Antti Pertovaara[3], Armando Almeida[1,2] and Filipa Pinto-Ribeiro[1,2#]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), Campus of Gualtar, University of Minho, 4750-057 Braga, Portugal

[2]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

[3]Institute of Biomedicine/Physiology, University of Helsinki, Helsinki, Finland

Diana Amorim (dianaamorim@ecsaude.uminho.pt)

Ana David-Pereira (anapereira@ecsaude.uminho.pt)

Patrícia Marques (parmarques87@gmail.com)

Sónia Puga (soniapuga@ecsaude.uminho.pt)

Patrícia Rebelo (patriciarebelo@ecsaude.uminho.pt)

Patrício Costa (pcosta@ecsaude.uminho.pt)

Antti Pertovaara (antti.pertovaara@helsinki.fi)

Armando Almeida (aalmeida@ecsaude.uminho.pt)

Filipa Pinto-Ribeiro (filiparibeiro@ecsaude.uminho.pt)

#Corresponding author:

Professor Filipa Pinto-Ribeiro

Email: filiparibeiro@ecsaude.uminho.pt

Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS),

Campus de Gualtar, University of Minho, 4750-057 Braga, Portugal

Telf: +351 253604852 Fax: +351 253604809

Abstract

In chronic pain disorders, galanin (GAL) is able to either facilitate or inhibit nociception in the spinal cord but the contribution of supraspinal galanin to pain signalling is mostly unknown. The dorsomedial nucleus of the hypothalamus (DMH) is rich in galanin receptors (GALR) and is involved in behavioural hyperalgesia. In this study, we evaluated the contribution of supraspinal GAL to behavioural hyperalgesia in experimental monoarthritis.

In Wistar-Han males with a four week kaolin/carrageenan-induced monoarthritis (ARTH), paw-withdrawal latency (PWL) was assessed before and after DMH administration of exogenous GAL, a non-specific GALR antagonist (M40), a specific GALR1 agonist (M617) and a specific GALR2 antagonist (M871). Additionally, the analysis of c-Fos expression after GAL injection in the DMH was used to investigate the potential involvement of brainstem pain control centres. Finally, electrophysiological recordings were performed to evaluate whether pronociceptive On- or antinociceptive Off-like cells in the rostral ventromedial medulla (RVM) relay the effect of GAL.

Exogenous GAL in the DMH decreased PWL in ARTH and SHAM animals, an effect that was mimicked by a GALR1 agonist (M617). In SHAM animals, an unselective GALR antagonist (M40) increased PWL, while a GALR2 antagonist (M871) decreased PWL. M40 or M871 failed to influence PWL in ARTH animals. Exogenous GAL increased c-Fos expression in the RVM and dorsal raphe nucleus (DRN), with effects being more prominent in SHAM than ARTH animals. Exogenous GAL failed to influence activity of RVM On- or Off-like cells of SHAM and ARTH animals.

Overall, exogenous GAL in the DMH had a pronociceptive effect that is mediated by GALR1 in healthy and arthritic animals and is associated with alterations of c-Fos expression in RVM and DRN that are serotoninergic brainstem nuclei known to be involved in the regulation of pain.

Keywords: experimental monoarthritis, pronociception, galanin, galanin receptor type-1, dorsomedial hypothalamic nucleus, rostral ventromedial medulla.

1. Introduction

Galanin (GAL) is an injury-responsive peptide that is dramatically upregulated in the dorsal root ganglia and spinal dorsal horn interneurones during inflammation (Jimenez-Andrade et al., 2004) or after nerve injury (Liu and Hökfelt, 2002). In healthy animals, GAL's action on nociceptive processing in the spinal cord is bidirectional, with low concentrations eliciting pronociceptive actions (Reeve et al., 2000) and high concentrations promoting antinociception (Yue et al., 2011). Differences in spinal actions of GAL also vary with the differential availability/activation of GAL receptor (GALR) subtypes. GALR1 has an inhibitory action and is more abundant than GALR2 (excitatory) and GALR3 (inhibitory) in the superficial dorsal horn (Brumovsky et al., 2006). Despite the considerable number of works evaluating its action in the peripheral nervous system and at the spinal cord level, the role of GAL in pain modulation at the supraspinal level is mostly unknown.

In basal conditions several studies showed that, both in humans and rodents, GAL is expressed in the supraoptic nucleus, the paraventricular nucleus of the hypothalamus, the dorsomedial hypothalamic nucleus (DMH), the arcuate nuclei, the lateral hypothalamic area, the locus coeruleus (LC), the amygdala (AMY) and the median raphe nucleus (Ch'ng et al., 1985), all areas involved in supraspinal pain modulation (Millan, 2002; Almeida et al., 2006; Pertovaara and Almeida, 2006; Pinto-Ribeiro et al., 2008, 2013). In relation to receptor expression, GALR1 is greatly expressed in the LC, dorsal raphe nucleus (DRN), the paraventricular nucleus of the hypothalamus, DMH, AMY, thalamus and medulla oblongata (Mitchell et al., 1997; Waters and Krause, 2000; Lu et al., 2005; Webling et al., 2012). However, in the AMY, GALR2/R3 are also significantly expressed (Lu et al., 2005). Similarly, all types of GAL receptors are expressed in the prefrontal cortex and the hippocampus but to a lesser extent (Waters and Krause, 2000; Lu et al., 2005; Webling et al., 2012). GALR2 is highly expressed in the hypothalamus, dentate gyrus, piriform cortex and mammillary nuclei (Waters and Krause, 2000; Webling et al., 2012), while the expression of GALR3 has been reported mainly in the hypothalamus (preoptic, DMH, lateral and posterior hypothalamic, ventromedial and premammillary nuclei) (Webling et al., 2012), the bed nucleus of the stria terminalis, periaqueductal grey matter (PAG), lateral parabrachial nucleus and medial reticular formation (Mennicken et al., 2002). Again, most brain areas mentioned above are involved in the codification and modulation of nociceptive inputs (Millan, 2002; Pertovaara and Almeida, 2006).

The administration of exogenous GAL to the arcuate (Sun et al., 2003), tuberomammillary (Sun et al., 2004), nucleus accumbens (Xu et al., 2012), central nucleus of the AMY (Jin et al., 2010; Li et al., 2012) and PAG (Wang et al., 2000) decreases nociception in healthy rats, an effect that is mediated by GalR1 in rodents (Kong and Yu, 2013). A similar effect is observed in some pathological conditions, such as acute inflammation or mononeuropathy (Wang et al., 2000), where the microinjection of supraspinal exogenous GAL also decreases nociception. Albeit the apparent antinociceptive role of supraspinal GAL in pain modulation, the intracerebroventricular administration of a GALR1 agonist in rats increased c-Fos expression in the DMH (Blackshear et al., 2007), an area that facilitates nociception by promoting behavioural hyperalgesia (Martenson et al., 2009; Pinto-Ribeiro et al., 2013). As hyperalgesia is one of the hallmarks of chronic pain, activation of the DMH promotes behavioural hyperalgesia and GAL receptors are strongly expressed in the DMH, here we evaluated the contribution of GAL receptors in the DMH to the descending control of inflammatory hyperalgesia in monoarthritis as well as nociception in healthy controls.

2. Methods

2.1. Animals, ethical issues and anaesthesia

The experiments were performed in adult male Wistar Han rats with 175–250g (Charles Rivers, Barcelona, Spain). A total of 96 animals (SHAM, n=48 and ARTH, n=48) were used in the experiments herein, 40 animals (SHAM, n=20 and ARTH, n=20) were used in the behavioural assessment, 32 animals (SHAM, n=16 and ARTH, n=16) in the c-Fos protocol and 24 animals (SHAM, n=12 and ARTH, n=12) in the electrophysiological evaluation. Animals were randomly assigned two by two to boxes upon arrival; a blue line was painted in the tail of one rat and a red line in the tail of the other. Each box was numbered from 1 to 48, no indication concerning if the animals were assigned to the SHAM or ARTH group was displayed. The list discriminating the boxes corresponding to the SHAM or ARTH groups was kept by an independent party. Each animal was considered a single unit within its experimental group. Animals were housed two per cage, except for animals with chronic intracerebral cannulae implanted that were housed individually. Food and water were available *ad libitum* and animals were maintained in a climate-controlled room, under 22±2°C of temperature, 55±5% of humidity and under a 12h light/dark cycle with lights on at 8:00am. The experimental protocol followed the European Community

Council Directive 86/609/EEC and 2010/63/EU concerning the use of animals for scientific purposes and was approved by the Institutional Ethical Commission (Permit Number: 23248). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

For cannula implantation the animals were anaesthetized i.p. with a mixture 1:1.5 of ketamine (Imalgene®, Merial, Oeiras, Portugal) and medetomidine (Dorbene®, Esteve, Carnaxide, Portugal). After the surgical procedure, the anaesthesia was reversed using atipamezole (Antisedan®, Pfizer, Oeiras, Portugal, i.p.) and the animals were monitored until fully awake (grooming and eating).

Anaesthesia was induced by administering pentobarbitone (50 mg/kg, i.p., Eutasil®, CEVA, Algés, Portugal) and maintained by infusing pentobarbitone (15–20 mg/kg/h, i.p.). The level of anaesthesia was frequently assessed by determining behavioural responses to noxious pinching. Body temperature was maintained within physiological range with the help of a warming blanket (DC Temperature Controller, FHC, Bowdoin, ME, USA). At the end of the experiment, animals received a lethal dose of pentobarbitone.

2.2. Induction of arthritis

The induction of monoarthritis (ARTH) was performed four weeks before the actual experiments, as described in detail elsewhere (Ansah and Pertovaara, 2007; Pinto-Ribeiro et al., 2013). In order to maintain the researcher blind in relation to whether the animals from a specific box were assigned to the SHAM or ARTH groups, the animals were anaesthetized (section 2.1) by a third party in an adjacent room and then brought to the chirurgical table in groups of two for the injection of SAL or K/C in the right knee joint. Briefly, in anaesthetised animals a mixture of 3% kaolin and 3% carrageenan (K/C, Sigma-Aldrich, St. Louis, MO, USA) dissolved in saline was injected into the synovial cavity of the right knee joint at a volume of 0.1 mL. This model produces mechanical hyperalgesia, which begins a few hours after surgery and extends up to 8 weeks (Radhakrishnan et al., 2003). After the procedure, animals returned to the adjacent room, the anaesthesia was reversed and animals were monitored until fully recovered (eating and grooming). At the end of the induction session all boxes were returned to the animal house. In each animal, development of arthritis was verified again 1 h prior to each behavioural session. While confirming the arthritic status of the animals, through the flexion and extension of the right

leg, the experimenter was handed the animals by a third party without any specific order and without prior knowledge of the box number. Only those rats that vocalized every time after five flexion–extension movements of the knee joint were considered to have arthritis, and they were included in the ARTH group. SHAM animals were injected with 0.1 mL saline in the synovial cavity of the right knee joint. SHAM animals did not vocalize to any of the five consecutive flexion–extension movements of the knee joint. After the test, the animals were returned to their home cages by a person other than the evaluator.

2.3. Behavioural assessment of nociception

All behavioural tests were performed during the day time, starting at 9:30am and ending at 1:30pm after which the animals were returned to the animal house.

2.3.1. Mechanical hyperalgesia

The application of noxious pressure to the primary site of injury is a classical approach to measure mechanical hyperalgesia (Randall and Selitto, 1957), both in humans and animals (Rivat et al., 2008). Here, the pressure application measurement (PAM; Ugo Basile, Comerio, Italy) method was used. It allows an accurate behavioural measurement of mechanical hypersensitivity in rodents with chronic inflammatory joint pain (Barton et al., 2007) by the application of a force range of 0–1500g. To perform the test and with the animal securely held, the force transducer unit (fitted to the experimenter's thumb) is placed on one side of the animal's knee joint and the forefinger on the other and an increasingly force is applied across the joint until a behavioural response is observed (limb-withdrawal, freezing of whisker movement, wriggling or vocalization) with a cut-off of 5 s. The peak force applied immediately prior to the behavioural response is recorded as the response threshold (RT). RT was measured twice in the ipsilateral and contralateral limbs at 1 min intervals. The mean RTs were calculated per animal. At the end of the session animals were returned to their home cage.

2.3.2. Thermal hyperalgesia (heat)

Heat hyperalgesia was evaluated using the Hargreaves test (Hargreaves et al., 1988). The rats were habituated to the experimental conditions by allowing them to spend 1–2 h daily in the

experimental room for the three days preceding any behavioural tests (Pinto-Ribeiro et al., 2013). For assessing heat hyperalgesia, a radiant heat source was placed under the hindpaws in awake animals and the time spent between the heat application and the withdrawal response (Plantar Test Instrument, Model 37370, Ugo Basile, Varese, Italy) was registered as the paw-withdrawal latency (PWL). In each session, the PWL was assessed prior to drug administration in the DMH and 20 min after. In each time point, the PWL was repeated twice at an interval of 1 min and the mean of these values was used in further calculations. Cut-off time was 15 s.

2.4. Procedures for intra-DMH microinjections

Before the placement of the guide cannulas the animals were anaesthetized (section 2.1) by a third party in an adjacent room and then brought to the chirurgical table one at the time. For intra-DMH drug administration, four weeks before the actual experiments (at the same time that arthritis was induced), animals were anaesthetised and placed in a stereotaxic frame, and one stainless steel guide cannula (26 gauge; PlasticsOne, Roanoke, VA, USA) was then implanted in the DMH according to the coordinates of the atlas by Paxinos and Watson (Paxinos and Watson, 2007). The tip of the guide cannula was positioned 1 mm above the desired injection site in the DMH [AP, -3.24 mm from bregma; LM, 0.4 mm lateral from the midline (right side); DV, 7.5 mm below the surface of the skull]. The guide cannula was kept in place through the use of two dental screws and dental cement. A dummy cannula was inserted into the guide cannula to close the top. After the procedure, the anaesthesia was reversed and animals were monitored until being fully recovered (eating and grooming) in the adjacent room and returned to the animal house.

In order for the experimenter to remain blinded in relation to which animals were SHAMs or ARTHs, prior to the beginning of the behavioural session, the cards displaying the number of the box were substituted by cards displaying letters. Test drugs were administered in the DMH through a 33-gauge injection cannula (PlasticsOne) inserted into and protruding 1 mm beyond the tip of the guide cannula. The microinjection was made using a 10.0-μL-Hamilton syringe connected to the injection cannula by a polyethylene catheter (PE-10; Plastics One). The injection volume was 0.5 μL and therefore, the spread of the injected drugs within the brain was expected to be 1 mm (Myers, 1966). The efficacy of injection was monitored by observing the movement of a small air bubble through the tubing. The injection lasted 20 s and the injection cannula was

left in place for additional 30 s to minimize the return of drug solution back to the injection cannula. Brain injection sites were histologically verified from post-mortem sections and plotted on standardized sections from the stereotaxic atlas (Paxinos and Watson, 2007) (**Fig. 1**). After the completion of the tests and animals were returned to the animal house, the cards were switch again. The attribution of the letter cards was recorded in a lab book separate from the one used to register the results. The order of attribution of the letter cards was random and changed in each experimental session. The order of the administration of the drugs to each animal was defined at the beginning of the experiment to avoid potential confounding effects related to this parameter. The results of the tests were only associated with the respective animal after the end of the experiment.

2.5. Drugs

Solutions for drug administration in the DMH were prepared in sterilized saline 0.9% (Unither, Amiens, France; pH 7.2). All the experimental drugs used in this work were acquired from Tocris (Bristol, UK). Each injection had a volume of 0.5 µL and contained either GAL (1.0 nmol), a non-specific GAL receptor antagonist (M40, 1.0 nmol), a specific GALR1 agonist (M617, 1.0 nmol) or a specific GALR2 antagonist (M871, 1.0 nmol) (Sun et al., 2003; Jin et al., 2010; Kong and Yu, 2013). Control injections were performed with SAL in order to avoid any confounding effect that might result from injecting the liquid itself.

2.6. Course of the pharmacological study

Four weeks following induction of arthritis and insertion of the guide cannula for DMH injections, the efficacy of DMH-induced phasic and tonic modulation of nociception was determined by assessing the effect of DMH injection of exogenous GAL, M40, M671 and M871 upon the PWL in awake SHAM and ARTH animals. SAL was used in control injections. The latency of the withdrawal response was assessed 20 min (Sun et al., 2004, 2007) following the intra-DMH injections. The interval between behavioural assessments of different drug treatment conditions in the same animal was at least two days. The order of testing different drugs varied between the animals.

2.7. Recording of neuronal activity in nociceptive RVM cells

For the electrophysiological study, animals were removed from the animal house in a random order, one per day, already anaesthetized, by a person other than the experimenter. Anaesthesia (section 2.1) was administered at 9:30am, the electrophysiological recordings started between 10am and 10:30 am and lasted for 3h. The order of the administration of the drugs varied between the animals. The electrophysiological recordings of the activity of RVM neurones followed a protocol described in Pinto-Ribeiro and colleagues (Pinto-Ribeiro et al., 2013). In anaesthetised animals, a recording electrode was placed in the RVM (AP: 5.88 mm rostral to the interaural line, ML: -0.6 to 0.6 mm lateral from the midline, and DV: 10.0 mm below the surface of the skull) (Paxinos and Watson, 2007). Single neurone activity was recorded extracellularly with tungsten electrodes (tip impedance 3–10 MΩ at 1 kHz), the signal was amplified and filtered and data sampling was performed through a CED Micro 1401 interface and Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

Recording of RVM neurones was started after the animal was under light anaesthesia; i.e., the animals gave a brief withdrawal response to noxious pinch, but the pinch did not produce any longer lasting motor activity, nor did the animals have spontaneous limb movements. RVM neurones were classified based on their response to noxious heating of the tail with a tail-flick device (Ugo Basile). Heat stimulation of the tail was applied during 10 s. Functional classification of RVM neurones followed the scheme developed earlier by Fields and colleagues (Fields et al, 1983) and by Fields and Heinricher (Fields and Heinricher, 1985). The neurones whose firing activity increased during heat stimulation of the tail were considered On-cells, those decreasing its activity were classified as Off-cells and finally, cells displaying only a negligible (< 10%) or no alteration in discharge rates during noxious stimulation were considered Neutral-cells and were not analysed in this study. However, a significant difference with the classification scheme of Fields and colleagues (Fields et al., 1983) is that in the present study the noxious stimulus-induced withdrawal reflex was not taken into account in the classification. Therefore, as in previous studies, RVM cells are here called On-like and Off-like cells (Sanoja et al., 2010; Pinto-Ribeiro et al., 2013; Song et al., 2013) rather than On- or Off-cells.

The characterization of the response properties of RVM cells consisted of the following assessments performed successively: (i) spontaneous activity; (ii) response to heating of the tail; (iii) recovery to the spontaneous activity level.

It should be noted that when analysing responses of RVM neurones to peripheral stimulation, the baseline discharge frequency (recorded just before the stimulation) was subtracted from the discharge frequency assessed during the stimulation using the following formula:

Evoked response = (cell activity during acute noxious stimulation) – (basal cell activity prior to stimulus application)

Thus, positive values represent an increase and negative ones represent a decrease in cell activity evoked by peripheral stimulation.

During the recordings, animals also had a guide cannula implanted for drug administration into the DMH. After determining the baseline spontaneous activity of RVM cells and their baseline noxious-evoked responses to peripheral stimulation, either exogenous GAL or a non-specific GALRs antagonist (M40) were microinjected in the DMH, in order to assess its phasic or tonic effect, respectively, upon the discharge rate of RVM neurones. All results from drug administrations were plotted for the variation in activity comparing baseline (before drug administration) and values obtained 20 min after the injection into the DMH. The results of the electrophysiological analysis were only associated with the respective animal after all recordings were performed.

2.8. Course of the electrophysiological study

Electrophysiological recordings of RVM neurones (SHAM: On-like cells, n=58 and Off-like cells, n=40; ARTH: On-like cells, n=46 and Off-like cells, n=47) were performed under pentobarbitone anaesthesia four weeks after the administration of K/C (ARTH) or SAL (SHAM) in the right knee of animals. In RVM recordings, the response properties of nociceptive neurones were assessed by determining their spontaneous activity and the response to noxious heating of the tail. Search for the next neurone to be studied started about 30 min after testing of the previous one was completed. At the end of the recording session, electrolytic lesions were made in the recording sites, the animals were given a lethal dose of pentobarbitone and the brains were removed for histological verification of the recording and injection sites.

2.9. c-Fos study

For the c-Fos induction protocol, animals were removed from the animal house in a random order, one per day, already anaesthetized, by a person other than the experimenter. Anaesthesia (section 2.1) was administered at 9:30am, the protocol started between 10am and 10:30am and lasted for 2h. To evaluate changes in brain activation after exogenous GAL administration in the DMH and/or peripheral noxious stimulation in SHAM and ARTH animals, c-Fos immunoreaction was performed following the protocol described elsewhere (Morgado and Tavares, 2007). Animals were held in a stereotaxic frame. For drug administration, a guide cannula was placed in the DMH according to the coordinates of the atlas by Paxinos and Watson (Paxinos and Watson, 2007) and one of the following protocols was performed: (i) SAL microinjection in the DMH of SHAM animals; (ii) exogenous GAL microinjection in the DMH of SHAM animals; (iii) SAL microinjection in the DMH and extension of right limb of SHAM animals; (iv) exogenous GAL microinjection in the DMH and extension of right limb of SHAM animals; (v) SAL microinjection in the DMH of ARTH animals; (vi) exogenous GAL microinjection in the DMH of SHAM animals; (vii) extension of right limb of ARTH animals; (viii) exogenous GAL microinjection in the DMH and extension of right limb of ARTH animals. Two exogenous GAL (or SAL) doses were injected in the DMH with a 15 min interval (**Fig. 2**). Extension of the paw was performed 5 times every 2 minutes for 2 h. Two hours after the first injection and first knee extension (beginning of the protocol), the animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH=7.4), brains were removed and then post-fixed overnight in the same fixative and kept in a solution of 8% sucrose in PBS. One in three coronal vibratome (Leica, Carnaxide, Portugal) sections (50 μm thick) were treated with a solution of 3.3% H2O2 in PBS (30 min) to inhibit endogenous peroxidase activity, and then sequentially washed thrice (10 min) in PBS and PBS-Triton (PBS-T; 0.3% triton X-100; Sigma-Aldrich, Sintra, Portugal). Sections were then incubated in a blocking solution of 2.5% fetal bovine serum (FBS; Biochrom, Cambridge, United Kingdom) in PBS for 2 h, followed by the incubation overnight at 4°C in rabbit anti-Fos antibody (1:2000 in PBST and 2% FBS; Calbiochem, Merck, Algés, Portugal). The following day, after three washes (10 min) in PBST, sections were incubated in biotinylated polyclonal swine anti-rabbit antibody (1:200 in PBST; Dako, Denmark) for 1 h and again washed thrice (10 min) in PBS-T. Sections were then incubated in avidin–biotin complex (ABC; 1:200 in PBST; Vectastain, Vector Laboratories, Peterborough, USA) for 1 h followed by a series of washing steps with PBST (twice, 10min), PBS (twice, 10min) and Tris-HCl (0.05 M, pH 7.6) (twice, 10min). Finally,

sections were stained with diaminobenzidine (0.0125% in a solution of Tris-HCl with 0.02% H2O2; Sigma Aldrich) and washed twice (10min) with Tris-HCl and PBS. After staining, the sections were mounted on SuperFrost slides (Braunschweig, Germany). c-Fos levels were determined by counting the number of Fos-immunoreactive neurones occurring bilaterally in the brainstem with the aid of a Stereo Investigator 10 Software® (Microbrigthfield Bioscience, Madgedurg, Germany) using a video camera (Microbrigthfield Bioscience) attached to a microscope (BX51, Olympus Iberia, Lisboa, Portugal).

2.10. Statistics

The minimum number of animals needed to evaluate the effect of arthritis induction upon RT was determined à priori using the G power software (version 3.1.9.2, University of Kiel, Germany) considering a t-test between two independent means, two tailed analysis, α err probability of 0.05, power of 0.95 and an effect size of 2 (n=16). For the effect of drugs upon PWL, the RVM neuronal activity and c-Fos expression, the minimum number of animals needed was determine à priori using the G power software considering a ANOVA-2-way test, α err probability of 0.05, power of 0.95 and an effect size of 1 (n=15). The results of the RT analysis correspond to the mean±SEM of raw data, no method of data normalization was used. To assess the effect of the drugs upon PWL for each behavioural session, the value of the basal withdrawal latency (withdrawal latency prior to drug administration) was subtracted from the value of the withdrawal latency at the peak effect of the drug, a negative value indicated the withdrawal latency decreased while a positive value corresponded to an increase in withdrawal latency after drug administration to the DMH. To perform this evaluation, raw data was used. To assess the effect of the drugs upon spontaneous neuronal activity, the value of the activity of RVM On- and Off-like cells without noxious peripheral stimulation prior to the administration of drugs in the DMH was subtracted from the activity of these cells without noxious peripheral stimulation at the peak effect of the drug. Similarly, to assess the effect of the drugs upon the noxious-evoked neuronal activity the value of the activity of RVM On- and Off-like cells during noxious peripheral stimulation prior to the administration of drugs in the DMH was subtracted from the activity of these cells during noxious peripheral stimulation at the peak effect of the drug. Only raw data was used in this analysis. To compare the level of c-fos in each area, the total number of cells stained was registered per area studied and only raw data was used in this analysis. The GraphPad Prism® 6

software (GraphPad Software Inc, La Jolla, CA, USA) was used to perform the statistical analysis. The comparison of differences between RT in the PAM test and between the baseline of RVM neuronal spontaneous and heat-evoked activities of SHAM and ARTH animals were performed using a student's t-test for unpaired data. To compare differences in RT between the ipsilateral and the contralateral side in SHAM and ARTH animals a student's t-test for paired data was used. All other comparisons between groups were performed using a two-way ANOVA followed by a Bonferroni correction for multiple comparisons post-hoc test. Statistical significance was accepted for $P<0.05$.

3. Results

3.1. Monoarthritic animals developed ipsilateral mechanical allodynia

Three days after the intrasynovial injection, all animals in the ARTH group developed a clear swelling of the treated knee joint and all gave a vocalization response during a minor extension and flexion of the affected limb by the experimenter. SHAM animals displayed no obvious swelling of the knee joint and did not vocalize when the limb was flexed.

Mechanical hyperalgesia in the knee joint was assessed by determining RT to mechanical pressure over the knee joint. No differences were found between the RT of the ipsilateral and contralateral hindpaws in SHAM animals (t_7=1.535, P=0.169) while in ARTH animals the ipsilateral RT was significantly lower than the contralateral (t_7=3.377, P=0.0118). No differences were found between the contralateral RT of SHAM and ARTH animals (t_{14}=0.000, P>0.999). Four weeks after induction of monoarthritis RT was significantly different between SHAM and ARTH animals (t_{14}=2.883, P=0.012). This result indicates that K/C induced a significant RT decrease, i.e., mechanical hyperalgesia (**Fig. 3**).

3.2. Exogenous GAL in the DMH decreases paw-withdrawal latency, an effect reversed by the administration of a GAL receptors antagonist.

To investigate a possible role of supraspinal GAL in phasic and tonic pain facilitation in SHAM and ARTH animals, paw withdrawal latencies (PWL) were assessed after exogenous GAL or M40 microinjection, respectively, in the DMH. The PWL of SHAM and ARTH animals 20 min after exogenous GAL microinjection in the DMH was significantly decreased when compared with SAL

injection (main effect of the drug: $F_{1,76}$=61.880, P<0.001). The exogenous GAL-induced decrease in PWL was of the same magnitude in the SHAM and ARTH groups (main effect of the group: $F_{1,76}$=2.704, P=0.104). *Post hoc* tests confirmed that the PWL of SHAM and ARTH animals treated with exogenous GAL was significantly lower than the PWL of SHAM and ARTH animals treated with SAL (**Fig. 4A**).

Non-specific inhibition of GAL receptors induced by administration of M40 in the DMH significantly altered the PWL when compared with SAL injection (main effect of the drug: $F_{1,76}$=13.830, P<0.001). The effect of M40 was significantly different between SHAM and ARTH animals (main effect of the group: $F_{1,76}$=10.070, P=0.002). The M40-induced effect on PWL varied with the experimental group (interaction between group and drug: $F_{1,76}$=8.048, P=0.006). *Post hoc* tests indicated that M40 significantly increased PWL in SHAM animals, but did not alter PWL in ARTH animals (**Fig. 4B**).

3.3. Nociceptive facilitation after exogenous GAL in the DMH is mediated by GAL receptors type-1

To determine which GAL receptor is involved in pain facilitation induced by exogenous GAL in the DMH, PWL was assessed after the administration of M617 (a specific agonist of GAL receptor type-1 - GalR1) and M871 (a specific antagonist of GAL receptor type-2 - GalR2) into the DMH. Twenty minutes after microinjecting M617 in the DMH, PWL was significantly decreased when compared with SAL injection (main effect of the drug: $F_{1,76}$=39.530, P<0.001). The effect of M617 was not different between SHAM and ARTH groups (main effect of the group: $F_{1,76}$=0.357, P=0.552). *Post hoc* tests confirmed that PWL significantly decreased after M617 administration in the DMH both in SHAM and ARTH animals when compared to PWL after SAL administration (**Fig. 4C**).

Administration of M871 in the DMH had a significant effect on PWL (main effect of the drug: $F_{1,76}$=29.820, P<0.001), and the effect of M871 varied with the experimental group (interaction group x drug: $F_{1,76}$=5.089, P=0.027). *Post hoc* tests showed that M871 significantly decreased the PWL in SHAM animals but did not alter significantly the PWL of ARTH animals (**Fig. 4D**).

3.4. Expression of c-Fos in brainstem areas involved in pain control is altered by exogenous GAL in the DMH

Descending pain modulatory drive from the forebrain to the spinal cord may be relayed by multiple areas in the brainstem. To determine which brainstem areas mediate exogenous GAL-driven descending pain modulatory effects originating in the DMH, c-Fos expression was investigated in caudal brain areas that not only expressed GAL and/or its receptors but that are also involved in the descending modulation of nociception. Hence, we compared changes in c-Fos expression in the ventrolateral periaqueductal grey matter (VLPAG), the LC, the dorsal raphe nucleus (DRN) and the rostral ventromedial medulla (RVM) between SHAM and ARTH animals after (i) SAL microinjection in the DMH of SHAM animals; (ii) GAL microinjection in the DMH of SHAM animals; (iii) SAL microinjection in the DMH and extension of right limb of SHAM animals; (iv) GAL microinjection in the DMH and extension of right limb of SHAM animals; (v) SAL microinjection in the DMH of ARTH animals; (vi) GAL microinjection in the DMH of SHAM animals; (vii) extension of right limb of ARTH animals; (viii) GAL microinjection in the DMH and extension of right limb of ARTH animals.

Expression of c-Fos following injection of SAL in the DMH was considered to represent basal activation. The number of c-Fos positive neurones in the contralateral RVM varied with the stimulation protocol used (main effect of the protocol: $F_{3,24}$=22.570, P<0.001) with different effects on SHAM and ARTH animals (interaction group x protocol: $F_{3,24}$=42.280, P<0.001). *Post-hoc* testing showed that exogenous GAL in the DMH significantly increased c-Fos expression when compared to SAL-injected animals in both experimental groups, although a higher expression was observed in ARTH animals (**Fig. 5A**). The flexion-extension protocol (SAL+STI) increased c-Fos expression in SHAM animals when compared to SAL and GAL administration while it decreased its expression in ARTH animals when compared to GAL-Injected ARTH. The simultaneous infusion of GAL in the DMH and flexion-extension of the injected limb decreased c-Fos expression in SHAM when compared with its expression after the flexion-extension protocol and in ARTH when compared with GAL-injected ARTH (**Fig. 5A**). Similarly, in the ipsilateral RVM, the number of cells activated was significantly different depending on the protocols (main effect of the protocol: $F_{3,24}$=70.240, P<0.001), an effect that varied with the experimental group (interaction group x protocol: $F_{3,24}$=20.240, P<0.001). *Post-hoc* testing showed that GAL in the DMH increased the number of c-Fos expressing cells in both experimental groups when compared to SAL-injected animals, while its expression was different between SHAM and ARTH

animals after repeated flexion-extension of the injected limb, with increased c-Fos expression in SHAM animals alone when compared with SAL and GAL-injected SHAM (**Fig. 5B**). The simultaneous injection of GAL in the DMH and flexion-extension of the injected limb significantly decreased the number of c-Fos positive cells in SHAM when compared to the flexion-extension protocol and in ARTH animals when compared with GAL administration and the flexion-extension protocols (**Fig. 5B**).

The number of c-Fos expressing neurones in the contralateral LC did not vary with the stimulation protocols (main effect of the protocol: $F_{3,24}$=0.413, P=0.745) although it was significantly different between experimental groups (main effect of the group: $F_{3,24}$=16.410, P<0.001). *Post-hoc* testing did not show a specific alteration between each stimulation protocol (**Fig. 5C**). In the ipsilateral LC, the number of c-Fos positive cells varied with the stimulation protocol (main effect of the protocol: $F_{3,24}$=7.462, P=0.001), an effect that depended on the experimental group (interaction group x protocol: $F_{3,24}$=14.310, P<0.001). *Post-hoc* testing showed an increase in the number of c-Fos expressing neurones after the simultaneous infusion of GAL in the DMH and flexion-extension of the injected limb in ARTH animals when compared with the same protocol in SHAM and with the SAL/GAL/flexion-extension protocols in ARTH (**Fig. 5D**).

In the contralateral vlPAG, the number of c-Fos expressing cells varied with the stimulation protocol (main effect of the protocol: $F_{3,24}$=19.200, P<0.001), an effect that depended on the experimental group (interaction group x protocol: $F_{3,24}$=40.030, P<0.001). *Post-hoc* testing showed a significant increase in the number of c-Fos positive cells in ARTH animals when compared to SHAM and after exogenous GAL in the DMH of SHAM animals when compared to SAL injected SHAM (**Fig. 5E**). In addition, in ARTH animals the number of c-fos expressing neurones was significantly lower in all protocols when compared to SAL injected ARTH animals (**Fig. 5E**). Similarly, the number of cells activated in the ipsilateral vlPAG was different after the stimulation protocols (main effect of the protocol: $F_{3,24}$=22.920, P<0.001) and depended on the experimental group (interaction group x protocol: $F_{3,24}$=47.100, P<0.001). *Post-hoc* testing (**Fig. 5F**) showed increased c-Fos expression in SAL-injected ARTH animals when compared with SAL-injected SHAM. GAL in the DMH significantly increased c-Fos expression in SHAM animals while it significantly decreased its expression in ARTH animals. c-Fos expression after the flexion-extension of the injected limb was not significantly different when compared to SAL-injected SHAM although it was significantly decreased when compared to GAL-injected SHAM (**Fig. 5F**). This protocol also significantly decreased c-Fos expression in ARTH animals when compared to

SAL-injected ARTH although its expression was significantly higher when compared to GAL-injected ARTH. The simultaneous infusion of GAL in the DMH and flexion-extension of the injected limb did not significantly alter c-Fos expression when compared to SAL-injected SHAM but was significantly decreased when compared to its expression after GAL injection in the DMH. In ARTH animals, c-Fos expression was significantly decreased after the simultaneous infusion of GAL in the DMH and flexion-extension of the injected limb when compared to SAL-injected ARTH and the flexion-extension protocol (**Fig.5F**).

Finally, In the DRN, the number of c-Fos positive cells varied with the stimulation protocol (main effect of the protocol: $F_{3,24}$=24.690, P<0.001) and this effect was dependent of the experimental group (interaction group x protocol: $F_{3,24}$=14.140, P<0.001). *Post-hoc* testing showed an increased DRN activation after GAL in the DMH in both experimental groups when compared to SAL-injected animals (**Fig.5G**). The flexion-extension of the injured limb significantly increased c-Fos expression in SHAM animals when compared to ARTH animals and when compared with SAL- and GAL-injected SHAM. The simultaneous infusion of GAL in the DMH and flexion-extension of the injected limb increased c-Fos expression in SHAM animals when compared to ARTH and to its expression after SAL, but decreased when compared to the flexion-extension protocol. Additionally, it decreased c-Fos expression in ARTH animals when compared to GAL-injected ARTH (**Fig. 5G**).

3.5. The activity of pain modulatory On- or Off-like cells in the RVM is not altered by exogenous GAL in the DMH

To evaluate the effect of exogenous GAL administration in the DMH upon the activity of RVM neurones, the spontaneous and heat-evoked activities of presumably pronociceptive RVM On-like cells and antinociceptive RVM Off-like cells were recorded in SHAM and ARTH animals before and after the administration of exogenous GAL, M40 or SAL.

Before drug administration, the spontaneous activity of RVM On-like cells was significantly decreased in ARTH animals when compared to SHAM animals (**Table 1**). The magnitude of the response evoked by noxious heating of the tail in RVM On-like cells was not different between SHAM and ARTH animals. In RVM Off-like cells, the spontaneous activity before drug treatments was significantly decreased in ARTH animals when compared to SHAM animals. Similarly, the

magnitude of the heat-evoked response in RVM Off-like cells of ARTH animals was significantly lower when compared to that in SHAM animals (**Table 1**).

Microinjection of drugs into the DMH did not alter the spontaneous activity of RVM On-like cells (main effect of the drug: $F_{2,98}$=0.262, P=0.770). Overall, after drug injection, On-like cell spontaneous activity was different between ARTH and SHAM animals (main effect of the group: $F_{1,98}$=6.510, P=0.012) (**Fig. 5A**), although *post-hoc* tests failed to show a significant difference between experimental groups at a specific time point. The administration of drugs to the DMH did not alter the spontaneous activity of RVM Off-like cells (main effect of the drug: $F_{2,81}$=0.616, P=0.543) and the spontaneous activity was not different between experimental groups (main effect of the group: $F_{1,81}$=1.200, P=0.277) (**Fig. 5B**) neurones 20 min after its administration.

Microinjection of drugs into the DMH altered the heat-evoked activity of RVM On-like cells (main effect of the drug: $F_{2,98}$=5.010, P=0.009) but this effect did not vary with the experimental group (interaction group x drug: $F_{2,98}$=1.318, P=0.272). *Post hoc* testing failed to find significant drug treatment-induced effects on the heat-evoked response of On-like cells (**Fig. 5C**). Similarly, drug administration in the DMH changed the heat-evoked activity of RVM Off-like cells (main effect of the drug: $F_{2,81}$=4.967, P=0.009) but these differences did not vary with the experimental group (interaction group x drug: $F_{2,81}$=2.230, P=0.114), 20 min after administration. Again, *post hoc* testing failed to find significant drug treatment-induced effects on the heat-evoked response of Off-like cells, except for the increase of response after exogenous GAL treatment in the SHAM group (**Fig. 5D**).

4. Discussion

This study demonstrates, for the first time, a pronociceptive role for supraspinal GAL, as the administration of this neuropeptide to the DMH significantly increased spinal nociception (as indicated by the decrease in PWL) in awake healthy and arthritic animals. Moreover, the microinjection of GAL receptor agonist/antagonist in the DMH showed that the exogenous GAL's pronociceptive effect was mediated by GALR1 but not GALR2. The analysis of c-Fos expression revealed the serotoninergic RVM and DRN, particularly in SHAM animals, as caudal areas potentially involved in signalling this descending pronociceptive effect. The exogenous GAL-induced increase of c-Fos expression in the RVM may not be explained by action on RVM On-like

or Off-like pain modulatory cells, as the discharge rates of these two non-serotoninergic cell types remained unaltered during pharmacological manipulations in the present study.

4.1. Novel pronociceptive effect of supraspinal GAL

Administration of exogenous GAL into the DMH induced behavioural hyperalgesia (decreased PWL) in healthy and ARTH animals. This is a novel effect for GAL as previous studies had only reported an antinociceptive role of this neuropeptide after its administration in brain areas involved in pain modulation, such as the hypothalamic arcuate nucleus (Gu et al., 2007), central AMY (Jin et al., 2010) and the PAG (Wang et al., 2000). Thus, and similarly to what is observed at the spinal cord level (Liu et al., 2001; Hulse et al., 2012), GAL appears to have a bidirectional role in supraspinal descending pain modulation depending on the area where GALRs are activated. The demonstration of a tonic pronociceptive effect of GAL, by treatment of the DMH with a non-specific GALR antagonist, supports the proposal that the pronociceptive effect of GAL was mediated by GALRs.

Administration of exogenous GAL in the DMH facilitated nociception in both ARTH and SHAM animals. This finding contrasts with the results of previous studies indicating that exogenous GAL is antinociceptive when administered in the hypothalamic arcuate nucleus of animals with inflammation (Sun et al., 2003), or in the PAG of animals with mononeuropathy (Wang et al., 2000). More importantly, the present results show that of the descending pathways originating in the DMH, the GAL-driven pathway, unlike the glutamate-driven pathway (Pinto-Ribeiro et al., 2013), remains functional in animals with experimental monoarthritis. Administration of a non-specific GALR antagonist alone into the DMH of ARTH animals had no effect on nociception while it produced antinociception in SHAM controls. This finding suggests that the GAL-driven pathway descending from the DMH is not tonically active in ARTH as in SHAM animals, but its activation in ARTH animals depends on the activation of upstream pathways inducing the release of GAL in the DMH.

4.2. GAL-driven nociceptive facilitation is mediated by GALR1

Further analysis on the contributions of GALR1 and GALR2 to the pain modulatory role of GAL in the DMH demonstrated that the facilitatory effect of GAL is mediated by GALR1, a receptor that

couples to the Gi/Go pathway to decrease adenylyl cyclase activity (Wang et al., 1998). Once more, this result contrasts with the available literature, where the activation of this receptor at spinal and supraspinal levels is reported to elicit an antinociceptive effect (Blakeman et al., 2003; Hua et al., 2004; Fu et al., 2011). In fact, it is GALR2 in the spinal cord, which was reported to have a pronociceptive effect (Liu et al., 2001); however, the results on administration of a GALR2 antagonist in the present study indicated that endogenous GAL acting on GALR2 had a tonic antinociceptive action in SHAM animals, whereas blocking GALR2 did not alter nociception in ARTH animals. Another possibility would be that the differential distribution of GALR1 and GALR2 receptors in the DMH could contribute to enhance GALR1-dependent effects, however as demonstrated by Mitchell et al. (Mitchell et al., 1999) not only does mRNAs analysis confirm an overlapping of GAL-R1 and GAL-R2 in the DMH but both receptors are also highly expressed in this nuclei. On the other hand, the expression of both receptors in the DMH does not account for the GAL/DMH pronociceptive effect since these receptors are also highly expressed and overlapping in the arcuate nuclei, an area where the intracerebral administration of exogenous GAL promotes antinociception (Sun et al., 2003). Overall, it is probable that the facilitation of nociceptive behaviour by GAL in the DMH of ARTH animals results (i) from disinhibition of pronociceptive pathways driven by GALR1 and/or (ii) from a decrease in the activity of antinociceptive GALR2-driven circuits.

It is also possible that behavioural hyperalgesia in ARTH animals is reinforced by their emotional-like status. A recent study from our group (Amorim et al., 2014) showed that animals with experimental monoarthritis displayed depressive-like behaviour. Interestingly, Blackshear *et al.* (Blackshear et al., 2007) showed that the intracerebroventricular injection of GAL and M617 increased c-fos expression in the DMH and the AMY, a nuclei involved the modulation of the emotional component of pain. Another work (Kuteeva et al., 2008) showed that acute activation of GALR1 promoted the expression of 'prodepressive-like' behaviours, while GALR2 mediated the 'antidepressant-like' effects of GAL. Hence, taking into account that depressive states heighten pain perception in humans (Murphy et al., 2012) and rodents (Wang et al., 2012), the pronociceptive GALR1 and the antinociceptive GALR2 effects observed in this study may be related to comorbid mood alterations known to be associated with chronic pain (Verdu et al., 2008; Neugebauer et al., 2009).

4.3. Activation of serotonergic nuclei is influenced both by exogenous GAL in the DMH and noxious peripheral stimulation.

The analysis of c-Fos expression was restricted to the VLPAG, DR, LC and RVM since these areas have been previously demonstrated to be strongly modulated by the DMH (Aston-Jones et al., 2001; Johnson et al., 2008; Wagner et al., 2013), while simultaneously implicated in nociceptive processing (Wang and Nakai, 1994; de Luca et al., 2003; Hickey et al., 2014). The limb extension-induced increase in c-Fos expression in the VLPAG and RVM of SHAM animals suggests that repetitive extension of a non-arthritic knee joint for a period of two hours can be considered a noxious stimulus (Galbán et al., 2007). In addition, the increased c-for expression in the VLPAG and RVM also suggests that repetitive knee joint extension activated the feedback loop of nociception involving the PAG-RVM-spinal dorsal horn circuitry, which may either inhibit or facilitate nociception (Almeida et al., 2006; Heinricher et al., 2009; Ossipov et al., 2010).

Administration of exogenous GAL into the DMH increased the expression of c-Fos ipsilaterally in the VLPAG and bilaterally in the RVM, which suggests that DMH neurones expressing GALR are able to activate descending nociceptive controls. However, our electrophysiological data shows that exogenous GAL in the DMH did not alter the activity of RVM On- and Off-cells that are non-serotoninergic pain control neurones. Therefore, we propose that the RVM cells expressing c-Fos following exogenous GAL treatment may have been RVM Neutral-cells, a subpopulation of which are serotoninergic (Potrebic et al., 1994) and which were not studied in the present electrophysiological experiment. The fact that the DMH GAL-driven descending pronociceptive drive is independent of RVM On- and Off-like cell activity is very interesting in terms of pain management, since many centrally acting analgesic compounds (opioids, cannabinoids and non-steroidal anti-inflammatory drugs) reduce pain by increasing the discharge rate of antinociceptive RVM Off-cells and/or by inhibiting the discharge rate of pronociceptive RVM On-cells (Ossipov et al., 2010).

In SHAM animals, repetitive limb extension alone or exogenous GAL administration alone in the DMH activated the descending PAG-RVM-spinal cord pathway as revealed by c-Fos expression. However, application of exogenous GAL simultaneously with repetitive extension of the limb failed to increase c-Fos expression in the PAG-RVM circuitry of SHAM animals, suggesting that together the two stimulation procedures counteracted each other's effect, leading to a general inhibition of this circuitry. The increased c-Fos expression in the RVM by the pronociceptive exogenous GAL

treatment alone might reflect activation of RVM serotoninergic cells. While the serotoninergic system has a complex role in pain control, there is evidence suggesting that the net effect induced by RVM serotonergic neurones is facilitation of nociception (Wei et al., 2010). It should be noted here that serotonergic RVM neurones are not On- or Off- cells (Potrebic et al., 1994) that were studied in the present electrophysiological experiment using noxious heat and shown not to be influenced by exogenous GAL. We propose that the GAL-induced descending action may have induced activation of medullo-spinal serotoninergic neurones shown as increased c-Fos expression in the RVM and resulting in the relay of pronociceptive action to the spinal cord.

The increased expression of c-Fos in the serotoninergic DRN after limb extensions is in line with a role of this nucleus in ascending (Qiao and Dafny, 1988) and descending (Prado and Faganello, 2000) pain modulatory pathways. Similarly, increased expression of c-Fos of DRN after exogenous GAL in the DMH is not unexpected as the DMH projects directly to the DRN (ter Horst and Luiten, 1986) and the activity of DRN serotonergic neurones is influenced by GALR1 present on their soma and proximal dendrites (Larm et al., 2003). It still remains to be studied through which mechanisms the DRN might be involved in the relay of the descending pronociceptive effect driven by exogenous GAL in the DMH.

4.4. Activation of the noradrenergic LC by exogenous GAL in the DMH and noxious peripheral stimulation varies between SHAM and ARTH animals

Previous studies have demonstrated that the LC responds to noxious stimulation, as revealed e.g. by c-Fos expression (Voisin et al., 2005), while it is a major source of spinal noradrenaline and descending noradrenergic control of nociception (Jones, 1991; Pertovaara, 2006). In the present study, exogenous GAL treatment of DMH alone failed to influence c-Fos expression of LC in SHAM or ARTH animals. However, following repetitive limb extensions, c-Fos expression of LC was increased in SHAM but not ARTH animals. Interestingly, the peripheral stimulation-induced increase of c-Fos expression in the LC was predominantly ipsilateral, while ascending nociceptive signals activate the LC contra- or bilaterally (Voisin et al., 2005). A potential explanation for the ipsilaterally increased c-Fos expression after peripheral stimulation in the present study is that it reflected activation of descending pain modulation pathways descending predominantly ipsilaterally rather than processing of the ascending afferent volley that is expected to be contra- or bilateral. The DMH has a strong galaninergic output (Jacobowitz et al., 2004) to various brain

areas, including the LC (Thompson et al., 1996), and GAL has been shown to decrease neuronal firing in LC (Sevcik et al., 1993). While these findings suggest that the DMH may directly modulate activity of the LC, they still leave open what is the underlying mechanism and functional significance of the finding that exogenous GAL treatment of the DMH together with repetitive limb extensions increased c-Fos activity in the LC of ARTH but not SHAM animals.

4.5. Influence of arthritis and repetitive limb movement

The increase of c-Fos expression in the VLPAG and to a lesser extent in the RVM of SAL-treated ARTH animals indicates an overall increase in the tonic activity of the PAG-RVM-spinal cord pathways after the induction of experimental monoarthritis, which is in accordance with the enhancement of descending inhibitory circuits during chronic inflammation (Cervero et al., 1991; Ren and Dubner, 1996, 2002; Terayama et al., 2000). Interestingly, limb extension in the ARTH group decreased c-Fos expression in the VLPAG suggesting that acute noxious mechanical stimulation of the injured knee dampens tonic descending inhibition mediated by the VLPAG. On the other hand, the increase in c-Fos expression in the RVM, taking into account that the RVM can either facilitate or inhibit nociception (Heinricher et al., 1989), could indicate that this nucleus is engaged in descending facilitation during acute noxious stimulation of ARTH animals, as shown for other chronic pain disorders (Herrero and Cervero, 1996; Kovelowski et al., 2000; Porreca et al., 2002).

Our electrophysiological results showed that before any drug treatments both the baseline and the peripheral stimulus-evoked response in antinociceptive RVM Off-like cells were lower in ARTH than SHAM animals, while there was no difference in the pre-treatment heat-evoked activity of pronociceptive RVM On-like neurones of ARTH and SHAM animals. This finding suggests that a decreased activity of RVM Off-like cells contributes to hyperalgesia in ARTH animals. However, it does not exclude the possibility that among descending facilitatory mechanisms contributing to hyperalgesia in ARTH animals were other cell types of the RVM, in particular medullospinal serotoninergic neurones, or other brainstem nuclei.

Concerning the DRN, the expression of c-Fos after repetitive limb extensions was increased, when compared with SAL-treated ARTH animals, but similar to the expression in c-Fos in SHAM after limb extensions, suggesting that the nociceptive processing through this pathway is not enhanced after the induction of experimental monoarthritis. By contrast, it is possible that the noxious

stimulation-evoked activation of the LC is impaired in ARTH animals, since c-Fos expression was decreased when compared to SHAM animals after limb extensions and unaltered when compared to SAL-treated ARTH animals.

Without noxious stimulation, exogenous GAL in the DMH of ARTH animals appeared to dampen tonic descending inhibition (as indicated by decreased c-Fos expression in the VLPAG) while it enhanced the tonic activity of pronociceptive serotonergic (indicated by increased c-Fos expression in the DRN and RVM), but not noradrenergic (unaltered c-Fos expression in the LC) circuits. However, when combined with limb extensions, both tonic descending inhibition (decreased c-Fos expression in the VLPAG) and the activity of pronociceptive serotonergic areas (decreased c-Fos expression in the RVM and DRN) were diminished. This finding indicates that in ARTH animals, exogenous GAL in the DMH exerts differential effects under basal and noxious stimulation-evoked conditions. A differential effect has also been reported while studying the role of GAL in the presence/absence of stress (Kuteeva et al., 2008).

By contrast, only the combination of exogenous GAL injection in the DMH and limb stimulation was able to enhance the activity of the noradrenergic pain system as evidenced by the strong increase of c-Fos expression in the LC. Although noradrenergic pathways were up to recently considered to exert mostly inhibitory influences on spinal nociception, Hickey and collegues (Hickey et al., 2014) recently demonstrated that a specific subpopulation of LC neurones enhances the processing of nociceptive information and could thus partly contribute to behavioural hyperalgesia in chronic inflammation. Further studies are needed to find out whether LC is involved in mediating the descending pronociceptive effect elicited by exogenous GAL in the DMH.

5. Conclusion

In the present study, we demonstrate a pronociceptive GALR1-mediated role for hypothalamic GAL in experimental monoarthritis. Exogenous GAL in the DMH appeared to exert differential effects upon the brainstem pain modulatory areas; the effect varied between the experimental group (healthy or arthritic animals), brainstem nucleus (PAG, RVM, DRN, or LC), and the presence or absence of concomitant noxious stimulation. Finally, the results suggest that further studies evaluating the potential applicability of GALR1 antagonists in the control of chronic inflammatory pain are needed.

Abbreviations

AMY – amygdala

ARTH – monoarthritis/monoarthritic

DMH – dorsomedial nucleus of the hypothalamus

DRN – dorsal raphe nucleus

GAL – galanin

GALR – galanin receptor

GALR1 – galanin receptor type-1

GALR2 – galanin receptor type-2

gf – gram force

K/C – kaolin/carrageenan

LC – locus coeruleus

RT – response threshold

M40 – non-specific galanin receptor antagonist

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Figures

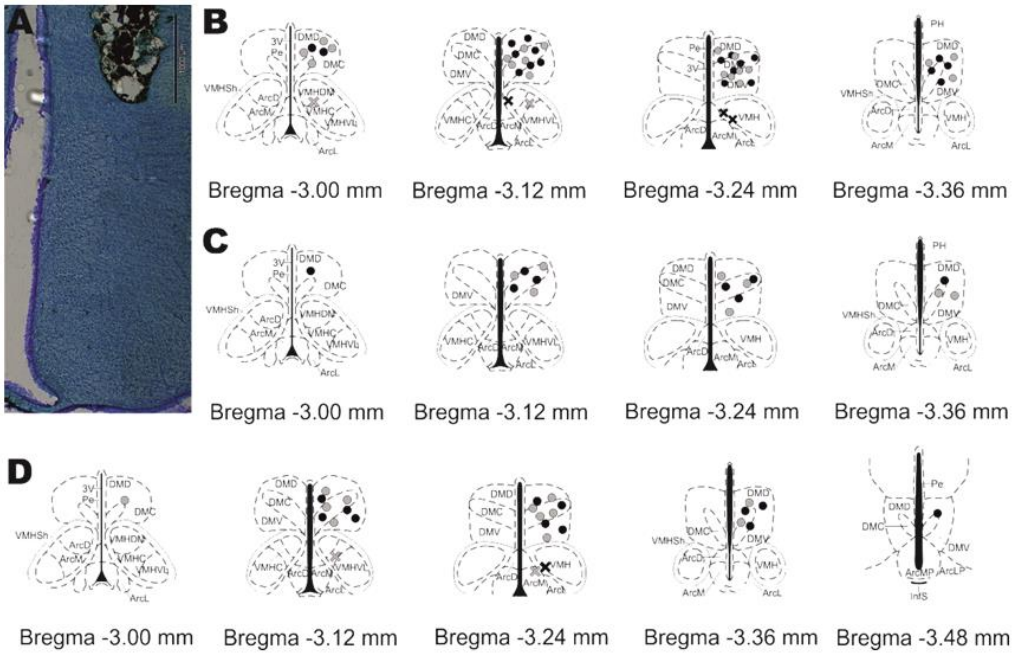


Figure 1. Anatomical confirmation of drug injection sites in the dorsomedial nucleus of the hypothalamus (DMH). (A) Photomicrograph of an example of the drug injection site in the DMH of the rat brain (AP: -3.24 mm from bregma) superimposed with the appropriate plate of the Paxinos and Watson (2007) stereotaxic atlas. **(B)** Schematic representation of injection sites in the DMH during the behavioural study. The coordinates for the injection sites are as follow -3.00 mm, -3.12 mm, -3.24 mm and -3.36 mm from bregma. **(C)** Schematic representation of injection sites in the DMH during the protocol for the induction of c-Fos expression. The coordinates for the injection sites are as follow -3.00 mm, -3.12 mm, -3.24 mm and -3.36 mm from bregma. **(D)** Schematic representation of injection sites in the DMH during the electrophysiological study. The coordinates for the injection sites are as follow -3.00 mm, -3.12 mm, -3.24 mm, -3.36 mm and -3.48 mm from bregma. (Grey dots correspond to injection sites in the DMH of control (SHAM) animals and black dots show injection sites in the DMH of arthritic (ARTH) animals; grey and black crosses correspond to injection sites outside the DMH of SHAM and ARTH animals, respectively) ArcD- arcuate hypothalamic nucleus, dorsal; ArcL- arcuate hypothalamic nucleus, lateral; ArcM- arcuate hypothalamic nucleus, medial; DMC - dorsomedial hypothalamic nucleus, compact; DMD – dorsomedial hypothalamic nucleus, dorsal; DMV - dorsomedial hypothalamic nucleus, ventral; VMHDM - ventromedial hypothalamic nucleus, dorsomedial; VMHSh - ventromedial hypothalamic nucleus, shell; VMHVL - ventromedial hypothalamic nucleus, ventrolateral.

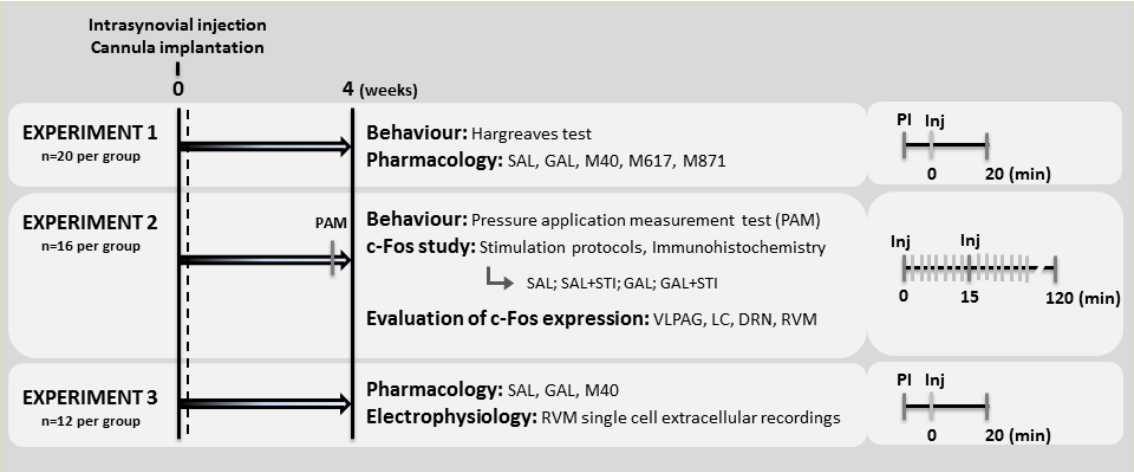


Figure 2. Schematic representation of the experimental design. In all experiments, animals were divided in two groups, control (SHAM) when injected with saline and arthritic (ARTH) when injected with a mixture of kaolin and carrageenan in the synovial capsule of the right knee joint. Three days after the intrasynovial injection, arthritis was confirmed by performing five consecutive movements of flexion/extension of the knee (dashed line). Animals in the ARTH group developed a clear swelling of the treated knee joint and all gave a vocalization response during a minor extension and flexion of the affected limb by the experimenter. SHAM animals displayed no obvious swelling of the knee joint and did not vocalize when the limb was flexed. Four weeks after the induction of monoarthritis animals were tested in three independent experiments. In experiment 1, the Hargreaves test was used to study the role of exogenous galanin (GAL), a non-specific GAL receptor antagonist (M40), a specific GAL receptor-1 agonist (M617) and a specific GAL receptor-2 antagonist (M871) in the dorsomedial nucleus of the hypothalamus (DMH) upon paw-withdrawal latency (PWL) (n=20 per experimental group). In each animal, the development of arthritis was confirmed again 1 h prior to each behavioural session by performing five consecutive movements of flexion/extension of the knee. During the experimental sessions, PWL was assessed before and 20 min after the administration of the drugs to the DMH. In experiment 2, two days prior the c-Fos study, the pressure application measurement (PAM) test was performed to confirm the arthritic state of the animals. c-Fos expression was evaluated in SHAM and ARTH animals after exogenous GAL or saline (SAL) administration in the DMH, peripheral noxious mechanical stimulation and the simultaneous application of noxious mechanical stimulation after the microinjection of exogenous GAL in the DMH (n=16 per experimental group). Neurones expressing c-Fos were quantified bilaterally in the periaqueductal grey matter (PAG), locus coeruleus (LC), dorsal raphe nucleus (DRN) and rostral ventromedial medulla (RVM). In experiment 3, RVM neurones were recorded before and after the administration of exogenous GAL and M40 in the DMH. The assessment of neuronal activity includes a preliminary evaluation of spontaneous and noxious-evoked activity followed by the recording of these parameters 20 min after drug administration to the DMH (n=12 animals per experimental group). PI – Pre-injection; Inj – Injection.

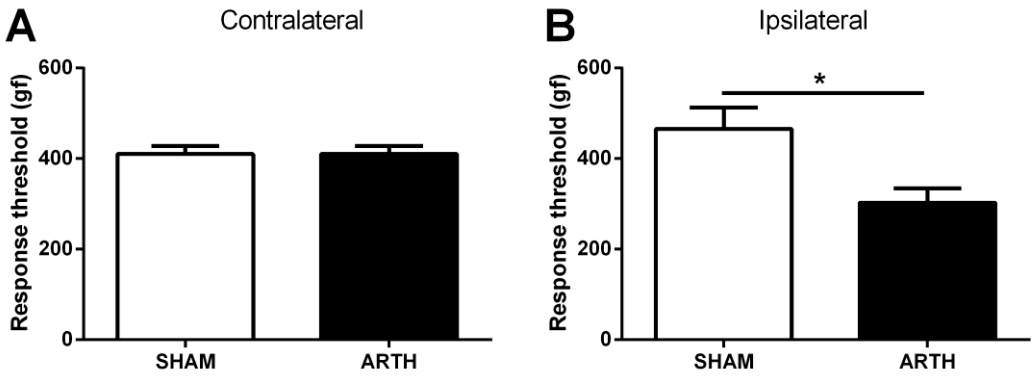


Figure 3. Response threshold. Four weeks after the induction of monoarthritis in the right hind limb, no differences were observed in the response threshold (RT) of the contralateral hindpaws between control (SHAM) and arthritic (ARTH) animals **(A)** in the pressure application measurement. In the ipsilateral side however ARTH animals displayed a decrease in RT during the pressure application measurement when compared to SHAM animals **(B)**. Mean response threshold is presented as mean + SEM. (*P<0.05, t-test for unpaired data). gf – gram force.

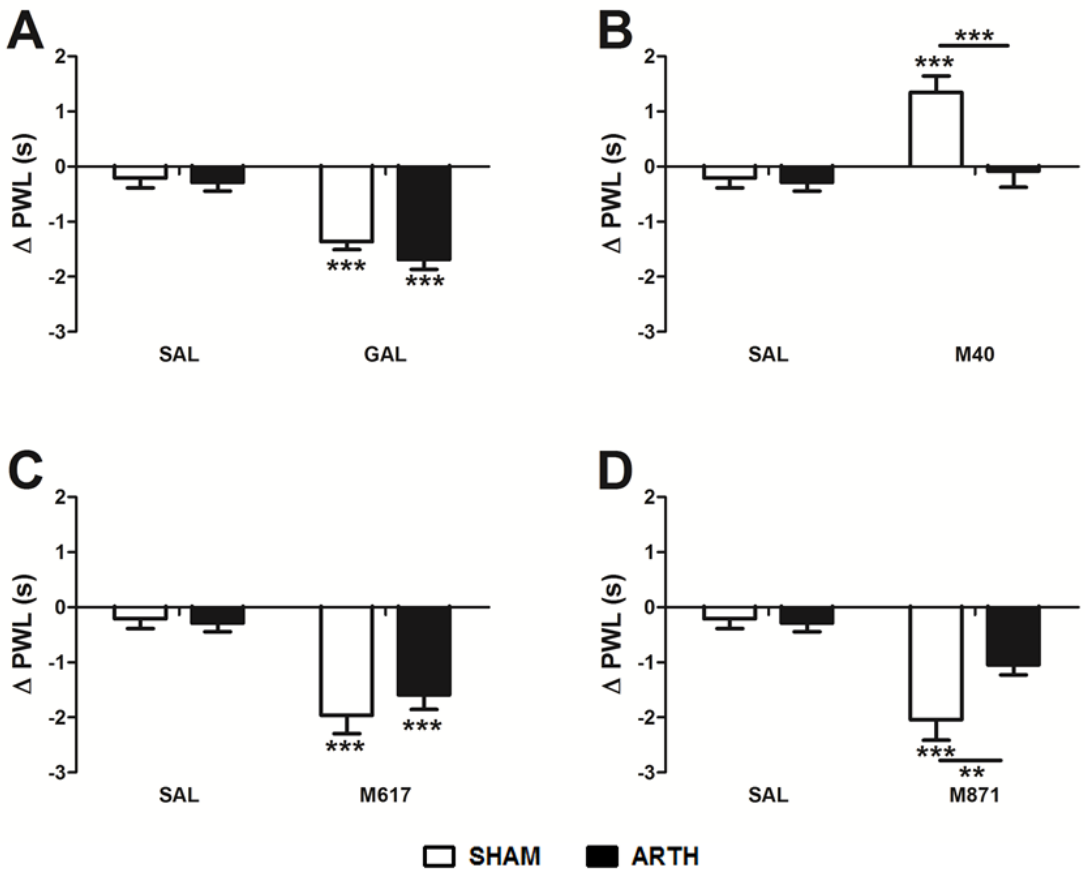


Figure 4. Paw-withdrawal latency after drug administration in the dorsomedial nucleus of the hypothalamus (DMH). In this experiment the analysis of the paw-withdrawal latencies in control (SHAM) and arthritic (ARTH) animals was performed 20 minutes after the intracerebral microinjection of either exogenous galanin (GAL) **(A)**, a non-specific antagonist of GAL receptors (M40; **B**), a specific agonist of GAL receptor-1 (M617; **C**) or a specific antagonist of GAL receptor-2 (M871; **D**). Note that the pronociceptive action of exogenous GAL administration in the DMH in SHAM and ARTH animals **(A)** is only mimicked by the microinjection of M617 **(C)** (n=20 animals per group). Mean response latency is presented as mean + SEM. (*P<0.05, **P<0.01, ***P<0.001, two-way ANOVA with a Bonferroni correction for multiple comparisons).

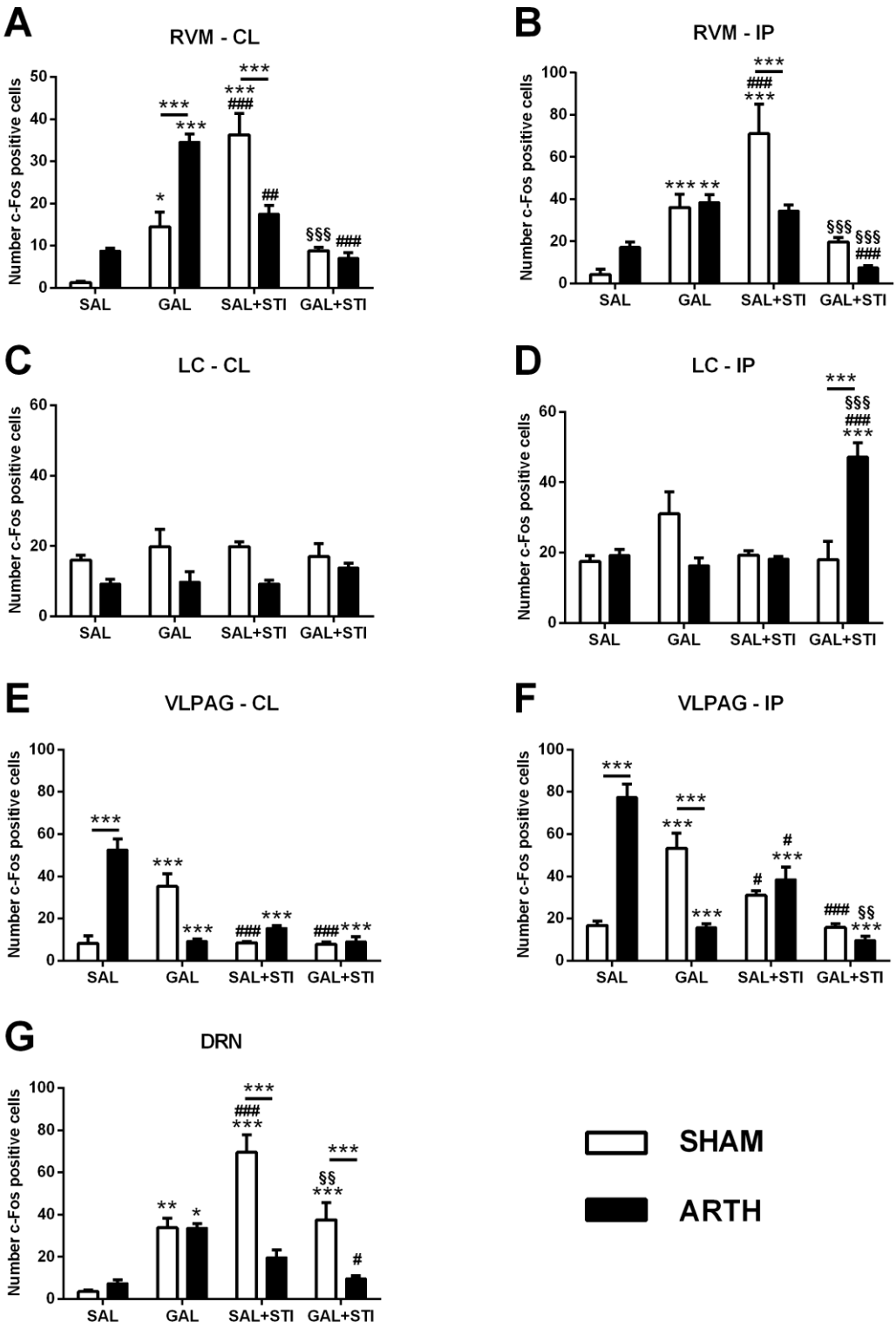


Figure 5. Brainstem c-Fos expression. Number of c-Fos positive cells in the contralateral (A,C,E) and ipsilateral (B,D,F) sides of the rostral ventromedial medulla (RVM; **A,B**), locus coeruleus (LC; **C,D**), ventrolateral periaqueductal matter (vlPAG; **E,F**) and dorsal raphe nucleus (DRN; **G**) after the administration of saline or exogenous galanin in the dorsomedial nucleus of

the hypothalamus (DMH) with and without peripheral noxious stimulation. Data is presented as mean + SEM. SAL – Saline; GAL – Galanin; SAL+STI – SAL and limb flexion-extension; GAL+STI – GAL and limb flexion-extension. * indicates significant differences in c-Fos expression when compared to SAL injection in the DMH; * over line indicates differences in c-Fos expression between experimental groups; # indicates significant differences in c-Fos expression when compared to GAL injection in the DMH; § indicates significant differences in c-Fos expression when compared to the flexion-extension protocol (*, #$P<0.05$; **, ##, §§$P<0.01$; ***, ###, §§§$P<0.001$)**.**

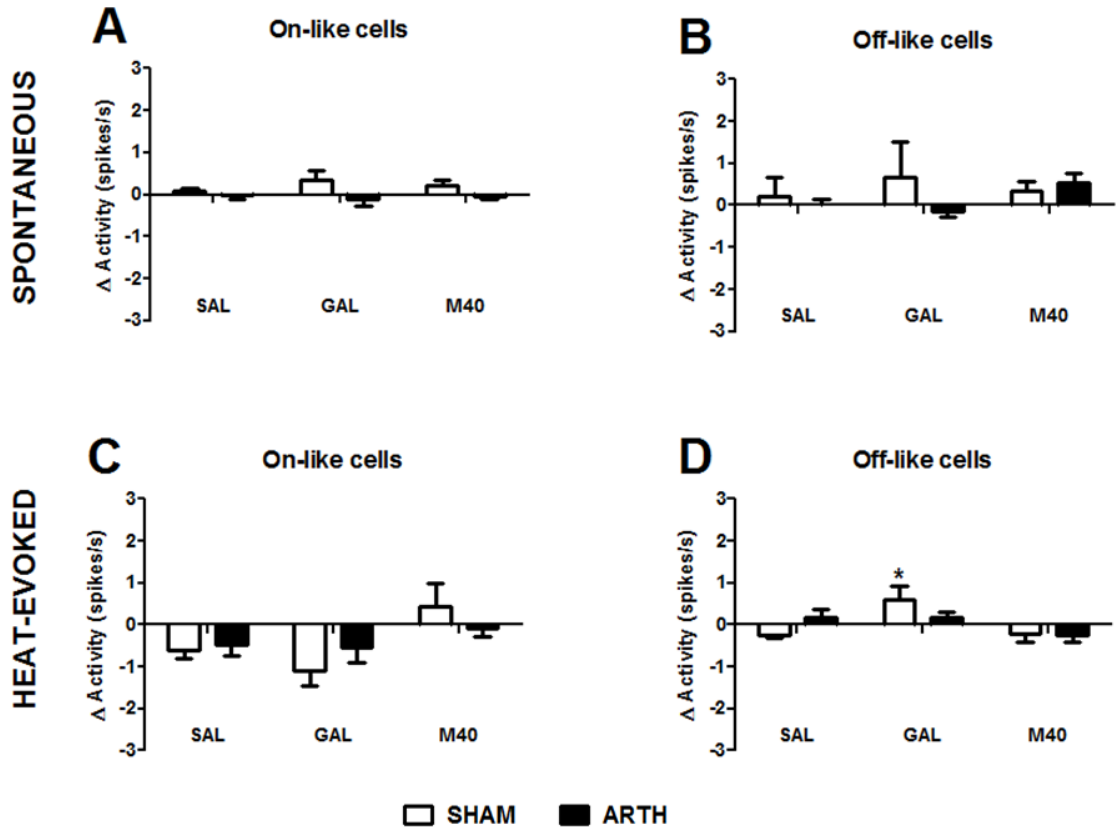


Figure 6. Spontaneous and noxious-evoked rostral ventromedial medulla (RVM) cell activity after the administration of exogenous galanin (GAL) or a non-selective GAL receptor antagonist (M40) in the dorsomedial nucleus of the hypothalamus (DMH). Overall, no changes were observed in the spontaneous **(A,B)** and noxious-evoked **(C,D)** activity of RVM pronociceptive On-like **(A,C)** and antinociceptive Off-like cells **(B,D)** before and 20 min after the intracerebral microinjection of exogenous GAL **(A-D)** and M40 **(A-D)**. Data is presented as mean + SEM. (two-way ANOVA with a Bonferroni correction for multiple comparisons). *$P<0.05$.

Table 1. Rostral ventromedial medullar (RVM) On- and Off-like cells spontaneous and heat-evoked baseline activities.

	Cell type	SHAM	ARTH	*t*	*P*
Spontaneous	On-like	1.86 ± 0.39 Hz	0.77 ± 0.19 Hz	t_{102}=2.689	*P*=0.008**
	Off-like	4.77 ± 0.46 Hz	3.37 ± 0.26 Hz	t_{85}=2.833	*P*=0.006**
Evoked	On-like	2.51 ± 0.31 Hz	2.82 ± 0.37 Hz	t_{102}=0.601	*P*=0.549
	Off-like	-2.45 ± 0.25 Hz	-1.72 ± 0.26 Hz	t_{85}=2.782	*P*=0.007**

Data presented as mean ± SEM.

** $P<0.01$

Chapter 2.4

Amorim D, Viisanen H, Wei H, Almeida A, Pertovaara A, Pinto-Ribeiro F

Galanin-mediated behavioural hyperalgesia from the dorsomedial nucleus of the hypothalamus involves two independent descending pronociceptive pathways

(Manuscript submitted)

Galanin-mediated behavioural hyperalgesia from the dorsomedial nucleus of the hypothalamus involves two independent descending pronociceptive pathways

Diana Amorim[1,2,3], Hanna Viisanen[3], Hong Wei[3], Armando Almeida[1,2], Antti Pertovaara[3], Filipa Pinto-Ribeiro[1,2#]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), Campus of Gualtar, University of Minho, 4750-057 Braga, Portugal

[2]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

[3]Institute of Biomedicine/Physiology, University of Helsinki, Helsinki, Finland

Diana Amorim (dianaamorim@ecsaude.uminho.pt)

Hanna Viisanen (hanna.viisanen@helsinki.fi)

Hong Wei (hong.wei@helsinki.fi)

Armando Almeida (aalmeida@ecsaude.uminho.pt)

Antti Pertovaara (antti.pertovaara@helsinki.fi)

Filipa Pinto-Ribeiro (filiparibeiro@ecsaude.uminho.pt)

#Corresponding author:

Professor Filipa Pinto-Ribeiro

Email: filiparibeiro@ecsaude.uminho.pt

Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS),

Campus de Gualtar, University of Minho, 4750-057 Braga, Portugal

Telf: +351 253604852 Fax: +351 253604809

Abstract

The activation of the dorsomedial nucleus of the hypothalamus (DMH) by galanin (GAL) induces behavioural hyperalgesia. Since DMH neurones do not project directly to the spinal cord, we hypothesized that the medullary dorsal reticular nucleus (DRt), a pronociceptive region that strongly projects to the spinal dorsal horn (SDH) and/or the serotoninergic raphe-spinal pathway acting on the spinal 5-HT_3 receptor ($5HT_3R$) could relay descending nociceptive facilitation induced by GAL in the DMH. Heat-evoked paw-withdrawal latency (PWL) and activity of SDH neurones were assessed in monoarthritic (ARTH) and control (SHAM) animals after pharmacological manipulations of the DMH, DRt and spinal cord. The results showed that GAL in the DMH and glutamate in the DRt lead to behavioural hyperalgesia in both SHAM and ARTH animals, which is accompanied by increased stimulus-evoked activity of SDH nociceptive neurones. Facilitation of pain behaviour induced by GAL in the DMH was reversed by lidocaine in the DRt and by ondansetron, a $5HT_3R$ antagonist, in the spinal cord. However, the hyperalgesia induced by glutamate in the DRt was not blocked by spinal ondansetron. In addition, in ARTH but not SHAM animals PWL was increased after lidocaine in the DRt and ondansetron in the spinal cord. Our data demonstrate that GAL in the DMH activates two independent descending facilitatory pathways: (i) one relays in the DRt and (ii) the other one involves 5-HT neurones acting on spinal $5HT_3Rs$. In experimental ARTH, the tonic pain-facilitatory action is increased in both of these descending pathways.

Keywords: nociceptive facilitation, dorsomedial nucleus of the hypothalamus, dorsal reticular nucleus, spinal dorsal horn, galanin, ondansetron

1. Introduction

Galanin (GAL) inhibits or enhances nociceptive transmission in the spinal cord depending on (i) its concentration, with high concentrations being antinociceptive and low concentration pronociceptive (Landry et al., 2005) and (ii) the availability of its receptors, with GALR1/3 being inhibitory and GALR2 facilitatory (Landry et al., 2005). Although supraspinal GAL was considered antinociceptive (Wang et al., 2000; Sun et al., 2003, 2004; Jin et al., 2010), it was recently shown that its administration to the dorsomedial nucleus of the hypothalamus (DMH) induces heat hyperalgesia (Amorim et al., 2014). Moreover, GAL/DMH-mediated pronociception was shown to be dependent on the activation of GALR1 (Amorim et al., 2014). Interestingly, DMH activation by glutamate or disinhibition by bicuculline induced behavioural hyperalgesia mediated by the rostral ventromedial medulla (RVM) in controls (Martenson et al., 2009; Pinto-Ribeiro et al., 2013) but not in monoarthritic animals (Pinto-Ribeiro et al., 2013). However, when GAL was microinjected in the DMH, both control and monoarthritic animals displayed behavioural hyperalgesia, although this effect could not be correlated to the activity of RVM On- and Off-like cells involved in descending modulation of nociception (Amorim et al., 2014). These findings leave open which relay nuclei and descending pathways mediate the pronociception induced by GAL in the DMH.

Since DMH neurones do not project directly to the spinal cord (ter Horst and Luiten, 1986; Martins et al., 2008), a potential pronociceptive pathway mediating the descending action of GAL in the DMH is the dorsal reticular nucleus (DRt) (Lima and Almeida, 2002), a structure receiving strong afferent projections from the DMH (ter Horst and Luiten, 1986; Martins et al., 2008). Neurones in the DRt respond exclusively or mainly to noxious stimulation (Villanueva et al., 1988, 1990). DRt lesion decreased hyperalgesia in the acute and late phase of the formalin test (Almeida et al., 1999) and reduced the noxious heat-evoked behavioural responses (Almeida et

al., 1996). In line with this, Dugast and colleagues (Dugast et al., 2003) demonstrated that activation of the DRt enhanced the noxious stimulation-evoked activity of spinal nociceptive neurones. Although the role of the DRt in chronic pain is poorly understood, there is evidence suggesting that the DRt contributes to the maintenance of central sensitization in experimental neuropathy (Sotgiu et al., 2008), and this DRt-induced facilitatory effect is modulated by opioids (Pinto et al., 2008) and noradrenaline (Martins et al., 2010).

In our recent study (Amorim et al., 2014), GAL in the DMH induced behavioural hyperalgesia without an accompanying increase in the activity of presumably pronociceptive RVM On-cells or a decrease in the activity of antinociceptive RVM Off-cells, which are changes associated with pronociception (Fields et al., 1983; Neubert et al., 2004; Sanoja et al., 2010; Pinto-Ribeiro et al., 2013; Silva et al., 2013). However, the RVM has a subpopulation of serotoninergic neurones that are not On- or Off-cells and which potentially contribute to descending regulation of nociception through action on spinal 5-HT receptors (Potrebic et al., 1994). Spinally projecting serotonergic pathways are reported to enhance or inhibit nociception (Oyama et al., 1996; Silveira et al., 2010) and the direction of effect has varied from antinociception to pronociception with the subtype of 5-HT receptor. Suzuki and colleagues (Suzuki et al., 2005) showed that the activation of spinal serotonin type-3 receptors ($5HT_3R$) enhanced nociception in chronic pain states. Earlier results (Amorim et al., 2014) leave open the possibility that serotoninergic pathways originating in the RVM and acting on the spinal $5HT_3R$ contribute to the hyperalgesia induced by GAL in the DMH.

Here we tested the hypotheses that the hyperalgesic effect of GAL in the DMH is mediated by the DRt and/or descending serotoninergic pathways acting on spinal $5\text{-}HT_3Rs$. Experiments were performed in healthy controls and animals with experimental monoarthritis.

2. Methods

2.1. Animals, ethical issues and anaesthesia

The experiments were performed using adult male Hannover–Wistar rats (220–260 g; Harlan, Horst, The Netherlands) in the Biomedicum Helsinki, Finland. All experiments were approved by the ethical committee for experimental animals studies of the State Provincial Office of Southern Finland (Hämeenlinna, Finland) and the experiments were performed according to the guidelines of European Communities Council Directive of 22nd September 2010 (2010/63/EU). All efforts were done to minimize animal suffering and to reduce the number of animals used.

The animals were housed in polycarbonate cages with a deep layer of saw dust, one animal in each cage, in a thermo-statically controlled room at 24.0 ± 0.5 °C. The room was artificially illuminated from 8.30 a.m. to 8.30 p.m. The animals received commercial pelleted rat feed (CRM-P pellets, Special Diets Services, Witham, Essex, England) and water *ad libitum*.

The anaesthesia, for surgeries and electrophysiological sessions, was induced by administering pentobarbitone (60 mg/kg, intraperitoneal; OrionPharma, Espoo, Finland). The level of anaesthesia was monitored by verifying behavioural responses to noxious pinching. If the pinch-evoked response was stronger than a brief muscle twitch restricted to the stimulated limb, the animal was given an additional dose of pentobarbitone (15–20 mg/kg, intraperitoneal).

2.2. Induction of monoarthritis

The induction of monoarthritis (ARTH) was performed, under anaesthesia, four weeks before the behavioural experiments, as described elsewhere (Pinto-Ribeiro et al., 2013). Briefly, 3% kaolin and 3% carrageenan (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in sterile saline solution (0.9% NaCl) and injected into the synovial cavity of the right knee joint at a volume of 0.1 mL. This model produces mechanical hyperalgesia, which begins just a few hours after

kaolin/carrageenan (K/C) injection and lasts for several weeks (Radhakrishnan et al., 2003). Control animals (SHAM) were injected with 0.1 mL saline in the synovial cavity of the right knee joint.

In each animal, the development of monoarthritis was verified 1h prior to each experiment. Only those rats that vocalized every time during five flexion–extension movements of the knee joint were considered to have developed monoarthritis and were included in the ARTH group. SHAM animals did not vocalize to any of the five consecutive flexion–extension movements of the knee joint.

2.3. Surgical procedures for the installation of the intrathecal catheter

For intrathecal (i.t.) drug administration, a catheter (PE-10, Becton Dickinson and Company, Sparks, MD, USA) was implanted at the lumbar level of the spinal cord as described in detail elsewhere (Størkson et al., 1996). In the following day, the correct placement of the catheter was verified by administering lidocaine (7 μL, 4 %; OrionPharma, Espoo, Finland) with a 50 μl Hamilton syringe (Hamilton Company, Bonaduz, Switzerland). Only those rats that had no motor impairment before lidocaine injection but had a bilateral paralysis of hind limbs following i.t. administration of lidocaine (LIDO) were further studied. During the pharmacological sessions, the drugs were microinjected i.t. with a 50 μL Hamilton syringe in a volume of 5 μL. To minimize the number of times the animal was anesthetized, the installation of the i.t. catheter and the induction of monoarthritis were performed at the same time.

2.4. Surgical procedures for intracerebral cannula implantation

For intracerebral drug administration, two stainless steel guide cannulae (26 gauge; PlasticsOne, Roanoke, USA) were implanted in the DMH and DRt of each animal, according to the

coordinates of the rat brain atlas (Paxinos and Watson, 2007). The tips of the guide cannulae were positioned 1 mm above the desired injection site in the DMH [anteroposterior (AP), -3.24 mm from bregma; lateromedial (LM), 0.4 mm lateral from the midline (on the right side); dorsoventral (DV), 7.5 mm below the surface of the skull] and the DRt [AP, -14.04 mm from bregma; LM, 1.8 mm lateral from the midline (on the right side); DV, 7.6 mm below the surface of the skull]. The guide cannulae were kept in place with dental screws and dental cement. A dummy cannula was inserted into each guide cannula to prevent their blockade. The animals were allowed to recover from the surgery for at least one week after the behavioural tests were performed.

Test drugs were administered in the DMH and in the DRt through a 33-gauge injection cannula (PlasticsOne) with an injection volume of 0.5 μL. The spread of the injected drugs within the brain was expected to have a diameter of at least 1 mm (Myers, 1966). The efficacy of injections was monitored by watching the movement of a small air bubble through the tubing. The injection lasted 20 s and the injection cannula was left in place for additional 30 s to minimize the return of drug solution back to the injection cannula.

2.5. Pressure application measurement

The application of noxious pressure to the primary site of injury is a classical approach to measure mechanical hyperalgesia (Randall and Selitto, 1957), both in humans and animals (Rivat et al., 2008). Here, a new pressure application measurement (PAM) method was used to evaluate mechanical hyperalgesia after four weeks of ARTH induction. It allows an accurate behavioural measurement of primary mechanical hypersensitivity in rodents with chronic inflammatory joint pain (Barton et al., 2007) by the application of a force range of 0–1500 g. To perform the test and with the animal securely held, the force transducer unit (fitted to the

experimenter's thumb) is placed on one side of the animal's knee joint and the forefinger on the other. Then, an increasing force is applied across the joint at a rate of approximately 300 g/s until a behavioural response is observed (limb-withdrawal, freezing of whisker movement, wriggling or vocalization) with a cut-off of 5 s. The peak force applied immediately prior to the behavioural response is recorded as the limb withdrawal threshold (LWT). Three measurements of the ipsilateral and contralateral limbs were made at 1 min intervals. The mean LWTs were calculated per animal.

2.6. Drugs

Solutions for intracerebral drug injections in the DMH and DRt were prepared with sterilized saline 0.9 % (Unither, Amiens, France; pH 7.2) except for LIDO, which was acquired as a solution. Each injection had a volume of 0.5 µL and contained either 50 nmol of GLU (Merck, Darmstadt, Germany), LIDO 4 % (OrionPharma, Espoo, Finland), 1 nmol of GAL (Tocris, Bristol, UK) or 10 µg ondansetron (OND) (Tocris). In the results section, the behavioural and electrophysiological changes induced by a drug were considered at the time point of maximum effect of each drug: 1 min for GLU, 20 min for GAL and LIDO and 30 min for OND.

2.7. Course of the behavioural and pharmacological study

Rats were habituated to the experimental conditions by allowing them to spend 1-2 h daily in the experimental room during the three days preceding any testing. For assessing nociception in awake animals, radiant heat-induced latencies for withdrawing the hind paw (The plantar test instrument model 37370, Ugo Basile, Varese, Italy) were determined. In each pharmacological session paw-withdrawal latency (PWL) was assessed prior to drug administration and at the time of maximum effect of each drug according to previous studies (Sun et al., 2003; Suzuki et al.,

2004; Jin et al., 2010; Pinto-Ribeiro et al., 2013). The behavioural study started four weeks after the induction of ARTH and at least one week following the insertion of the guide cannulae in the DMH and DRt. Hind paw skin temperature was assessed with an electronic thermometer (BAT-12, Physitemp Instruments Inc., Clifton, NJ, USA) before and after drug administration in the DMH in order to exclude the confounding influence of changes in skin temperature upon PWL.

To study the effect of galanin (GAL) in the DMH upon the DMH-DRt-spinal cord pathway several pharmacological approaches were used: i) GAL administration in the DMH; ii) glutamate (GLU) administration in the DRt; iii) lidocaine (LIDO) administration in the DRt; iv) ondansetron (OND) administration in the spinal cord; v) LIDO in the DRt followed immediately by GAL in the DMH; vi) OND in the spinal cord followed 10 min later by GAL in the DMH and; vii) OND in the spinal cord followed 30 min later by GLU in the DRt. Saline solution (SAL) was used in control injections.

2.8. Course of the electrophysiological recordings in the spinal dorsal horn

Electrophysiological recordings of spinal dorsal horn (SDH) neurones were performed in anaesthetized rats. Lumbar vertebrae T12-L2 were located, a laminectomy was performed, and segments L4-L6 of the spinal cord were exposed. The dura was removed and dehydration of the spinal cord was prevented with mineral oil. The animal was placed in a standard stereotaxic frame and two guide cannulae were implanted in the DMH and DRt as described above (section 2.4). To stabilize the spinal cord two spinal clamps, one rostral and one caudal to the laminectomy, were used. Extracellular recordings of dorsal horn tungsten electrodes (tip impedance 3–10 MΩ at 1 kHz). The signal was amplified and filtered using neurones, ipsilateral to the arthritic knee or SAL-injected knee, were performed using lacquer-coated standard techniques. Data sampling was performed with a computer connected to a CED Micro 1401 interface and using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). In the

spinal dorsal horn, search and classification of spinal units was performed as described elsewhere (Pertovaara et al., 2001). The recording depth from the spinal cord surface was 0.4–1.0 mm (Willis and Coggeshall, 1991) and all the spinal neurones included in the study had their receptive field in the plantar skin of the right hind paw. The evaluation of the electrophysiological properties of a SDH neurone consisted of the following assessments performed sequentially: i) spontaneous activity; ii) response to brushing 5 times (1 Hz); iii) response to von Frey filaments (4, 26, 60, 100 g; Stoelting, Wood Dale, IL, USA) 5 times (1 Hz) at each stimulus force; iv) response to heating with a feedback-controlled contact thermostimulator (a heat ramp rising at the rate of 10 °C/s from the baseline temperature of 35 °C to the peak temperature of 50, 52 and 54 °C and peak duration of 10s after which the temperature was decreased to the baseline temperature of 35 °C at a rate of 4 °C/s; 82.8 mm^2, LTS-3 Stimulator, Thermal Devices Inc., Golden Valley, MN, USA) of the ipsilateral hind paw. Because GLU has a short time of action after its injection in the DRt, recordings on the effect of GLU in the DRt include only the response to the stimulus temperature of 54 °C. Body temperature was maintained within a physiological range with a Homeothermic Blanket System (Harvard Apparatus Ltd, Edenbridge, U.K.).

The neurones activated by innocuous brush as well as noxious heat were classified as wide-dynamic range (WDR) neurones, while neurones responsive only to peripheral noxious stimulation were classified as nociceptive-specific (NS) according to the criteria described earlier (Willis and Coggeshall, 1991). Spontaneous activity of neurones was recorded over a period of at least 10 min or until response stabilization. When analysing (before drug administration) the baseline properties of neurones, the response of a neurone was defined as the difference between neuronal activity during peripheral stimulation and the spontaneous activity of the neurone, i.e., positive values represent excitatory responses evoked by peripheral stimulation and negative ones inhibitory responses. The effect of a drug upon the spontaneous activity of a

neurone was calculated as the difference between spontaneous activity at the time of maximum effect of a drug and the spontaneous activity before drug administration (baseline spontaneous activity); i.e., positive values represent an increase and negative ones a decrease of the spontaneous activity induced by the drug. The effect of a drug upon the peripheral stimulation-evoked response is the difference between the neuronal response to a stimulus at the time of maximum effect of a drug and the response to a peripheral stimulation before drug administration (baseline response); i.e., positive values represent a drug-induced increase and negative ones a decrease of the stimulus-evoked response.

2.9. Histology

At the end of the experimental period, animals received a lethal dose of pentobarbitone and the brains were removed and preserved in 10 % formalin. Coronal sections were cut in a vibratome and stained with cresyl violet. Injection sites were histologically verified and plotted on standardized sections adapted from Paxinos and Watson (Paxinos and Watson, 2007). Injection sites in the DMH and DRt are shown in **Fig. 1A-C.**

2.10. Statistical analysis

The GraphPad Prism® 6 software (GraphPad Software Inc, La Jolla, CA, USA) was used to perform the statistical analyses. The comparison of differences between sides in the PAM and paw-flick tests and between the effects of SAL or GAL administration in the DMH on hind paws skin temperature were performed using a Student's t-test for paired data. Student's t-test for unpaired data was used to compare the baseline PWL of the right (ipsilateral) hind paws, the spontaneous activity of spinal neurones and the effect of GLU injection in the DRt upon the activity of spinal cord neurones between SHAM and ARTH groups. One sample t-test was used to

compare the difference of spinal neuronal activity before (pre-injection) versus after drug administration. To compare drug-induced differences in PWL between SHAM and ARTH groups, two-way-analysis of variance (2wANOVA) followed by a Bonferroni-corrected t-test was used. Statistical significance was accepted for $P < 0.05$. Data in the results section are expressed as mean + standard error of the mean (SEM).

3. Results

3.1. Behavioural evaluation of nociception

3.1.1. Kaolin/carrageenan treatment induced monoarthritis

Three days after the intrasynovial injection of K/C, all animals in the ARTH group developed a clear swelling of the treated right knee joint and all vocalized during a minor extension and flexion of the affected limb by the experimenter. SHAM animals displayed no obvious swelling of the knee joint and did not vocalize when the limb was extended.

3.1.2. Arthritic animals display ipsilateral mechanical hyperalgesia

Mechanical hyperalgesia in the knee joint was assessed by determining LWT during the application of pressure to the knee joint. LWT of ARTH animals was significantly different between the left (598.3 ± 69.0) and the right (433.7 ± 29.3) knee joints (t_4=5.845, P=0.004), with the K/C injected right knee showing a significant decrease in the LWT, i.e., mechanical hyperalgesia.

3.1.3. The pronociceptive effect of GAL in the DMH is at least partly mediated by the DRt

Baseline PWL evoked by noxious heating of the ipsilateral hind paw (i.e., distal to the inflamed knee joint in the ARTH group) was not significantly different between SHAM and ARTH animals (t_{10}=0.279, P=0.786; SHAM= 7.0 ± 0.3 s, ARTH= 7.1 ± 0.4 s). To study the phasic and tonic action of the DRt upon nociceptive modulation, PWLs on both contralateral and ipsilateral hind paws were evaluated after injecting GLU, LIDO or SAL in the right (ipsilateral to the inflamed knee joint in the ARTH group) DRt. In the contralateral (left) paw, drug injection in the DRt significantly altered PWL (main effect of drug: $F_{2,30}$=14.180, P<0.001). *Post hoc* tests indicate that PWL was decreased after GLU microinjection in the DRt of SHAM and ARTH animals (**Fig. 2A**). In the ipsilateral (right) paw, drug injection in the DRt also significantly altered PWL (main effect of drug: $F_{2,30}$=30.960, P<0.001) with *post hoc* tests indicating that PWL was significantly decreased after DRt activation by GLU in both SHAM and ARTH animals, and increased after DRt inhibition by LIDO in ARTH animals alone (**Fig. 2B**). Moreover, the effect of GLU in the DRt was more pronounced in the ipsilateral than in the contralateral paw in SHAM (t_6=8.118, P<0.001) but not in ARTH animals (t_4=1.786, P=0.149).

To investigate a possible role of the (right) DRt in the mediation of the pronociceptive effect of GAL in the (right) DMH, PWL was assessed after inhibition of the DRt by LIDO followed by GAL microinjection in the DMH. In the contralateral (left) paw, drug injections significantly altered PWL (main effect of drug: $F_{2,30}$=20.270, P<0.001) and this effect varied with the drug treatment (interaction between experimental group and drug treatment: $F_{2,56}$=10.900, P<0.001). *Post hoc* tests indicated that PWL was decreased after GAL microinjection in the DMH in both SHAM and ARTH animals and that LIDO in the DRt reversed the GAL/DMH-induced effect in SHAM animals only (**Fig. 2C**). In the ipsilateral (right) paw, drug injections significantly altered PWL (main effect

of drug: $F_{2,30}$=23.720, P<0.001). *Post hoc* testing indicated that PWL was decreased after GAL in the DMH in both SHAM and ARTH animals and that LIDO microinjection in the DRt was able to reverse this effect in both experimental groups (**Fig. 2D**). Importantly, skin temperature of the hind paws did not vary with drug (SAL or GAL) administration in the DMH of SHAM (t_6=0.819, P=0.444) or ARTH animals (t_4=0.270, P=0.800).

3.1.4. The pronociceptive effect of GAL in the DMH is at least partly mediated by $5HT_3R$ at the spinal cord level

To study the tonic action of the spinal $5HT_3R$ on nociception in SHAM and ARTH animals, the PWL was evaluated after injecting OND, a $5HT_3R$ antagonist, intrathecally at the lumbar spinal cord level. PWL was significantly altered after OND administration (main effect of drug: $F_{1,18}$=7.970, P=0.011) and this effect varied with the experimental group (interaction between drug treatment and experimental group: $F_{2,18}$=12.570, P=0.002). *Post hoc* tests indicated that PWL was increased after spinal OND treatment in ARTH animals alone (**Fig. 3A**).

To investigate a possible role of the spinal $5HT_3R$ in the mediation of the pronociceptive effect of GAL in the DMH, PWL was assessed after GAL microinjection in the DMH following spinal administration of OND. PWL was significantly altered by drug administrations (main effect of drug: $F_{2,30}$=23.860, P<0.001) and the drug effect varied with the experimental group (main effect of group: $F_{1,30}$=4.318, P=0.046). *Post hoc* tests indicated that PWL was decreased after GAL microinjection in the DMH; this hyperalgesic effect was stronger in the SHAM than ARTH group and it was reversed by spinal administration of OND in both SHAM and ARTH animals (**Fig. 3B**).

To study a possible role of the spinal $5HT_3R$ in the mediation of the pronociceptive effect of the DRt, PWL was assessed after activation of the (right) DRt by GLU and inhibition of the spinal $5HT_3R$ by OND. In the contralateral (left) paw, PWL was significantly altered after drug

administrations (main effect of drug: $F_{2,30}$=8.917, P<0.001). *Post hoc* tests indicated that PWL was decreased after GLU administration in the DRt but not after OND in the spinal cord followed by GLU in the DRt (**Fig. 3C**). However, *post hoc* tests also indicated that the attenuation of the GLU-induced pronociceptive effect by spinal administration of OND was not significant (**Fig. 3 C**). In the ipsilateral (right) paw, the PWL was significantly altered after drug administrations (main effect of drug: $F_{2,30}$=37.160, P<0.001). *Post hoc* tests indicated that PWL was decreased after GLU administration in the DRt and that OND in the spinal cord failed to reverse this pronociceptive effect (**Fig. 3D**).

3.2. Electrophysiological analysis

3.2.1. Arthritis increases the activity of spinal dorsal horn neurones

To evaluate the effect of arthritis upon SDH neurones, discharge properties of WDR and NS neurones were assessed in SHAM and ARTH animals (**Fig. 4**). ARTH induced an increase in spontaneous activity of WDR (**Fig. 5A**) and NS (**Fig. 5B**) neurones.

To study mechanical stimulation-evoked neuronal activity, innocuous brush or a series of calibrated von Frey hairs were applied to the right hind paw (i.e., distal to the inflamed knee joint in the ARTH group). Mechanical stimulation-evoked activity of WDR neurones was different between experimental groups (main effect of the group: $F_{1,630}$=42.970, P<0.001), which effect varied with the intensity of the stimulus (interaction between experimental group and stimulus intensity: $F_{4,630}$=2.438, P=0.046). *Post hoc* tests indicated that neuronal responses evoked by brushing and a 26g-von Frey filament were increased in the ARTH group (**Fig. 5C**). Overall, the mechanical stimulation-evoked responses of NS neurones were significantly different between the experimental groups (main effect of the group: $F_{1,100}$=4.386, P=0.003), although *post hoc* testing

failed to show a significant difference between ARTH and SHAM groups at any of the currently used stimulus intensities (**Fig. 5D**).

The stimulus intensity-related increase of the heat-evoked response in WDR neurones varied significantly with the experimental group (interaction between experimental group and stimulus intensity: $F_{2,318}$=5.800, P=0.003). *Post hoc* testing showed that the response of WDR neurones to a heat stimulus of 54°C was significantly increased in ARTH animals (**Fig. 5E**). The heat-evoked response of NS neurones was significantly altered by the intensity of the stimulus (main effect of the stimulus: $F_{2,60}$=5.946, P=0.004), but this effect did not vary with the experimental group (interaction between experimental group and stimulus intensity: $F_{2,60}$=0.163, P=0.850). In line with this, *post hoc* tests did not show any differences in heat-evoked responses of NS neurones between the experimental groups at any of the stimulus temperatures (**Fig. 5F**).

3.2.2. GAL in the DMH increases activity of spinal dorsal horn neurones

To evaluate the effect of GAL administration in the DMH upon the activity of SDH neurones, spontaneous, mechanically and heat-evoked activities of WDR and NS neurones were assessed in SHAM and ARTH animals. When compared with pre-injection values, GAL in the DMH increased spontaneous activity of WDR (**Fig. 6A**) but not NS (**Fig. 6B**) neurones.

Overall, GAL administration in the DMH increased the mechanically evoked activity of WDR neurones as revealed by comparison with the corresponding pre-injection values (**Fig. 6C**). The facilitation of mechanically evoked responses of WDR neurones by GAL did not vary either with the experimental group (main effect of the group: $F_{1,630}$=0.256, P=0.613) or with the stimulus intensity (main effect of the stimulus intensity: $F_{4,630}$=1.239, P=0.293) (**Fig. 6C**). In contrast, the mechanically evoked responses of NS neurones were not altered following GAL in the DMH as revealed by comparison with the corresponding pre-injection values (Fig. 6D). In line with this,

GAL treatment failed to have a significant main effects varying with the experimental group (main effect of the group: $F_{1,100}$=2.149, P=0.146) or with the intensity of mechanical stimulus force (main effect of the stimulus intensity: $F_{4,100}$=1.020, P=0.401) (**Fig. 6D**).

After GAL administration in the DMH, the heat-evoked activity of WDR neurones was increased in both experimental groups and at all heat stimulus intensities as revealed by comparisons with the corresponding pre-injection values (**Fig. 6E**). The GAL-induced facilitation of heat-evoked responses of WDR neurones did not vary either with the experimental group (main effect of the group: $F_{1,318}$=0.094, P=0.759) or with the stimulus intensity (main effect of the stimulus intensity: $F_{2,318}$=0.550, P=0.578) (**Fig. 6E**). However, the heat stimulation-evoked activity of NS neurones increased only after 54°C noxious stimulation in SHAM animals (main effect of the group: $F_{1,60}$=7.910, P=0.007)(**Fig. 6F**).

3.2.3. GLU injection in the DRt increases the activity of spinal dorsal horn neurones

To study the effect of GLU administration in the DRt upon the activity of SDH neurones, spontaneous and heat-evoked activities of WDR and NS neurones were assessed in SHAM and ARTH animals. Comparisons with the corresponding pre-injection values indicated that GLU administration in the DRt did not alter the spontaneous activities of WDR (**Fig. 7A**) or NS (**Fig. 7B**) neurones, while it increased the heat stimulation-evoked response of WDR neurones in both SHAM and ARTH animals, which effect was of similar magnitude in both experimental groups (**Fig. 7C**). GLU in the DRt increased the heat-evoked response of NS neurones only in ARTH animals (**Fig. 7D**).

4. Discussion

In line with our recent results (Amorim et al., 2014), GAL in the DMH induced behavioural hyperalgesia in monoarthritic (ARTH) as well as control (SHAM) animals. Importantly, here we show that the GAL-induced facilitation of pain behaviour was mediated by two independent descending pathways, one relaying in the DRt and the other acting on the spinal $5HT_3$Rs. Both of these descending pronociceptive pathways were tonically active in the ARTH but not SHAM animals. In the spinal dorsal horn, increased spontaneous activity of both WDR and NS neurones was the most prominent neuronal correlate of arthritis. Moreover, in the basal WDR neuronal activity, the increased mechanical-evoked activity to low intensity stimuli and increased heat-evoked activity to high intensity stimulation in ARTH animals correlates with the development of mechanical allodynia and thermal hyperalgesia, respectively, often observed in chronic pain conditions. GAL in the DMH increased stimulus-evoked responses of spinal WDR neurones both in SHAM and ARTH animals, while spontaneous activity of WDR neurones was increased only in the ARTH group. In contrast, GAL in the DMH failed to have a significant influence on activity of spinal NS neurones, except for an increase of the heat-evoked response in the SHAM group. Glutamatergic DRt activation indicated that DRt facilitates heat-evoked but not spontaneous activity of nociceptive spinal dorsal horn neurones. Moreover, while the heat-evoked response of WDR neurons was facilitated by DRt both in SHAM and ARTH animals, the heat-evoked response of NS neurones was increased by glutamatergic DRt activation only in the ARTH group. Comparison of behavioural and electrophysiological results suggests that both NS and WDR neurones may have contributed to the GAL/DMH-induced behavioural hyperalgesia, although the contributions of these two nociceptive neurone types vary with the descending pathway mediating the pronociceptive effect and the pathophysiological condition.

4.1. The GAL/DMH-DRt facilitatory pathway

Recently, we showed that activation of the DMH by GLU (Pinto-Ribeiro et al., 2013) or GAL (Amorim et al., 2014) induced behavioural hyperalgesia. Interestingly, while the pronociceptive effect of GLU in the DMH of healthy controls was mediated by the RVM (Pinto-Ribeiro et al., 2013), the circuits mediating GAL/DMH-induced descending facilitation remained unclear (Amorim et al., 2014). Moreover, the facilitatory pathway activated by GLU in the DMH was impaired in experimental monoarthritis (Pinto-Ribeiro et al., 2013), while behavioural hyperalgesia could still be evoked by GAL in the DMH of monoarthritic animals (Amorim et al., 2014). These results suggest that at least partly different circuits were mediating the descending facilitatory effects induced by GLU and GAL in the DMH.

By activating the DMH with GAL and inhibiting the DRt with LIDO, we demonstrated that the GAL/DMH-induced descending facilitation was, at least in part, mediated by the DRt, since local blocking of the DRt prevented the GAL/DMH-induced behavioural hyperalgesia. These results are in accordance with several earlier studies showing a facilitatory role of the DRt in nociceptive modulation (Almeida et al., 1996, 1999; Lima and Almeida, 2002; Dugast et al., 2003; Pinto et al., 2008; Sotgiu et al., 2008; Martins et al., 2010). Indeed, Almeida and colleagues (Almeida et al., 1996) demonstrated that activation of the DRt by GLU decreased tail-flick latency and, conversely, lesion of the DRt increased response latencies in the tail-flick test, hot-plate test and the acute and inflammatory phases of the formalin test (Almeida et al., 1996, 1999). In addition, the DMH does not project directly to the SDH (ter Horst and Luiten, 1986), but projects to the DRt (ter Horst and Luiten, 1986; Martins et al., 2008), which indicates that DRt neurones can mediate the descending (Tavares and Lima, 1994; Almeida et al., 2000) pain modulatory action of the DMH.

In the present study, the inhibition of the DRt induced an increase in PWL in ARTH but not SHAM animals indicating that the DRt contributes to tonic facilitation of nociception in monoarthritis. Enhanced tonic descending facilitation from the DRt is in accordance with earlier reports showing an increase in c-Fos expression and metabolic activity of DRt in experimental monoarthritis (Lantéri-Minet et al., 1994; Neto et al., 1999). Moreover, since the DRt projects directly to the superficial dorsal horn (Tavares and Lima, 1994; Almeida et al., 2000), the increase in spontaneous activity of spinal WDR and NS neurones in ARTH animals, a finding commonly reported in several models of neuropathic and inflammatory pain (Chapman et al., 1998; Chu et al., 2004; Sotgiu et al., 2008), might partly be a consequence of enhanced DRt-mediated tonic facilitation.

Interestingly, our data showed that in ARTH animals the facilitatory effect relayed by the DRt descends predominantly ipsilaterally as evidenced e.g. by the predominantly ipsilateral effects of the LIDO-induced inhibition of the DRt on PWL. Although DRt laterality has not often been studied, it was previously demonstrated that in the formalin test, the lesion of the ipsilateral DRt decreased nociceptive phase 1 and 2 behaviours. In contrast, contralateral lesion of the DRt only affected phase 2 behaviour in the formalin test and was accompanied by a decrease of c-Fos positive cells in the ipsilateral SDH laminae I-II and IV-V (Almeida et al., 1999).

4.2. The GAL/DMH-5HT$_3$R facilitatory pathway

The existence of descending serotonergic circuits that modulate nociceptive transmission is widely known (Millan, 2002; Porreca et al., 2002). Serotonergic pathways can either inhibit or enhance nociception depending of the receptor subtypes that are being expressed and activated in the SDH (Millan, 2002). Here, we focused on 5HT$_3$Rs, as they are mainly expressed on small diameter afferent terminals of the superficial dorsal horn (Kia et al., 1995; Morales et al., 1998).

Spinal $5HT_3$Rs are known to participate in modulation of nociception, although their role in nociceptive processing has been not completely clear since both antinociceptive and pronociceptive effects have been reported (Glaum et al., 1988, 1990; Danzebrink and Gebhart, 1991; Rodgers and Shepherd, 1992; Ali et al., 1996; Silveira et al., 2010; Gu et al., 2011).

We found that OND, a $5HT_3$R antagonist injected in the lumbar spinal cord, increased heat-evoked PWLs in ARTH but not SHAM control animals. This finding indicates that the spinal 5-HT_3R tonically facilitates nociception in experimental monoarthritis. This result is in accordance with earlier results (Green et al., 2000) showing that blocking the spinal $5HT_3$R had hardly any effect on nociception in healthy animals while it decreased nociception in inflamed (Oyama et al., 1996) or nerve-injured animals (Suzuki et al., 2004), and reduced nociceptive (second phase) behaviour in the formalin test (Oyama et al., 1996; Zeitz et al., 2002). Additionally, neuropathic hypersensitivity was profoundly reduced in $5HT_3$R-depleted rats (Suzuki et al., 2005). Conversely, the intrathecal administration of a $5HT_3$R agonist facilitated nociceptive behaviours in the late phase of the formalin test (Oyama et al., 1996). In humans, intravenous administration of OND significantly decreased pain scores in patients with chronic neuropathic pain, again suggesting that serotonergic circuits acting on the $5HT_3$R promote tonic pain facilitation in neuropathy (McCleane et al., 2003). Together these data suggest $5HT_3$Rs facilitate nociception in chronic neuropathic and inflammatory disorders.

Among several supraspinal centres that project to the spinal dorsal horn, the RVM, in particular the nucleus raphe magnus, has been proposed to play a key role in descending bidirectional modulation of nociception both in physiological conditions (Zhuo et al., 1997; Urban and Gebhart, 1999) and chronic pain states (Pertovaara et al., 1996; Porreca et al., 2002; Carlson et al., 2007). In neuropathic conditions, descending facilitatory action of RVM appears to be essential for the maintenance, but not the initiation, of chronic pain (Burgess et al., 2002).

Our recent electrophysiological results suggest that pronociceptive On-like or antinociceptive Off-like cells in the RVM do not mediate the descending pronociceptive action induced by GAL in the DMH (Amorim et al., 2014). It should be noted that RVM On- and Off-cells are non-serotonergic cells while a subpopulation of RVM Neutral-cells that do not respond to peripheral noxious stimulation have been shown to be 5-HT immunoreactive (Potrebic et al., 1994). Taking into account that the RVM is a major source of serotoninergic innervation of the SDH (Millan, 2002) and that blocking the spinal $5HT_3$Rs reversed the pronociceptive action induced by GAL in the DMH, it may be proposed that serotonergic RVM cells (a subpopulation of RVM Neutral cells) provide a relay to the GAL/DMH-induced descending facilitation. Future studies should be performed to confirm this hypothesis.

4.3. Conclusions

GAL in the DMH activates in parallel two descending pronociceptive pathways one of which relays in the DRt and the other one in serotoninergic brainstem neurons that have descending projections promoting nociception through action on the spinal $5HT_3$R. Both of these descending pronociceptive pathways are tonically active in monoarthritic but not healthy control animals.

Abbreviations:

$5HT_3R$, serotonin type 3 receptor; AP, anteroposterior; ARTH, arthritic animals; DMH, dorsomedial nucleus of the hypothalamus; DRt, dorsal reticular nucleus; DV, dorsoventral; GAL, galanin; GLU, glutamate; i.t., intrathecal; LIDO, lidocaine; LM, lateromedial; LWT, limb withdrawal threshold; NS, nociceptive specific neurones; OND, ondansetron; PWL, paw withdrawal latency; RVM, rostral ventromedial medulla; SAL, saline solution; SDH, spinal dorsal horn; SHAM, control animals; WDR, wide dynamic range neurones.

Conflict of interest statement

The authors report no conflict of interest.

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Figures

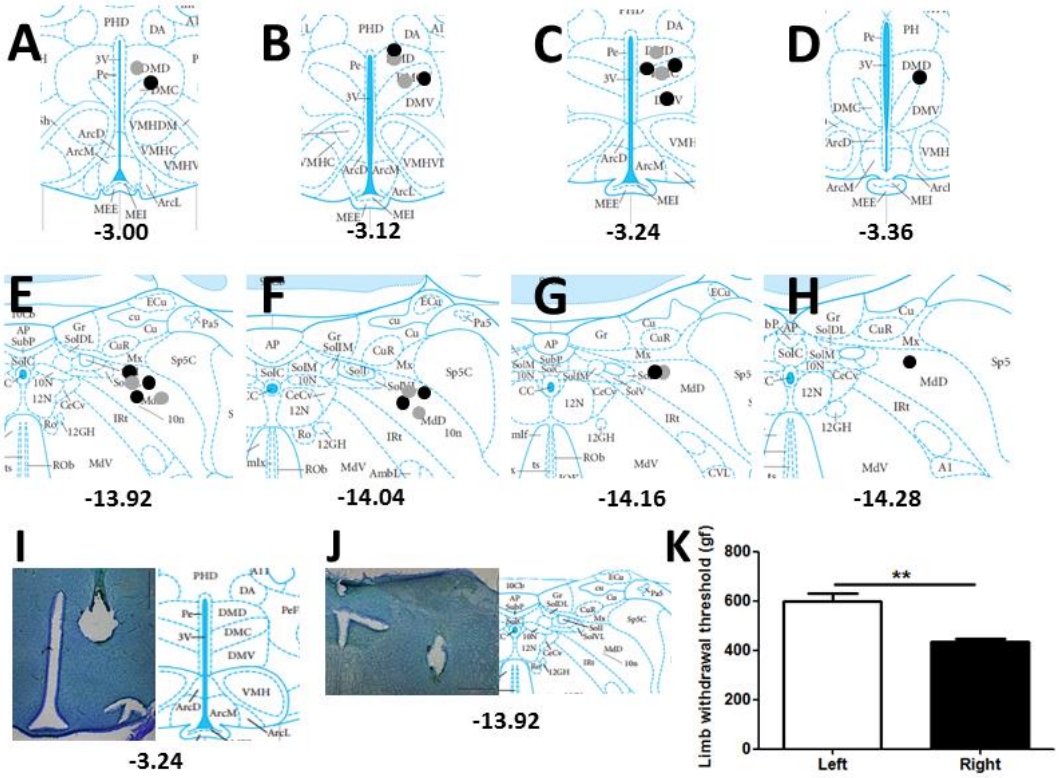


Figure 1. Schematic representation of the injection sites in the dorsomedial nucleus of the hypothalamus (DMH) (**A-D**: -3.00 mm, -3.12 mm, -3.24 mm and -3.36 mm from bregma) and medullary dorsal reticular nucleus (DRt) (**E-H**: -13.92 mm, -14.04 mm, -14.16 and -14.28 mm from bregma). Photomicrograph of an example of the drug injection site in the DMH (**I**: -3.24 mm from bregma) and the DRt (**J**: -14.16 mm from bregma). **(K)** Limb withdrawal threshold four weeks after the induction of monoarthritis in the right knee, arthritic animals display a decreased limb withdrawal threshold in the right hind limb in the pressure application measurement test when compared to the left hind limb. Limb withdrawal threshold is presented as mean + SEM. (**P<0.05, t-test for paired data). gf – gram force.

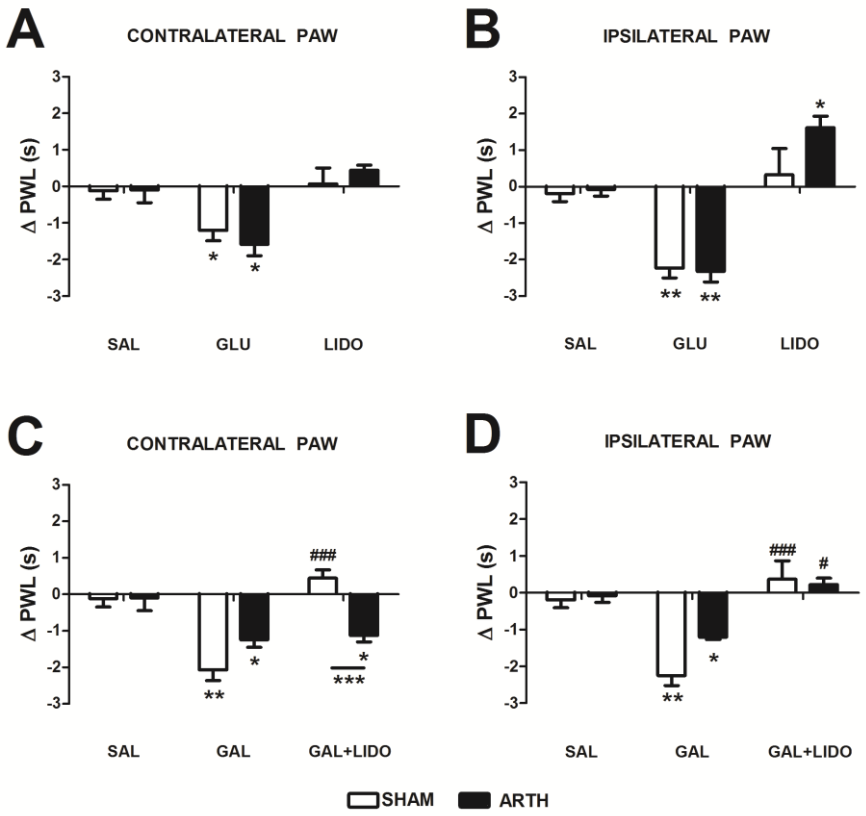


Figure 2. Behavioural evaluation of paw-withdrawal latencies (PWL) after drug microinjections in the dorsomedial nucleus of the hypothalamus (DMH) and the medullary dorsal reticular nucleus (DRt) of control (SHAM) and arthritic (ARTH) animals. PWL in the contralateral **(A)** and ipsilateral **(B)** hind limbs after glutamate (GLU) or lidocaine (LIDO) in the DRt. PWL in the contralateral **(C)** and ipsilateral **(D)** hind limbs after galanin (GAL) in the DMH or the combination of GAL in the DMH and LIDO in the DRt. Saline (SAL) injections were used as controls. Graphs show mean PWL + SEM (n_{SHAM}=7; n_{ARTH}=5). (*P<0.05, ***P<0.001: Comparison of the drug effect with the effect of SAL injection in the same experimental group; #P<0.05, ###P<0.001: Comparison of the effect of drug combination (GAL+LIDO) with the corresponding GAL alone group (t-test with a Bonferroni correction for multiple comparisons).

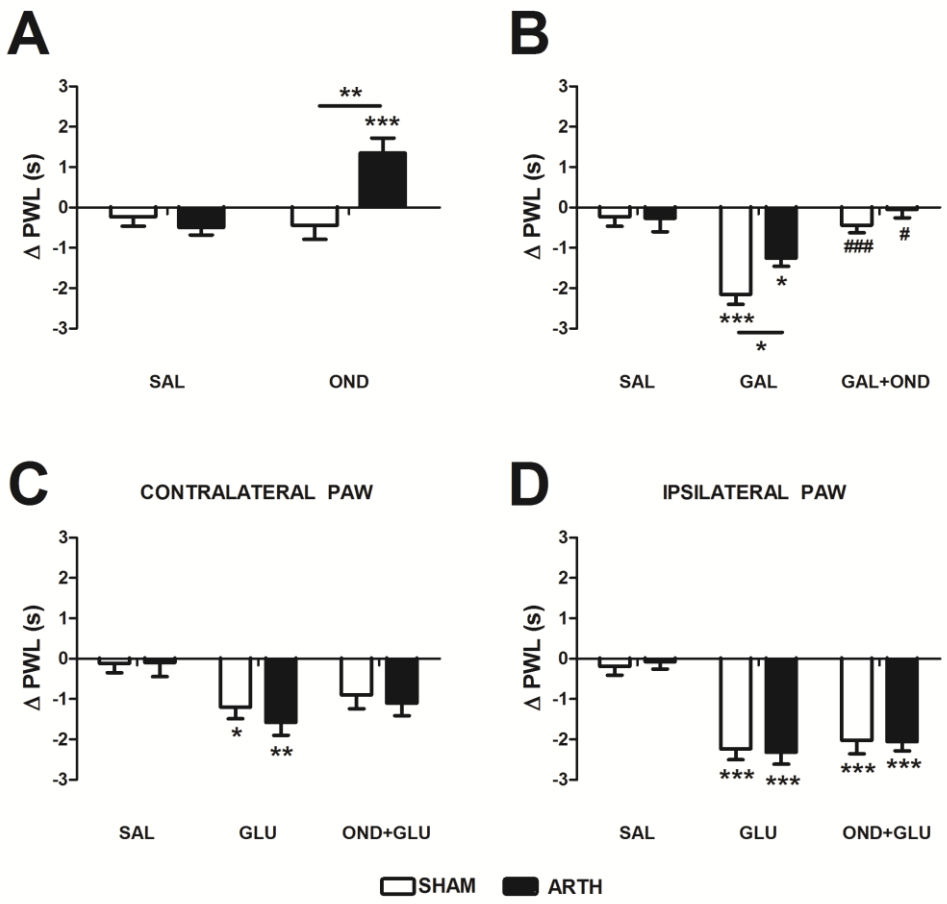


Figure 3. Behavioural evaluation of paw-withdrawal latencies (PWL) after the drug microinjection in the dorsomedial nucleus of the hypothalamus (DMH), medullary dorsal reticular nucleus (DRt) and spinal cord of control (SHAM) and arthritic (ARTH) animals. **(A)** PWL after spinal administration of ondansetron (OND). **(B)** PWL after administration of galanin (GAL) in the DMH or the combination of GAL in the DMH and OND in the spinal cord. **(C)** PWL in the contralateral **(D)** and ipsilateral hind paw after glutamate (GLU) administration in the DRt or after the combined administration of OND in the spinal cord and GLU in the DRt. Saline (SAL) injections were used as controls. Graphs show mean PWL + SEM (n_{SHAM}=7; n_{ARTH}=5). *P<0.05, **P<0.01, ***P<0.001: Comparison of the drug effect with the effect of SAL within the same experimental group; ##P<0.01, ###P<0.001: Comparison of the effect of drug combination with the corresponding drug alone (t-test with a Bonferroni correction for multiple comparisons).

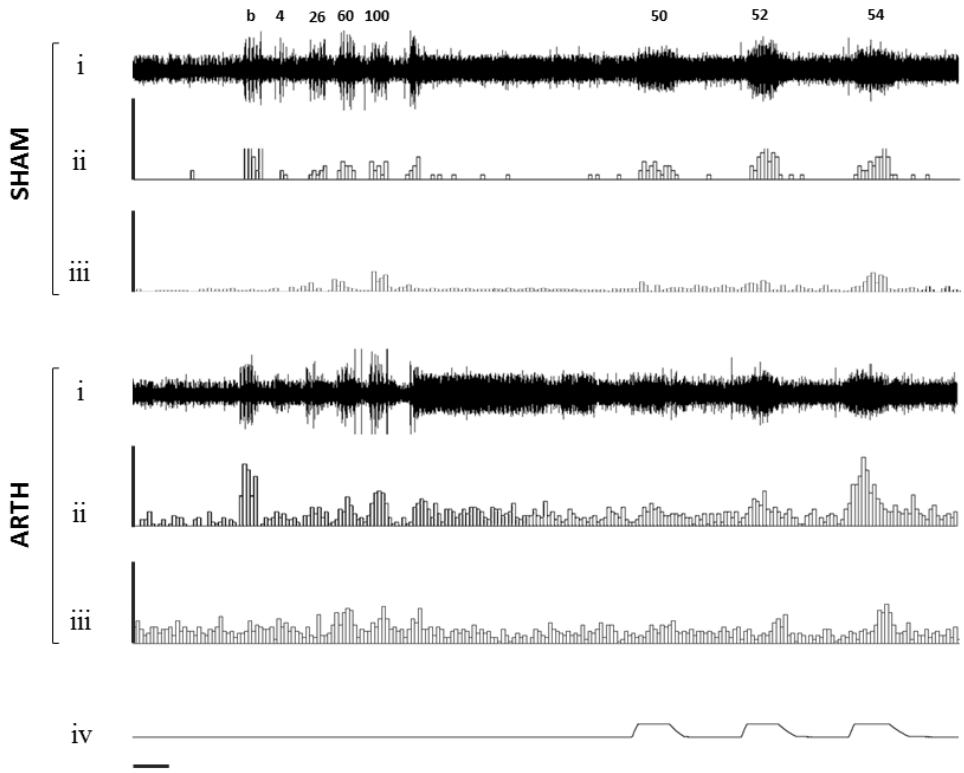


Figure 4. Examples of original recordings of spinal dorsal horn wide-dynamic-range (WDR) and nociceptive specific (NS) neuronal activity without peripheral stimulation and in response to mechanical stimulation [brush (B), 4, 26, 60 and 100 g von Frey filaments] and heating of the right hind paw of control (SHAM) and arthritic (ARTH) animals before drugs injection. **(i)** Raw data of neuronal responses; **(ii)** peristimulus time histogram showing the discharge of a WDR neurone; **(iii)** peristimulus time histogram showing the discharge of a NS neurone**; (iv)** heat stimulus that starts from the baseline temperature of 35 °C and peaks at 50, 52 and 54 °C. Vertical calibration bar for the neuronal activity represents 15 Hz (ii, iii) and the horizontal calibration bar represents 15 seconds.

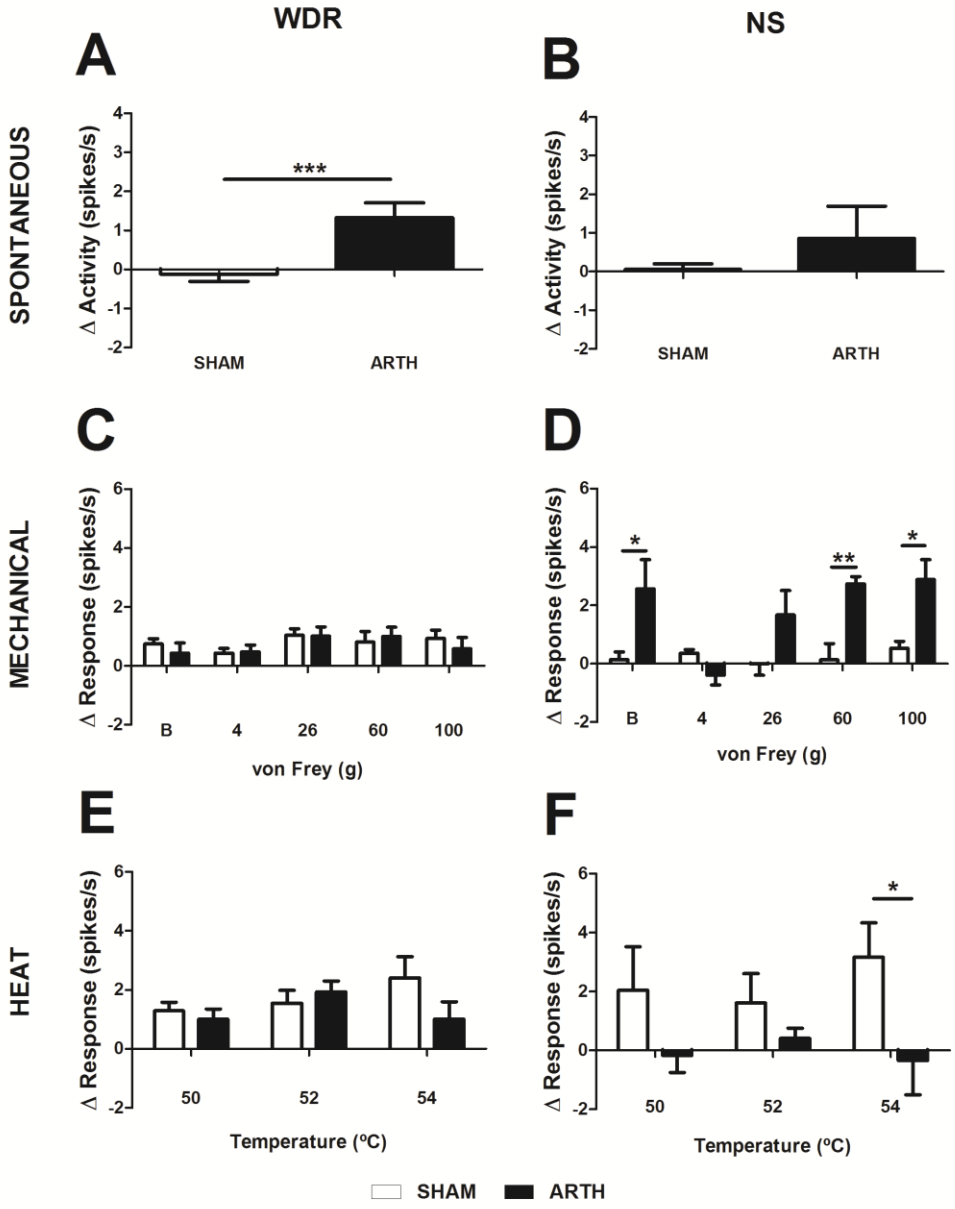


Figure 5. Baseline activity of spinal dorsal horn wide-dynamic-range (WDR) and nociceptive-specific (NS) neurons of control (SHAM) and arthritic (ARTH) animals. Spontaneous activity (WDR:**A**, NS:**B**), mechanical stimulation [brush (B), 4, 26, 60, 100 g von Frey filaments; WDR:**C**, NS:**D**] and heat-evoked responses (50, 52 and 54 °C; WDR:**E**, NS:**F**) in ARTH and SHAM groups. Graphs show mean neuronal activity + SEM (WDR: n_{SHAM}=60; n_{ARTH}=68; NS: n_{SHAM}=10; n_{ARTH}=12). *P<0.05, **P<0.01, ***P<0.001 (t-test with a Bonferroni correction for multiple comparisons).

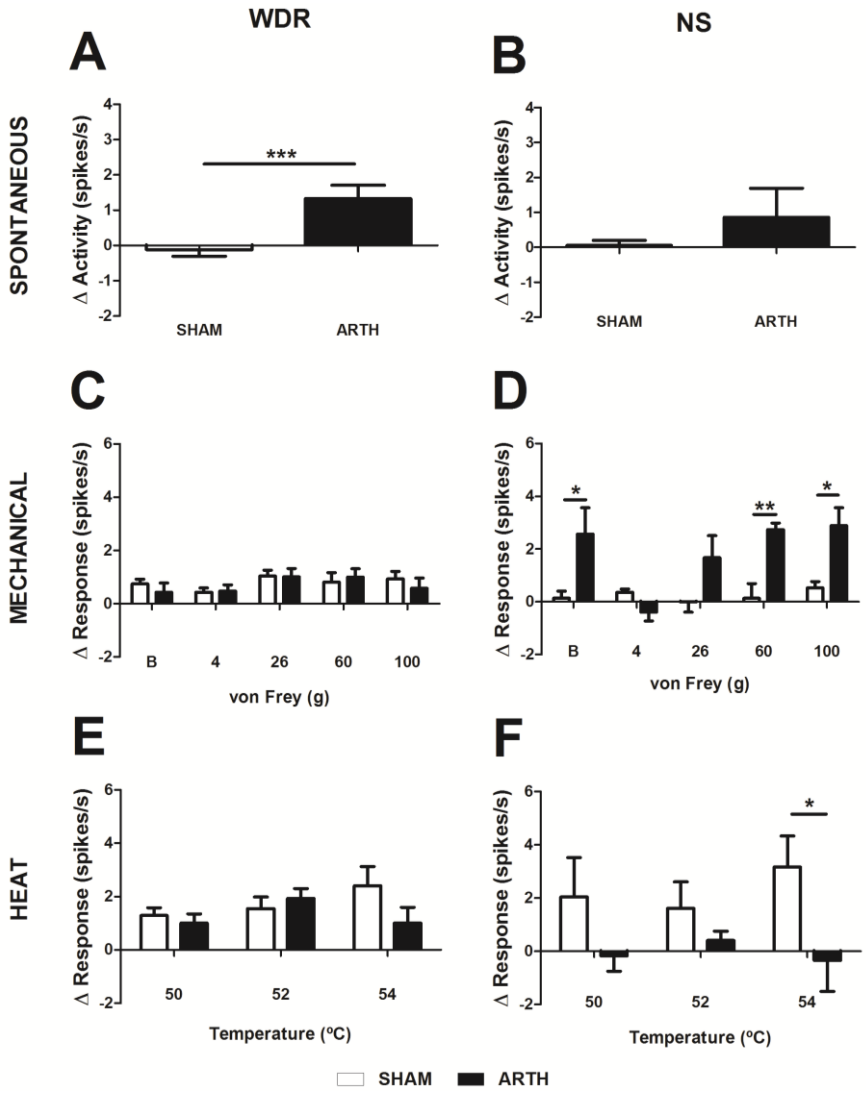


Figure 6. Effect of galanin (GAL) injection in dorsomedial nucleus of the hypothalamus (DMH) on spontaneous and stimulus-evoked activity in spinal dorsal horn wide-dynamic-range (WDR) and nociceptive specific (NS) neurones of control (SHAM) and arthritic (ARTH) animals. Mean changes in spontaneous (WDR:**A**, NS:**B**), mechanical stimulation [brush (B), 4, 26, 60, 100 g von Frey filaments; WDR:**C**, NS:**D**] and heat-evoked (50, 52 and 54 °C; WDR:**E**, NS:**F**) activities of WDR and NS neurones are shown 20 min after GAL administration in the DMH.

Δ Activity - represents the difference between spontaneous activity 20 min after GAL injection in the DMH and the spontaneous activity before GAL administration (baseline). Δ Response - represents the difference between neuronal response 20 min after GAL injection in the DMH and the response before GAL administration (baseline). Δ Activity or Δ response values > 0 represent GAL-induced increase in the neuronal activity or response. Graphs show mean activity + SEM (WDR: n_{SHAM}=60; n_{ARTH}=68; NS: n_{SHAM}=10; n_{ARTH}=12). *P<0.05, **P<0.01, ***P<0.001: Comparison of the effect of GAL with the corresponding pre-injection value (one sample t-test). ##P<0.01: Comparison of the effect of GAL between SHAM and ARTH groups (t-test with a Bonferroni correction for multiple comparisons).

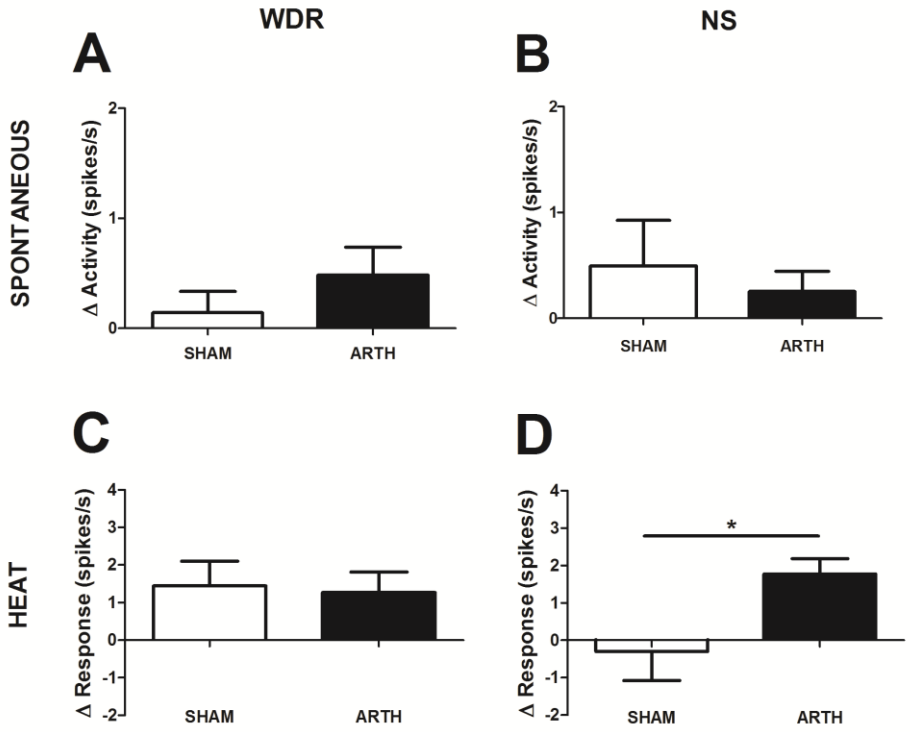


Figure 7. Effect of glutamate (GLU) injection in the medullary dorsal reticular nucleus (DRt) on activity of spinal dorsal horn wide-dynamic-range (WDR) and nociceptive specific (NS) neurons in control (SHAM) and arthritic (ARTH) animals. Mean changes in spontaneous (WDR:**A**, NS:**B**) and heat-evoked (54 °C. WDR:**C**, NS:**D**) activities of WDR and NS neurones are shown 1 min after GLU administration in the DRt.

Δ Activity - represents the difference between spontaneous activity 1 min after GLU injection in the DRt and the spontaneous activity before GLU administration (baseline). Δ Response - represents the difference between neuronal response 1 min after GLU injection in the DRt and the response before GLU administration (baseline). Δ Activity or Δ response values > 0 represent GAL-induced increase in the neuronal activity or response. Graphs show mean changes in neuronal activity + SEM (WDR: n_{SHAM}=31; n_{ARTH}=53; NS: n_{SHAM}=7; n_{ARTH}=9). $^{*}P<0.05$: Comparison of the effect of GLU with the corresponding pre-injection value (one sample t-test). $^{\#}P<0.05$: Comparison of the effect of GLU between SHAM and ARTH groups (t-test with a Bonferroni correction for multiple comparisons).

Chapter 2.5

Amorim D, Rebelo P, Almeida A, Pinto-Ribeiro F

The rostral ventromedial medulla as a brainstem mediator of behavioural hyperagesia induced by galanin in the dorsomedial nucleus of the hypothalamus

(Manuscript under preparation)

The rostral ventromedial medulla as a brainstem mediator of behavioural hyperagesia induced by galanin in the dorsomedial nucleus of the hypothalamus

Diana Amorim[1,2], Patrícia Rebelo[1,2], Armando Almeida[1,2], Filipa Pinto-Ribeiro[1,2]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), Campus of Gualtar, University of Minho, 4750-057 Braga, Portugal

[2]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

ABSTRACT

The rostral ventromedial medulla (RVM) is a serotonergic brainstem region highly implicated in descending pain modulation (Millan, 2002), as it receives inputs from frontal pain modulatory areas and modulates nociceptive transmission through direct projections to the spinal cord (Millan, 2002). Recently, we demonstrated that the activation by glutamate of the dorsomedial of the hypothalamus (DMH), a pronociceptive area, alters the activity of RVM On- and Off-like cells resulting in behavioural hyperalgesia (Pinto-Ribeiro et al., 2013). Behavioural hyperalgesia was also observed after galanin (GAL) microinjection in the DMH however this effect could not be correlated to changes in RVM On- and Off-like cell activity (Amorim et al., 2014). Further studies demonstrated that GAL in the DMH activates two independent descending pronociceptive pathways, one mediated by the dorsal reticular nucleus and a second that targets spinal 5HT3 receptors (Chapter 2.4). Thus, although GAL in the DMH pronociceptive effect was not dependent on RVM On- and Off-like cells, the participation of the RVM in the mediation of GAL/DMH-induced behavioural hyperalgesia cannot be excluded since RVM serotonin expressing neurones are Neutral-cells and not On- or Off-cells (Potrebic et al., 1994). To address this question, we chronically implanted two intracerebral guide cannulae, one in the RVM and another in the DMH, of control (SHAM) and arthritic (ARTH) animals and evaluated paw-withdrawal latencies (PWL) after each of the following protocols: (i) saline (SAL) injection in both the RVM and DMH; (ii) SAL injection in the RVM and GAL in the DMH; (iii) lidocaine (LIDO) injection in the RVM and SAL in the DMH, and; (iv) LIDO injection in the RVM followed by GAL in the DMH.

Drug injection in the DMH and RVM significantly altered the PWL of animals (main effect of drug: $F_{(3,40)}$=20.57, P<0.001), an effect that did not varied with the experimental group (interaction drug x group $F_{(3,40)}$=0.488, P=0.692). *Post-hoc* testing showed that SAL in the DMH and RVM of SHAM and ARTH animals did not alter PWL. GAL administration in the DMH decreased PWL of SHAM and ARTH animals. Similarly, PWL was also decrease after the LIDO in the RVM. Importantly, the

RVM blockage by LIDO prevented the behavioural hyperalgesia induced by GAL in the DMH (**Fig 1**). Since RVM On- and Off-like cells are not involved, our results indicate that a subpopulation of spinally-projecting RVM Neutral-cells have a pronociceptive role in the behavioural hyperalgesia induced by GAL in the DMH. Taking into account that GAL/DMH pronociception is mediated by 5HT3R, it is probable that spinally-projecting RVM serotonergic (Neutral) neurones target these receptors. As the role of RVM Neutral-cells in nociception is not clear, further studies using retrograde and anterograde tracers should be performed in order to demonstrate that DMH cells target spinally-projecting RVM serotonergic cells.

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Chapter 2.4. of this thesis

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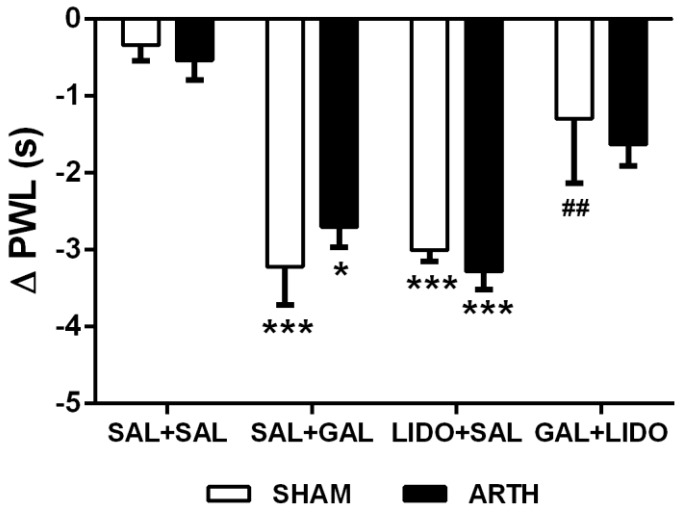


Figure 1: Paw-withdrawal latency (PWL) after drug administration in the rostral ventromedial medulla (RVM) and dorsomedial nucleus of the hypothalamus (DMH). Variations in PWL (ΔPWL) of control (SHAM) and arthritic (ARTH) animals between the behavioural response of the animals 20 min after each of the following pharmacological protocols and the response before drugs administration: (i) saline (SAL) injection in both the RVM and DMH; (ii) SAL injection in the RVM and galanin (GAL) in the DMH; (iii) lidocaine (LIDO) injection in the RVM and SAL in the DMH, and; (iv) LIDO injection in the RVM followed by GAL in the DMH. No differences were observed after SAL injections in both experimental groups. The administration of GAL in the DMH resulted in a decrease in the PWL of SHAM and ARTH animals. Similarly, a decrease in PWL was observed after the LIDO in the RVM. The RVM blockage by LIDO prevented the behavioural hyperalgesia induced by GAL in the DMH. (n=6 per protocol in each experimental group). The graph is presented as mean ΔPWL+SEM. $^{**}P<0.01$, $^{***}P<0.001$: Comparison of the drugs effect with the effect of SAL+SAL within the same experimental group; $\#\#P<0.01$: Comparison of the effect induced by the combination of LIDO-GAL with the corresponding SAL+GAL group (t-test with a Bonferroni correction for multiple comparisons).

Chapter 3

Discussion

3. DISCUSSION

In the present thesis with the aid of morphofunctional, behavioural, pharmacological and electrophysiological tools, we were able to demonstrate some of the underlying mechanisms of descending nociceptive facilitation associated to chronic pain and comorbid emotional impairments. In **Chapter 2.1** we show that prolonged monoarthritis in the knee results in the comorbid development of anxiety- and depression-like behaviours in a rat model. Additionally, the treatment with amitriptyline, a tricyclic antidepressant, results in the improvement of both pain- and mood-like disorders. In **Chapter 2.2** we demonstrate that the DMH is a pronociceptive nucleus whose facilitatory drive is at least partly mediated by RVM pain modulatory cells. Surprisingly, nociceptive facilitation from the DMH after its general activation by glutamate is absent in arthritic (ARTH) animals. Interestingly, when galanin-dependent pathways in the DMH are pharmacologically manipulated with specific GAL agonist and antagonists (**Chapter 2.3**) an increase in the nociceptive response of both healthy and ARTH animals is observed, showing, for the first time, a pronociceptive role for supraspinal GAL. In addition, we were able to associate the activation of GALR1 in the DMH with the behavioural hyperalgesia induced by GAL in this nucleus. Contrary to what was expected, GAL/DMH-induced behavioural hyperalgesia is not concomitant with changes in the activity of in RVM On- and/or Off-like cells. While (**Chapter 2.4**) searching for other possible mediators of the nociceptive facilitatory effect of GAL in the DMH, we showed that GAL in the DMH activates two distinct pain facilitatory pathways: (i) one relayed by the DRt and (ii) another involving 5HT neurones acting on 5HT3Rs in the spinal cord. Finally, in **Chapter 2.5** we demonstrate that, although RVM On- and Off-like cells do not appear to be involved in the GAL/DMH pronociceptive effect, this nucleus should not be excluded as a brainstem mediator of the GAL/DMH action, since RVM blockage with lidocaine prevente behavioural hyperalgesia after GAL in the DMH. In summary, the studies presented herein provide novel data regarding brain and spinal cord alterations elicited by a prolonged pain state (monoarthritis) and comorbid emotional-like disorders, and a pronociceptive role of supraspinal GAL (**Fig. 4**).

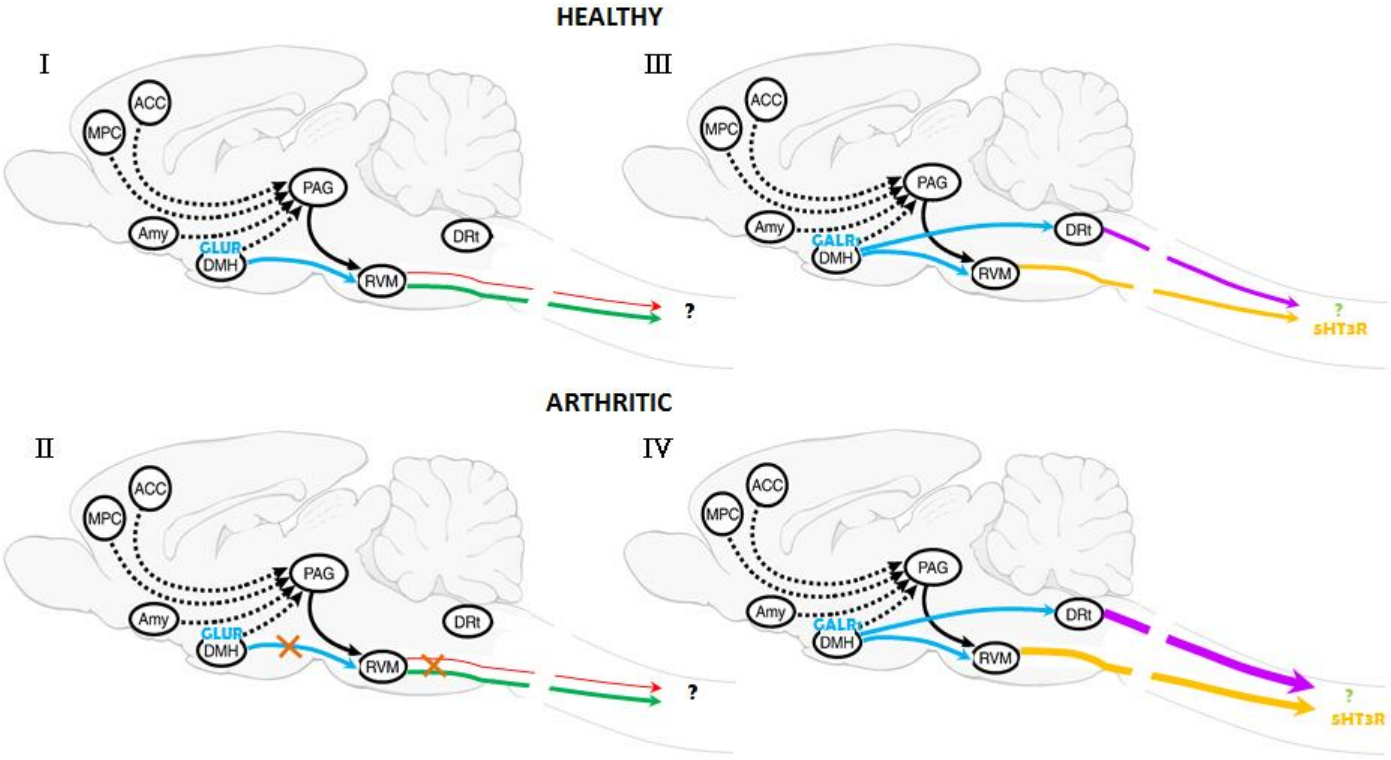


Figure 4. Pain facilitatory pathways from de dorsomedial nucleus of the hypothalamus (DMH) in healthy and arthritic animals. While glutamate receptors (GLUR) activation in the DMH stimulate rostral ventromedial medulla (RVM) On-like cells (pronociceptive; green line) and inhibit RVM Off-like cells (antinociceptive; red line) in healthy animals (I) these effects are absent in arthritic animals (II). Curiously, the activation of the galanin receptor 1 (GALR1) in the DMH stimulates two independent pain facilitatory pathways, one relayed by the DRt and another involving 5HT neurones, possibly from the RVM, acting on 5HT3Rs in the spinal cord in healthy (III) and arthritic animals (IV). Importantly, both galanin/DMH pronociceptive pathways are tonically activated only in arthritic animals (IV), which may correlate with the behavioural allodynia and hyperalgesia observed in these animals. [Modified from (Heinricher et al., 2009)].

3.1. Technical considerations

3.1.1. Ethical issues

All scientific research that includes the use of animals is sustained by several ethical recommendations that ensure an appropriate level of animal's well-being (Wolfensohn and Lloyd, 2013). These requirements not only protect the animals but also the research in general, as healthy animals will allow the reaching of valuable results. In this context, since pain by definition reduces dramatically the well-being of the animals, the research on the pain field follows the recommendations of ethical committees in general, the European Community Council Directive

86/609/EEC and 2010/63/EU, but also specific recommendations formulated by the IASP ethical committee (Zimmermann, 1983).

The awareness that the animal is a living being with sensations and not a mere laboratory object is the first step to ensure valid animal research. Moreover, protocols should minimize the number of animals needed to obtain valuable results and avoid unnecessary pain, discomfort or stress (Wolfensohn and Lloyd, 2013). Accordingly, in the work of this thesis great care was taken in order to give the animals the better conditions possible. Animals were daily supported by caretakers that guaranteed the availability of food and water and the cleaning of the home cages. The researchers and a veterinary followed the general health state of the animals throughout all the experiments. Anaesthesia was used whenever the experimental procedure was more invasive, such as arthritis induction, cannulae implantation and electrophysiological recordings. Euthanasia was carefully prepared to be performed as humanely as possible (Reilly, 2001).

3.1.2. The choice of the experimental model

Controlled research of joint structural and general physiological changes in arthritic patients presents ethical and practical limitations, such as the lack of precise non- or minimal-invasive methods to identify the early stages of the disease along with a variety of genetic, nutritional, biomechanical, and pharmacological patient backgrounds, hence experimental animal models that mimic the human disorders are commonly used to study arthritis.

Animal models are powerful tools for studying the pathogenesis and potential therapeutic intervention of specific diseases. The existence of numerous experimental models of arthritis allows the clarification of mechanisms underlying changes in functionality and degeneration of the cartilage, bone and synovium. Although *ex vivo* models are useful for understanding short-term biological events and events isolated from the physiological influences of adjacent structures and general metabolism, animal models are more helpful while studying complex changes over time and therapeutic strategies. However, since no model is able to completely replicate all the events occurring in the human disease, the choice of the animal model to use depends on its ability to mimic the phenomenon under study. Additionally, some practical matters such as the adequacy of animal supply, the presence of adequate controls, the ease of handling and environmental maintenance, as well as costs are also, to some extent, criteria while choosing an animal model.

In arthritis research, there are several animal models available (Pritzker, 1994; Little and Zaki, 2012), including spontaneous and experimentally-induced models. Within the latter, some affect specific joints (monoarthritis model) while others induce widespread effects (polyarthritis models). To study specific mechanisms as in the works presented herein, a monoarthritic model was more appropriate since it allowed a more precise evaluation of the type and severity of the injury as well as the time of onset and progression. Additionally, the contralateral joint can function as an internal control although local lesions may impact other parts of the body, thus joints of age-matched animals were preferably used as controls (Sham).

Studies on osteoarthritis (OA), the most common form of arthritis, can be divided in: (i) works investigating the pathophysiology and regulation of structural joint damage, often using models in which the induction method has some physiological relevance to naturally-occurring initiating events or risk factors such as aging, obesity and joint injury; and (ii) works focused on understanding pain mechanisms, using intra-articular injection models (Pritzker, 1994; Little and Zaki, 2012). In the latter, the initiating event is not typical of OA but, as mentioned above, since the injury is located in a single joint, these models are more appropriate to study pain modulatory pathways.

The intra-articular injection of monoiodoacetate (MIA) is the most used in OA pain research (Little and Zaki, 2012). This model results in articular degeneration but the pathophysiological processes are different from the ones observed in humans, namely in what concerns the cartilage transcriptome with about two-third transcripts being up-regulated in MIA contrasting with the two-third being down-regulated in OA patients (Barve et al., 2007). The kaolin/carrageenan (K/C) model of OA although less studied also induces joint degeneration and pain (Little and Zaki, 2012). Importantly, most of the studies using this experimental monoarthritic model focus on short post induction time-frames in which the inflammatory component is its main feature and structural changes in the joint less significant (Lu and Westlund, 1999; Ji et al., 2007). In this thesis we choose to prolong the post-induction time before testing the animals (four weeks), demonstrating that, at this time point, animals presented significant knee degeneration that is comparable to grade 3 of human OA (Pearson et al., 2011), with irregular cartilage surface, cartilage detachment, narrowing of the joint space and subchondral cyst formation (**Chapter 2.1**).

3.1.3. Behavioural assessment of locomotion

The use of behavioural paradigms to assess locomotion and anxiety in rodents is common; however, the unavailability of tests that allow the evaluation of pure emotional behaviour is a major drawback. In fact, many of the behavioural tests are dependent on locomotion, which can bias the behavioural analysis if this parameter is affected by the model itself. Hence, the assessment of the emotional state of the animals should always be accompanied by the assessment of eventual locomotory impairments.

In this context, the open field (OF) is a good complementary test when assessing anxiety, since it combines both the analysis of locomotion and anxiety (Sousa et al., 2006; Gonçalves et al., 2008; Grégoire et al., 2012). The total distance travelled by the animal in the OF arena is used as an indication of the locomotory status of the animal. In addition, several parameters can be used to evaluate the anxiety-like status, such as the number of fecal boli and the time spent in the centre of the arena. Importantly, the protocols to assess anxiety using the OF test should guarantee that the intensity of the light in the centre of the arena is high enough to be considered anxiolytic but not excessively intense to affect the normal locomotory activity of the animals (Bouwknecht et al., 2007).

In addition, motor skills can also be assessed using the rotarod test (Monville et al., 2006; Bohlen et al., 2009). In this test animals are placed in a rotating rod and the latency to fall is a measure of balance, coordination, physical condition, and motor-planning. It is important to take into account that prolonged monoarthritis affects the performance of animals mainly during intense physical activity does not altering the total distance travelled by the animals in the OF test. Hence, as the test used to assess anxiety-like behaviour (elevated plus maze) requires only normal walking activity, the OF test was preferred to evaluate the locomotor conditions of the animals.

3.1.4. Behavioural assessment of anxiety and depression

Emotional impairments, such as anxiety and depression, are common comorbities of patients with arthritis (Murphy et al., 2012). In animals these emotional disorders can only be assessed indirectly and are thus often referred to as anxiety-like and depressive-like behaviours.

To assess the anxiety-like behaviour of animals, the elevated plus maze (EPM) test is currently the first-choice test and its validity has been extensively documented (Cruz et al., 1994; Rodgers and Dalvi, 1997; Wall, 2001; Sousa et al., 2006). In this test the animal has to overcome a natural conflict between the innate drive to explore novel environments and the tendency to avoid a potential dangerous place. It should be noted that the behaviours measured in the EPM test depend on locomotor activity, which should be also evaluated. An alternative method to assess anxiety-like behaviour in rats is the elevated zero maze. This test consists on a circular runway elevated from the floor with alternate open/brightly light areas and enclosed/dark paths. This test has the advantage of eliminating the ambiguous interpretation of animal location in the centre area of the EPM (Shepherd et al., 1994). The choice of the behavioural test used in our work was based on the availability of the apparatus and the fact that the EPM is the gold standard test to assess anxiety.

While assessing depressive-like behaviour in our animals we opted to evaluate two different components, anhedonia-like behaviour and learned helplessness. There are no perfect behavioural tests to assess hedonic-like behaviour in rodents, however the sucrose preference test (SPT) is a standard and validated test to measure this component of depression in rats (Duman, 2010; Overstreet, 2012). As for most experimental procedures, there is a variety of SPT protocols available that differ in terms of prior deprivation of food and water, duration of the evaluation session and time at which the animals are tested (light/dark cyle). In our studies we opted to perform food and water deprivation for 24h before testing the animals in order to guarantee they would be driven to drink. This choice also allowed us to reduce the evaluation time to one hour per animal. In addition, the animals were tested during their activity period which also contributed to more reliable results (Tõnissaar et al., 2006). Finally, one of the major advantages of this test is its ability to assess hedonic behaviour independently of the locomotory activity.

To assess learned helplessness in rats, the test of choice is the forced swimming test (FST) (Cryan et al., 2002; Duman, 2010; Overstreet, 2012). In this paradigm, rats are exposed to an inescapable aversive scenario (a cylinder filled with warm water) for two consecutive days. In the first day, the animals learn that they cannot escape the cylinder - learned helplessness - and in the second day a decrease in the latency to immobility and an increase in the time spent immobile are the outcomes associated to learned helplessness. In addition we opted to use a modified FST analysis procedure that also evaluates active behaviours such as the time spent

swimming and climbing during the test. This upgrade has been proposed to distinguish animal performance between behaviours that are dependent from serotonergic (swimming) or noradrenergic (climbing) pathways (Detke et al., 1995; Lucki, 1997). Since this test is highly dependent on motor activity it should be complemented by other tests assessing depressive-like behaviour, such as the SPT and analysed in light of the results concerning motor performance.

3.1.5. Behavioural assessment of nociception

The biggest challenge of animal experiments involving pain is the lack of verbal communication. Hence, while the application of acute noxious stimuli results in the display of a series of standardized behavioural responses, its absence does not prove the animals are not in pain (Le Bars et al., 2001). The acknowledgement of these differences lead to the adaptation of the IASP definition of pain in order to be applied to animals, as "an aversive sensory experience caused by actual or potential injury that elicits progressive motor and vegetative reactions, results in learned avoidance behaviour, and may modify species specific behaviour, including social behaviour" (Zimmermann, 1986). Consequently, nociceptive behaviour is mainly ascertained by observing physical reactions of animals to a stimulus known to cause pain to humans. Importantly, the assessment of other impairments, such as ongoing pain and imbalanced of sleep patterns and mood, might better reflect the diverse symptomotology of chronic pain in humans (Blackburn-Munro, 2004).

3.1.5.1. Mechanical allodynia

Mechanical allodynia is a high constraint symptom in chronic pain. In animal models, mechanical allodynia is often addressed using von Frey filaments applied to the plantar surface of the hindpaws (Neugebauer et al., 2007). However, to study primary mechanical allodynia in joints different from hindpaw joints, the von Frey test is not appropriate. In that sense, a normal flexion/extension of the arthritic joint could be used as a measure of mechanical allodynia (Yu et al., 2002; Neugebauer et al., 2007). This is a qualitative analysis that requires the habituation of the animals to the experimenter in order to reduce stress confounding effect, since the animals need to be restrained. In our work, we tested the animals at 3 days post intra-articular injection of saline or K/C in the right knee and at least 1h before performing any behavioural test. Animals

that vocalized to every 5 flexion/extension movements of the right knee were included in the ARTH group.

3.1.5.2. Mechanical hyperalgesia

Patients with arthritic joints usually report mechanical hyperalgesia (Farrell et al., 2000; Imamura et al., 2008); however, in laboratory conditions, we only rely on a small variety of apparatus to measure this symptom. The Randall-Selitto paw-pressure device is a good approach to assess mechanical hyperalgesia in rodents, giving accurate and consistent results (Randall and Selitto, 1957). The drawback of this test is that it can be used only in the paws and is not applicable to other parts of the body, namely the knee joint.

More recently, a new technology, the pressure application measurement (PAM) test (Barton et al., 2007), was developed to allow assessing mechanical hyperalgesia in any joint of the body. In this test the experimenter securely holds the animal and applies an increasing force to the joint being tested. The peak force applied, immediately before the first signs of discomfort, such as vocalization, freezing and withdrawal is recorded. Two of the disadvantages of the PAM test are that this test is highly dependent on the expertise of the experimenter and that is required the restraining/handling of the animals while the test is being performed, nonetheless the results obtained were consistent and PAM is an useful tool in the study of arthritis.

3.1.5.3. Thermal hyperalgesia

Thermal hyperalgesia, namely heat hyperalgesia, is also usually assessed in pain research using rodents. There are numerous behavioural tests available to evaluate this feature, involving the assessment of a response to the application of either a specific temperature or a gradually increasing temperature. In both the tail-flick (TF) and paw-flick (PF) tests (Hargreaves et al., 1988; Le Bars et al., 2001) a radiant heat source is applied to the skin, of the tail and hind paw respectively, and the latency to withdrawal is recorded. The fact that in these tests the stimulus is applied to a small surface of the skin instead of a large area or several different points, as in the hot plate, increases the tests accuracy and decreases the chance of evoking DNIC phenomena (Le Bars et al., 2001).

The TF and PF tests are simple, reproducible and present low inter-animal variability, all of which are essential when pharmacologically manipulating areas within the pain matrix. In our studies, we opted to use especially the PF test has it presents some advantages in relation to the TF test, such as: (i) the area of stimulation is the paw, which is not a thermoregulatory organ and hence is less prone to bias when using temperature as a stimulus (Hole and Tjølsen, 1993); (ii) the animals are not restrained thus eliminating the additional stress associated to animal manipulation; (iii) this test was specifically developed in order to allow the study of hyperalgesia in acute and chronic inflammatory conditions (Hargreaves et al., 1988).

3.1.6. Electrophysiological recordings

Electrophysiological recordings in anaesthetised rats have been widely used to clarify changes in spinal and supraspinal activity of nociceptive neurones (Ali et al., 1996; Chapman et al., 1998; Pertovaara et al., 2001; Martenson et al., 2009; Pinto-Ribeiro et al., 2011). The type and level of anaesthesia is a factor that should be taken into account during the electrophysiological sessions since it might influence the activity of neurones (Vahle-Hinz and Detsch, 2002). A series of anaesthetics, inhalational - isoflurane and sevoflurane, or injectable - ketamine and pentobarbitone, is currently used in this area making it difficult to compare results among works.

Recordings performed in rats under inhalational anaesthetics allow a stable level of unconsciousness but require extensively investments such as the installation of a specific delivery system and air circulation systems. By contrast, injectable anaesthetics are less stable but have less equipment requirements and are less expensive. Our recordings were performed using the latter thus, to counteract its instability, the anaesthesia level was continuously monitored and reinforced when necessary, the duration of the recordings limited to 4h and basal neuronal activity frequently evaluated.

Another relevant point is the type of electrophysiological recordings used. Although several electrophysiological works in neuroscience research use whole field recording, when studying pain it is essential to discriminate functionally-different cell types, such as those recorded in the RVM. In the pain field, the RVM is the brain region most studied electrophysiologically (Fields et al., 1983; Kincaid et al., 2006; Silva et al., 2013). Indeed, the classification of RVM neurones as On-, Off- and Neutral-cells based on their response to the application of peripheral noxious stimuli using *in vivo* extracellular single-cell electrophysiological recordings was a major step for pain

research. Moreover, the association of behaviour data with changes in RVM cell activity was vital to demonstrate the existence of descending inhibitory and facilitatory pathways, and their anatomical and physiological substrate (mechanism).

Similarly, this technique has also been used to study the activity of nociceptive spinal neurones, namely WDR and NS neurones, since they encode information about noxious peripheral stimulation (D'Mello and Dickenson, 2008). In terms of chronic pain disorders, the electrophysiological studies substantially contribute to the understanding of persistent nociceptive signalling towards the development of central sensitization, hyperalgesia and allodynia.

An additional alternative approach would be the chronic implantation of recording electrodes both in supraspinal pain modulatory areas and in the spinal cord (Berg et al., 2009; Chorev et al., 2009). Although chronic electrodes have been used mainly to record local field neuronal activity, single-unit recordings are possible (Chorev et al., 2009). Since single-unit recordings require the proximity of the electro to the target neurones, thus it is difficult to guarantee a long-term recording of the same neurone using chronic electrode implants, as any small variation of the electrode position, due to the movement of the animal, can damage or move away the neurone being recorded; however, it has several advantages in relation to *in vivo* extracellular single-cell recordings in anesthetised animals, firstly because animals is awake thus eliminating the potential confounding effect of anaesthetics, and secondly because it allows a follow-up analysis of the processes underlying pain chronification in the same animal.

3.1.7. *c-fos* as a neuronal marker in nociception

c-fos expression has been widely used as a marker of neuronal activation, either by quantifying the accumulation of its mRNA, which reach its peak 30 to 40 min after the stimulation, or by counting the number of positive-cells to the nuclear protein Fos that peaks around 2 h after induction of gene transcription (Harris, 1998).

The association between *c-fos* expression and nociception emerged based on observations showing that peripheral noxious stimulation induces *c-fos* expression in neurones of spinal dorsal horn laminae I, IIo, V and VI (Hunt et al., 1987; Presley et al., 1990; Bullitt, 1991), where nociceptors synapses, and importantly, this pattern of activation is suppressed by the administration of analgesic drugs (Presley et al., 1990; Gogas et al., 1991; Jasmin et al., 1994) or by activating endogenous pain antinociceptive pathways (Jones and Light, 1990; Lee and

Beitz, 1992). Moreover, in animal models of inflammatory or neuropathic pain, spinal *c-fos* expression increases even in the absence of additional stimuli or after innocuous stimulation (Abbadie and Besson, 1992; Lantéri-Minet et al., 1993; Bellavance and Beitz, 1996; Leah et al., 1996; Ma and Woolf, 1996), which correlates with the increased nociceptive sensitization observed in these animals. Besides the association of *c-fos* expression and nociception at spinal cord level, its expression in the brain has also been used to investigate nociceptive processing, namely in the brainstem, hypothalamus, and thalamus (Bullitt, 1989, 1990; Keay and Bandler, 1993; Pertovaara et al., 1993; Lantéri-Minet et al., 1994; Pan et al., 1994; Carstens et al., 1995; Bellavance and Beitz, 1996).

Among other advantages, *c-fos* expression allow an easy identification of the precise location of neuronal populations that respond to noxious stimulation and can be readily analyzed quantitatively by simply counting the number of neurones immunoreactively labelled for Fos or labeled for *c-fos* mRNA by in situ hybridisation. However, to reach quantifiable levels of Fos expression, stimulation must be intense and prolonged (Bullitt et al., 1992; Lima and Avelino, 1994) and the absence of *c-fos* expression cannot be taken to identify the absence of neuronal activity, since not all neurones express the gene when activated (Dragunow and Faull, 1989), namely dorsal root ganglion cells (Hunt et al., 1987) and neurones of ventroposterolateral nucleus in the thalamus. Important is also the risk of false positive results, since c-*fos* expression may be related to other factors, such as the stress response caused by pain itself, motor activity elicited by the stimulation, or anaesthesia (Harris, 1998).

3.1.8. Functional modulation of brain regions

The understanding of the relationship between the neuronal function of a brain region and a specific behaviour is a major goal of neurosciences, especially so when studying pain. The electrical or pharmacological lesion of a specific brain area has often been used to clarify the role the region in nociception. However these techniques might result in a permanent loss of function and, more importantly, is not specific for a neuronal population or neurotransmitter system within the area being evaluated (Almeida et al., 1996; Tsuruoka and Willis, 1996).

The opposite strategy activates an area either by electrical stimulation or pharmacologically, through local GLU injection (Mayer et al., 1971; Gallo, 1981; Zhuo et al., 1997), which presents identical limitations but the advantage of being reversible. While using the latter, pharmacological

studies are preferable when compared to electrical stimulation as it is possible to dissect specific neuronal pathways when using selective agonists/antagonists of neurotransmitters receptors.

Genetic models are another possible approach to understand the role of specific neurotransmitters in the brain but most of these models present constitutive and early life changes leading to confounding results due to developmental problems or compensatory mechanisms. Importantly, genetics models are almost non-existing in rats, a fact that greatly constrains their use.

In an attempt to overcome the above mentioned limitations optogenetics in rat models emerged as a technique that allows pathway-specific manipulation of brain circuitry over a range of timescales (Fenno et al., 2011), while avoiding the technical limitations of genetic models. In this context, simultaneously performing optical stimulation and multi-site extracellular neuronal recordings in free moving rats would greatly deepen our knowledge on pain mechanisms (Wang et al., 2012).

3.2. Arthritis and comorbid emotional disorders

3.2.1. Prolonged arthritis in the knee and associated emotional pathologies

A reciprocal potentiation between chronic pain and mood disorders is currently well established (Bair et al., 2003; Murphy et al., 2012; Gerrits et al., 2014), raising the need to consider chronic pain not as an independent disorder with independent neuronal circuits and neurotransmitter systems but as a physiological and psychological state that coexists with other pathologies. In this context, the treatment of chronic pain patients should target not only pain relief by itself but also provide preventive or actual treatment of its comorbid disorders, such as anxiety and depression.

In our work (**Chapter 2.1**), we demonstrated that animals with monoarthritis vocalize during a normal flexion/extension movement of the affected knee joint (mechanical allodynia) and displayed a decreased withdrawal threshold to noxious mechanical stimulation of the same joint (mechanical hyperalgesia). Importantly, when a prolonged pain condition (four weeks) was allowed to evolve, ARTH animals exhibited both anxiety-like, with increased number of fecal boli in the OF arena and low exploration of the open arms in the EPM tests, and depressive-like symptoms, as shown by reduced preference for a sweet solution in the SPT and increased immobility time in the FST.

In preclinical studies, anxiety- and depressive-like impairments associated to the development of chronic pain have been almost exclusively demonstrated in neuropathic pain animals (Gonçalves et al., 2008; Matsuzawa-Yanagida et al., 2008; Wallace et al., 2008; Hu et al., 2009; Yalcin et al., 2011; Wang et al., 2011; Bravo et al., 2012; Goffer et al., 2013; Caspani et al., 2014; Li et al., 2014; Saito et al., 2014). In arthritic pain models anxiety-like changes have been shown in some studies (Fernández-Guasti et al., 2005; Narita et al., 2006; Jiménez-Velázquez et al., 2010; Parent et al., 2012; Liu and Chen, 2014) while depression has been less studied (Borges et al., 2014).

Interestingly, in arthritic models, the assessment of emotional behaviour was mostly performed shortly after induction (Lu and Westlund, 1999; Ji et al., 2007; Kim et al., 2012), which is paradoxal *per se* as time is needed for the establishment of emotional comorbidities in chronic animals pain models as well as in humans (Burgess et al., 2002b; Yalcin et al., 2011; Mifflin and Kerr, 2014). In fact, a time-dependent profile in the development of anxiety and depression in an experimental model of neuropathic pain has been shown (Yalcin et al., 2011), in which the expression of an anxiety-like phenotype required four weeks while depressive-like behaviour was only observed 6 to 8 weeks post-induction. By extending the post-induction period to four weeks (**Chapter 2.1**) we were able to demonstrate the development of both anxiety- and depression-like behaviours in the K/C model of arthritis.

3.2.2. The role of 5HT and NA in pain and emotions

Our findings are in accordance with the definition of chronic pain, as pain is not only a sensory experience but also comprises a strong negative affective component (Calvino and Grilo, 2006; Loeser and Treede, 2008; Bie et al., 2011). This concept implies the existence of a close relationship between the neuronal circuits mediating the sensory-discriminative component of pain and the circuits mediating emotional processing. Nonetheless, the mechanisms underlying the development of anxiety and/or depression during chronic pain are still mostly unknown.

In the last decades, some monoamines (noradrenaline and serotonin) have slowly emerged as potential actors in this interplay. The results of the FST test support this hypothesis since a decrease in both the time spent climbing and swimming was observed (**Chapter 2.1**), showing potential impairments in 5HT and NA pathways (Detke et al., 1995), an effect that was partly reversed after treatment with a tricyclic antidepressant (amitriptyline). In fact, the monoaminergic

system seems to play a critical role in both affective and chronic pain disorders (Harro and Oreland, 2001; Stahl and Briley, 2004; Dray, 2008; Lin et al., 2011; Hamon and Blier, 2013). Changes in 5HT and/or NA content in certain brain regions, such as the raphe nuclei and the LC are correlated with the development of pain and emotional pathologies (Ressler and Nemeroff, 1999; Harro and Oreland, 2001; Stahl and Briley, 2004).

In the LC, dysfunction of NA neurones has been widely reported in depressed patients; however, inconsistent data regarding the decreased- or increased- (Ressler and Nemeroff, 1999) activation of their function difficult the development of a clear mechanism. The NA hypothesis of depression was raised based on the fact that drugs that deplete monoamines can lead to depressive symptoms (Ressler and Nemeroff, 1999) and antidepressants strongly target NA neurones, increasing the levels of NA in the synaptic cleft and improving these symptoms (Hamon and Blier, 2013). However, the analyses of samples of cerebral spinal fluid from depressed patients disagree with such observations, demonstrating either an increase or unchanged concentrations of NA or its metabolites in the central nervous system (Ressler and Nemeroff, 1999).

In parallel, the role of the LC in pain modulation is bidirectional, as it can either inhibit or facilitate nociception depending on the pathophysiological condition (Pertovaara, 2006). The damage of LC NA neurones in animals with inflammatory pain prevented the development of hyperalgesia (Tsuruoka and Willis, 1996), which is in line with a facilitatory role of this area upon the development of pain syndromes. More recently, two subpopulation of NA neurones in the LC has been demonstrated, one that inhibit and another that facilitates nociception, suggesting a bidirectional influence of the LC upon thermal nociception (Hickey et al., 2014).

Identically, although the RVM is a key brain region in of the endogenous pain inhibitory system (Millan, 2002; Pertovaara and Almeida, 2006), its role in nociceptive facilitation has also been described in prolonged pain conditions (Millan, 2002). The facilitatory role of the RVM was proposed based on the observations that its excitotoxic lesion in animals with arthritis attenuated both behavioural hyperalgesia and spinal neuronal activation (Wei et al., 1999; Porreca et al., 2002). In addition, the chemical lesion of the RVM in animals with knee monoarthritis also blocked secondary hyperalgesia (Urban et al., 1999; Vanderah et al., 2001). The mechanisms underlying the RVM nociceptive facilitatory effect during persistent pain is not yet clear, with some studies showing an imbalance between the activity of RVM pronociceptive On-cells and antinociceptive Off-cells (Kaplan and Fields, 1991; Heinricher et al., 2009). Alternatively, an

increase in 5HT signalling mediated by RVM Neutral-cells and targeting spinal 5HT3Rs has also been proposed (Potrebic et al., 1994; Ali et al., 1996; Rahman et al., 2009; Yanarates et al., 2010; Gu et al., 2011) but is yet to be demonstrated.

Another potential player is the AMY as this area not only receives extensive projections from pain modulatory regions but also mediates emotional behaviour, expressing and releasing 5HT and NA (Asan et al., 2013; Strobel et al., 2014). In fact, the AMY plays a key role in the processing (formation and storage) of memories associated with emotional events and is recruited during a painful experience during which it attributes an emotional significance to pain (Strobel et al., 2014). Interestingly, an identical increase in the activity of the AMY has been reported in depression (Drevets et al., 2002), anxiety (Etkin and Wager, 2007; Forster et al., 2012) and in patients with knee arthritis (Kulkarni et al., 2007). A similar pattern of AMY activity was also observed in animals with experimental arthritis (Neugebauer and Li, 2003; Han et al., 2004, 2006; Bird et al., 2005), a model were the AMY plays a role in pain-related anxiety-like behaviour and nocifensive responses (Ji et al., 2007).

Finally, in our work, we demonstrated that amitriptyline, a tricyclic antidepressant that is also used in the management of human chronic pain (Jann and Slade, 2007; Verdu et al., 2008), is able to reverse mechanical hyperalgesia in animals with experimental monoarthritis in addition to partly improving mood-associated impairments (**Chapter 2.1**). The fact that amitriptyline inhibits the re-uptake of 5HT and NA and that behavioural hyperalgesia was reversed in ARTH animals after treatment with amitriptyline indirectly suggests that a dysfunction of the monoaminergic system might be involved in pain-depression comorbidity. Thus, although to the best of our knowledge no available data could be found concerning the use of amitriptyline in the management of arthritic pain, this antidepressant could potentially ameliorate pain and some of its associated emotional impairments in arthritic patients.

3.2.3. The DMH as a potential mediator of chronic pain and emotional comorbidities

A strong bidirectional connection between the AMY and the DMH has been described (ter Horst and Luiten, 1986; Thompson, 1998). Moreover, this reciprocal projections result mostly in mutual activation as the disinhibition of the AMY, with a GABAA receptor antagonist, increased the activity of the DMH (Zhang et al., 2009) and the electrical stimulation of the DMH also

increased the activation of the AMY (Silveira et al., 1995). Furthermore, the fact that the projecting neurones from the central nucleus of the AMY that target the DMH are GABAergic (Myers et al., 2014) together with the knowledge that most DMH neurones express GABA (Okamura et al., 1990; Cullinan et al., 2008), suggest that the AMY-DMH loop functions as a disinhibiting mechanism.

Concomitantly, GABA receptors in the DMH have been associated with the regulation of anxiety in the EPM test and conflict and social interaction in rats, with GABA receptors blockage evoking anxiogenic- and its activation eliciting anxiolytic-like behaviours (Shekhar et al., 1990; Shekhar, 1993; Shekhar and Katner, 1995). In addition, inhibiting the DMH with muscimol, a GABAA receptor agonist, reversed the cardiovascular changes observed during anxiety events (Sévoz-Couche et al., 2013).

A possible role of the DMH in depression has also been suggested since increased activity of DMH orexinergic neurones was observed in an animal model of depression, an effect that was reversed by a treatment with fluoxetine (Nollet et al., 2011). In parallel, the chronic treatment of depressive-like animals with an oxytocin receptors antagonist reversed the depression-like behaviour during the tail suspension test (Nollet et al., 2011), suggesting a role for these receptors in the development of depressive disorders.

Reciprocal neuronal projections between the AMY and the DMH are not however restricted to GABAergic or orexinergic pathways, as GAL has recently been shown to also participate in this circuit. GAL involvement was first suggested by Blackshear and colleagues (Blackshear et al., 2007) that showed that the i.c.v. administration of GAL induces an increase in c-Fos levels specifically in the DMH and the central nucleus of the AMY, an effect that was mimicked by the administration of a GALR1 agonist (M617). Secondly, several studies have implicated GAL in the pathophysiology of depression (Weiss et al., 1998, 2005; Lu et al., 2005; Swanson et al., 2005; Barr et al., 2006; Karlsson and Holmes, 2006; Kuteeva et al., 2007, 2008; Yoshitake et al., 2014), acting as a prodepressive agent (Weiss et al., 1998, 2005; Kuteeva et al., 2007).

In this context, Kuteeva and co-workers (Kuteeva et al., 2008), showed that the i.c.v infusion of GAL resulted in increased immobility time in the FST, a behaviour that was also mimicked after the infusion of a GALR1 agonist. Also, the administration of GAL in the ventral tegmental area increased the immobility time in the FST, which was reversed by the administration of a non-selective GAL receptor antagonist (Weiss et al., 1998; Kuteeva et al., 2007). Furthermore, in

humans, variants of genes for GAL and its receptors (GALR1, GALR2, GALR3) were reported to confer increased risk to depression and anxiety in people who experienced childhood adversity or recent negative life events (Juhasz et al., 2014).

Based on these observations we hypothesised that AMY or DMH GALR1 might induce a prodepressive phenotype. Taking this into account, in a preliminary study (**Chapter 2.3**), we tested if GAL administration in the DMH was able to induce a depressive-like behaviour in the FST. In fact, in healthy rats, GAL in the DMH increased immobility time and decreased the latency to immobility and time spent active. By contrast, GAL administration in the DMH of ARTH animals did not alter significantly the behaviour of the animals, possibly because these animals are already depressed.

Interestingly, in our work, we also demonstrated a pronociceptive role of GALR1 in the DMH (**Chapter 2.3**), suggesting that this GALR subtype in this specific hypothalamic region is potentially involved in a neuronal circuit that links nociceptive facilitation and depression.

3.3. The role of the DMH in descending nociceptive modulation

3.3.1. The DMH as a pronociceptive nucleus

The integration of the DMH and the RVM as part of a common brain circuit have been hinted not only by anatomical works (ter Horst and Luiten, 1986; Thompson et al., 1996) but also by observations showing an increased Fos expression in the RVM after the disinhibition of the DMH with the GABAA antagonist bicuculline (Zaretskaia et al., 2008). In our study (**Chapter 2.2**), we demonstrated that the DMH undergoes plastic changes after prolonged arthritis. In fact, a phasic and tonic pronociceptive action of this nucleus was demonstrated in healthy but not in ARTH animals. A decrease in the paw-withdrawal latency (PWL) was observed after the administration of GLU in the DMH while the opposing effect was shown after LIDO injection. Moreover, these behavioural observations correlate with an increase in RVM On- and decrease in Off-like cells in SHAM animals.

Our results were in accordance with the work of Martenson and colleagues (Martenson et al., 2009) where the disinhibition of the DMH with bicuculline resulted in nociceptive facilitation in healthy animals, with concomitant changes in the activity of RVM pain-modulatory cells. More recently, DMH-containing cholecystokinin neurones that project to the RVM were proposed to play

a role in the DMH-mediated behavioural hyperalgesia, an effect that was suggested to be related to stress-induced hyperalgesia (Wagner et al., 2013). By contrast, other studies reported an antinociceptive role of the DMH, especially in innate fear-induced antinociception (Freitas et al., 2009, 2013; Biagioni et al., 2012, 2013) although none reported the RVM as a brainstem mediator of this effect.

3.3.2. The GAL-dependent DMH descending nociceptive facilitation

Curiously, contrary to the absence of an effect when generally activating (GLU) or inhibiting (LIDO) the DMH of ARTH animals, in another work we demonstrated that a GAL-dependent pain facilitatory pathway from the DMH remains functional after prolonged monoarthritis (**Chapter 2.3**), as the administration of GAL in the DMH decreased PWL in both SHAM and ARTH animals. This study showed for the first time, a pronociceptive role for supraspinal GAL. In fact, in the brain GAL administration was always associated to antinociception since its i.c.v. administration was reported to suppress nociception in healthy animals (Fu et al., 2011) with the same effect being observed after its infusion directly in the PAG, hypothalamic arcuate and tuberomammillary nuclei, nucleus accumbens and central AMY (Wang et al., 1999; Imbe et al., 2004; Sun et al., 2004; Gu et al., 2007; Jin et al., 2010; Li et al., 2012; Xu et al., 2012).

In an attempt to correlate the GAL/DMH nociceptive facilitatory role with alterations in the activity of RVM pain modulatory neurones, we recorded the activity of RVM On- and Off-like cells before and after the administration of GAL or a non-selective GALRs antagonist (M40) in the DMH. Our electrophysiological data showed that neither GAL nor M40 in the DMH influence the activity of RVM On- and Off-like cells contrary to what was observed when generally activating the DMH with GLU or inhibiting with LIDO. These results suggest that the DMH pronociceptive effect would be generated by different pain facilitatory pathways, some mediated by RVM On- and Off-cells and others mediated by RVM cells Neutral-cells or different brain regions.

In order to evaluate which subtype of GALR would be implicated in this nociceptive facilitatory effect evoked by GAL in the DMH, we tested the influence of GALRs agonists/antagonists in this nucleus upon the nociceptive response of the animals (**Chapter 2.3**). We observed that the GALR1 agonists mimicked GAL pronociceptive effect showing that these DMH receptors mediate GAL facilitatory action in SHAM and ARTH animals. By contrast, GALR2 appears to be associated to an antinociceptive pathway from the DMH, since the administration of a GALR2 antagonist

reduced the PWL of the animals. These results contrast with previous works studying the role of supraspinal GAL receptors, which associate the GALR1 to the antinociceptive effect of GAL (Sun et al., 2004; Fu et al., 2011; Li et al., 2012; Kong and Yu, 2013).

3.3.3. The DRt as a brainstem relay of the GAL/DMH-induced pronociception

Since the DMH does not directly project to the spinal cord (ter Horst and Luiten, 1986) and no changes in the activity of RVM On- and Off-like cells were detected after GAL or M40 was administrated in the DMH, we hypothesized that another brain region could be relaying the GAL/DMH pronociceptive action.

The DRt emerged as the most likely candidate as anatomical evidences show the existence of reciprocal projections between the DMH and the DRt (ter Horst and Luiten, 1986; Martins et al., 2008) and this nucleus has been widely recognized as a pronociceptive brainstem nucleus (Almeida et al., 1996, 1999; Lima and Almeida, 2002; Sotgiu et al., 2008). Neurones in the DRt respond almost exclusively to peripheral noxious stimulation (Villanueva et al., 1990) and its activation by GLU enhanced the post-discharge rate of spinal nociceptive neurones to noxious stimulation (Dugast et al., 2003). Furthermore, the lesion of the DRt was reported to prevent the development of behavioural hyperalgesia in the late phase of the formalin test (Almeida et al., 1999) and to reduce noxious heat-evoked behavioural responses in the tail-flick and hot plate tests (Almeida et al., 1996).

Although its role in chronic pain has been less explored, the DRt contributes to the maintenance of central sensitization in experimental models of neuropathic pain (Sotgiu et al., 2008) and DRt c-Fos expression as well as its metabolic activity are enhanced in experimental monoarthritis (Lantéri-Minet et al., 1994; Neto et al., 1999). In our work (**Chapter 2.4**) we demonstrated a tonic nociceptive facilitatory role of the DRt in prolonged monoarthritis since hipoalgesia was observed after inhibiting the DRt with lidocaine. In addition, we also demonstrated that pronociception after GAL in the DMH is mediated by the DRt as the inhibition of the DRt prevented GAL/DMH-induced behavioural hyperalgesia.

In what concerns the spontaneous and stimuli-evoked activity of spinal WDR and NS neurones in ARTH animals, our results show that experimental monoarthritis not only increased the spontaneous activity of spinal neurones but also the evoked-activity after innocuous mechanical (brush) and noxious thermal (heat) stimulation of the ipsilateral hindpaw. More importantly, these

changes in the phenotype of spinal neurones correlate with the behavioural mechanical allodynia and thermal hyperalgesia, two hallmarks of chronic pain (Loeser and Treede, 2008). The infusion of GAL in the DMH increased the spontaneous activity of spinal WDR neurones, while it increased the noxious heat and mechanical -evoked activity of spinal WDR and NS neurones (**Chapter 2.4**). Future studies should evaluate the contribution of the DRt and the RVM to these alterations in spinal nociceptive neurones induced by arthritis.

3.3.4. The 5HT3Rs as spinal mediators of the GAL/DMH-induced pronociception

In parallel, the role of a 5HT facilitatory drive activated by GAL in the DMH and acting on 5HT3Rs in the spinal cord was also demonstrated (**Chapter 2.4**). Interestingly, this pronociceptive pathway is independent of the DRt although it is also tonically activated during prolonged monoarthritis, as the administration of a 5HT3R antagonist (ondansetron; OND) in the lumbar spinal cord increased heat-evoked PWLs in ARTH but not SHAM control animals.

The implication of 5HT in nociceptive modulation in the spinal cord was not surprising since 5HT descending drives have already been reported (Kia et al., 1995; Porreca et al., 2002). However, 5HT effect upon nociception is bidirectional as this neurotransmitter can either inhibit or facilitate nociceptive transmission depending on the spinal receptors being activated. 5HT-mediated nociceptive facilitation has been associated to the activation of 5HT3Rs (Dogrul et al., 2009) that are mainly expressed in small diameter afferent terminals of the superficial dorsal horn (Glaum et al., 1988; Morales et al., 1998). Their localization suggests that descending 5HT pathways acts as a presynaptic modulators activating the terminals of primary afferents and consequently enhancing the transmission of the nociceptive information from peripheral neurones to the spinal cord.

Moreover, our data shows that 5HT3Rs-mediated tonic nociceptive facilitation occurs only in ARTH animals (McCleane et al., 2003). In fact, some studies have shown that the blockage of these spinal receptors had no effect in healthy animals (Green et al., 2000) while it decreased nociceptive responses in animals with inflammation (Bravo-Hernández et al., 2012). Accordingly, both the intrathecal infusion of OND and the depletion of 5HT3Rs in neuropathic animals reduced the pain-related behaviours in these animals (Suzuki et al., 2004; Rahman et al., 2006). Importantly, 5HT3Rs-mediated nociceptive facilitation in chronic pain conditions is not restricted to experimental animal models but has also been reported in humans (McCleane et al., 2003) as

the intravenous administration of OND significantly decreases pain scores of patients with chronic neuropathic pain (McCleane et al., 2003).

3.3.5. The RVM serotonergic cells as potential mediators of the GAL/DMH-induced pronociception

Although earlier (**Chapter 2.3**) we have excluded the RVM as a mediator of GAL/DMH-induced pronociception as no change in the activity of its On- and Off-cells was observed, in light of the 5HT3R results we had to consider the possibility that 5HT pathways originating in the RVM and acting on the spinal 5HT3R might be involved in GAL/DMH-induced nociceptive facilitation. One evidence that supports this hypothesis is the fact that the RVM has a subpopulation of spinally-projecting serotoninergic neurones that are not On- or Off-cells, but Neutral-cells (Potrebic et al., 1994). In addition, the RVM plays a key role in nociceptive facilitation in chronic pain states (Kia et al., 1995; Burgess et al., 2002a; Carlson et al., 2007). Thus, one of the mechanisms that could underlie the RVM mediation of the GAL/DMH-induced pronociceptive role implicates the activation of its serotonergic Neutral-cells instead of On- or Off-cells. Interestingly, in a preliminary work (**Chapter 2.5**), we showed that the pharmacological inhibition of the RVM with LIDO was able to reverse, at least in part, the pronociceptive effect evoked by GAL in the DMH.

In summary, we demonstrated that GAL in the DMH, through the activation of its GALR1, promotes behavioural hyperalgesia in SHAM and ARTH animals, an effect relayed by two independent circuits (**Chapters 2.3 and 2.4**), one mediated by the DRt and a second that targets spinal 5HT3R (**Chapters 2.5**). Some questions however remain unanswered such as which upstream areas are activating GALR in the DMH. In light of the anatomical studies by Thompson and Swanson (1998) some likely candidates are the prefrontal cortex, the bed nucleus of the stria terminalis, the AMY, most of the hypothalamic nuclei, the PAG, the DRN and the LC (Jacobowitz et al., 2004). In addition, it would be interesting to evaluate changes in the function of the DMH/AMY loop during the establishment of experimental monoarthritis in relation to the development of hyperalgesia and allodynia as well as anxiety- and depressive-like phenotypes.

3.4. References

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Chapter 4

Conclusions and Future Perspectives

4. CONCLUSIONS AND FUTURE PERSPECTIVES

In the present thesis, by pharmacologically manipulating the neuronal circuits associated to the modulation of nociception while assessing the response of animals to specific paradigms as well as by directly measuring the activity of pain modulatory neurones in the brainstem and spinal areas, we were able to identify some of the biochemical and anatomical substrates that could underlie the mechanisms by which prolonged experimental arthritis can affect pain and, eventually, emotions. More specifically, we demonstrated that:

(i) The K/C animal model of prolonged monoarthritis of the knee is a valid method to study arthritic pain and its emotional comorbidities;

(ii) Amitriptyline can be a valuable therapeutic agent to reverse hyperalgesia and improve mood-like disorders associated to prolonged arthritis;

(iii) The DMH has a phasic and tonic pronociceptive role mediated by nociceptive modulatory RVM On- and Off-like cells;

(iv) GAL in the DMH is pronociceptive, an effect mediated by GALR1, a mechanism that activates two independent descending pain modulatory pathways: one relayed by the DRt at the brainstem level and another that acts upon 5HT3Rs in the spinal cord and is possibly mediated by RVM serotonergic neurones.

Further studies should be performed in order to better clarify the interaction between the development of chronic arthritis and comorbid emotional impairments, namely:

(i) To analyse the time course of neuronal and behavioural alterations during the development of arthritis by chronically implanting electrodes in key regions known to be implicated in both pain and emotions, such as the AMY, DMH and RVM, and by performing nociceptive and mood-related behavioural evaluations of the animals at several time points along the development of arthritis;

(ii) To modulate directly the function of brain regions by targeting more specific circuits, using for example optogenetical tools.

(iii) To identify the brain regions releasing GAL in the DMH and inducing the activation of both nociceptive facilitation and mood-like alterations in arthritic animals;

(iv) Clarifying the neurotransmitter system mediating descending nociceptive facilitation elicited by GAL through the DRt in healthy and arthritic animals;

(v) Confirming if RVM Neutral-cells are brainstem mediators of the nociceptive facilitatory pathway that is activated by GAL in the DMH and acts upon spinal 5HT3R.