Universidade do Minho

Escola de Ciências da Saúde

Sara Vieira Gonçalves

NOVEL ROLE OF INFRALIMBIC ASTROCYTIC TYPE-5 METABOTROPIC GLUTAMATE RECEPTORS IN DESCENDING FACILITATION IN MONOARTHRITIS

Dissertação de Mestrado

Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação da:

Professora Doutora Filipa Pinto-Ribeiro

Junho de 2016

DECLARAÇÃO

Nome: Sara Vieira Gonçalves

Endereço eletrónico: saravieirag@gmail.com

Telefone: 916037393

Número do Cartão de Cidadão: 14190670

Título da dissertação:

Novel role of infralimbic astrocytic type-5 metabotropic glutamate receptors in descending facilitatory monoarthritis

Orientador:

Professora Doutora Filipa Pinto-Ribeiro

Ano de conclusão: 2016

Designação do Mestrado:

Ciências da Saúde

Universidade do Minho, 8 de Junho de 2016

Assinatura

À Tété

"...Birth and death are only a door through which we go in and out

Birth and death are only a game of hide-and-seek

So smile to me and take my hand and wave good-bye

Tomorrow we shall meet again or even before..."

Thich Nhat Hanh

AGRADECIMENTOS

Gostaria de agradecer a todos aqueles que, direta ou indiretamente, ajudaram a que este trabalho fosse possível:

À minha orientadora Filipa Pinto-Ribeiro, pelo apoio e ensinamentos a todos os níveis. Mais do que permitir a realização deste projeto, foi uma pessoa fantástica sempre com uma risada contagiante. E, principalmente, obrigada pela apresentação, naquele verão de 2010, certamente o meu caminho não teria sido o mesmo se não fosse a maneira cativante como falou de ciência.

À Ana Pereira, a minha co-orientadora (fantasma), o mais sincero e profundo obrigada por tudo. Este espaço é demasiado limitado para escrever tudo pelo qual te tenho que agradecer, foste o meu pilar neste projeto. Obrigada pelos infinitos ensinamentos, pelo apoio e boa disposição. Obrigada por estares sempre presente e teres sempre um ombro amigo. Obrigada por puxares por mim e me fazeres dar sempre o melhor de mim. Obrigada também à Armanda e à Clara por te ajudarem a ter paciência, ou não, comigo. Foste uma orientadora exemplar e tenho certeza que serás uma amiga para a vida, mesmo sem aceitar devoluções.

À Diana Amorim e à Sónia Puga, por estarem sempre prontas a ajudar, mesmo com os pontapés acidentais.

Ao professor Armando Almeida por disponibilizar todas as ferramentas necessárias para o desenvolvimento deste trabalho.

À minha família, sem eles o caminho até aqui não seria possível. À Rita, por ser uma irmã fora de série, por estar sempre presente e me apoiar em todas as circunstâncias. Aos meus pais, por todo o apoio ao longo da vida e me ajudarem a lutar pelos meus sonhos. Ao Kika, o pai emprestado, obrigada por puxares sempre mais e mais por mim. Ao Vicente, o raio de luz nas nossas vidas.

Ao Master gang, pelo companheirismo. Pelos momentos de descontração e de choro, pelo ombro e sorriso amigo. Obrigada Ana, Bárbara, Diana, Eduardo, Joana, Leonor, Margarida e Mendanha. Tenho certeza que mais que boas recordações, levo amigos para a vida.

Às minha primas do coração, Ana e Lú, obrigada pelo apoio, pela presença e pela amizade.

Ao pessoal do Tea Time, obrigada pelo apoio nestes últimos anos. Em especial à Bia, a tagarela; à Jelly, a sorridente e à Marii, a otimista; três adjetivos que fizeram de mim uma pessoa melhor.

Ao Davide, o amigo de sempre.

À Mariana, a companheira de uma vida, obrigada pelo apoio e pela constante presença.

A todos os NeRDs pelo acolhimento, apoio nas imunos e discussões científicas.

Aos ratos.

E por fim, ao Filipe, obrigada pela gigante paciência, pelo apoio nos piores momentos e por todas as palavras de força e confiança. Obrigada por todos os "pkós" e por todos os sorrisos. Tu és a prova que mesmo longe é possível estar pertinho.

Novel role of infralimbic astrocytic type-5 metabotropic glutamate receptors in descending facilitatory monoarthritis

ABSTRACT

Pain arises as a vital defense mechanism, essential for survival. Unfortunately, it can become a disease and lose its biological value when prolonged in time, as in chronic disorders. Arthritis, a chronic inflammatory disease highly prevalent in the elderly, is characterized by progressive degradation of joints and persistent pain. Recent advances in brain imaging techniques allowed pinpointing specific brain areas whose activity is altered in arthritic patients. Amongst these areas, the prefrontal cortex (PFC) was reported to be functionally altered in arthritis. In addition, a previous work from our lab showed the infralimbic cortex (IL), a subarea of the PFC, plays a facilitatory role in the descending modulation of nociception after activation of its type-5 metabotropic glutamate receptors (mGluR5) in experimental monoarthritis.

As mGluR5 are expressed in neurons and astrocytes, to better understand which cell type might be mediating IL mGluR5-induced pronociception in healthy and monoarthritic animals, we selectively ablated IL astrocyte function using a gliotoxin (L-α-aminoadipate). Nociception was then evaluated before and after IL mGluR5 activation/inhibition during peripheral thermal noxious stimulation. In addition, to evaluate the role of the rostroventromedial medulla (RVM) as a potential downstream spinal-projecting effector of IL mGluR5-induced pronociception, the activity of its ON- and OFF-like cells was also assessed.

Our results suggest IL astrocytic mGluR5 are involved in nociceptive facilitation in experimental monoarthritis but not in healthy animals. Interestingly, RVM cells traditionally associated with nociceptive facilitation/inhibition are not involved in IL mGluR5-induced pronociception although a potential contribution of RVM NEUTRAL-cells cannot be discarded. Future studies should focus on the mechanisms between astrocytes and neurons that facilitate nociception in experimental monoarthritis.

O papel dos recetores metabotrópicos de glutamato do tipo 5 nos astrócitos do córtex infralímbico na facilitação descendente em monoartrite

RESUMO

A dor é um mecanismo de defesa essencial para a sobrevivência. Infelizmente, pode perder o seu valor biológico quando se prolonga no tempo, tornando-se ela própria numa doença crónica. A artrite, uma doença crónica inflamatória com elevada prevalência em idades mais avançadas. É caracterizada pela degradação progressiva das articulações e por dor persistente. Os avanços nas técnicas de imagiologia cerebral permitiram, nos últimos anos, a identificação de áreas específicas do cérebro que sofrem alterações na sua atividade em doentes com artrite. Entre estas áreas encontra-se o córtex pré-frontal (PFC), onde foram detetadas alterações funcionais na sua atividade em pacientes com artrite. Recentemente, um estudo do nosso laboratório demonstrou que o córtex infralímbico (IL), uma sub-região do PFC, desempenha um papel facilitador na modulação descendente da nocicepção após a ativação dos recetores metabotrópicos de glutamato do tipo 5 (mGluR5) em ratos com monoartrite experimental.

Dado que os mGluR5 são expressos em neurónios e astrócitos, e para perceber qual o tipo de célula que pode estar a mediar o efeito pronociceptivo dos mGluR5 no IL em ratos saudáveis e com monoartrite, a função astrocítica no IL foi selectivamente ablada com recurso a uma gliotoxina (L-α-aminoadipato). A nocicepção foi avaliada antes e depois da ativação ou inibição dos mGluR5 no IL através da aplicação periférica de estimulação térmica nóxica. Adicionalmente, para avaliar o papel do bolbo rostral ventromedial (RVM) como um potencial efetor da pronocicepção induzida pelos mGluR5 no IL, a atividade das suas células do tipo ON e OFF foi avaliada.

Os nossos resultados sugerem que a função dos mGluR5 nos astrócitos do IL está envolvida na facilitação descendente da nocicepção em monoartrite experimental. Curiosamente, as células do RVM tradicionalmente associadas à facilitação/inibição nociceptiva não estão envolvidas na pronocicepção induzida pelos mGluR5 no IL, no entanto, um potencial contributo das células NEUTRAS do RVM não deve ser descartado. Seria interessante em trabalhos posteriores estudar os mecanismos de interação entre astrócitos e neurónios que levam à facilitação da nocicepção em ratos com monoartrite experimental.

TABLE OF CONTENTS

ABBREVIATION LIST

ACC – anterior cingulate cortex

ARTH - arthritis

AMY – amygdala

ANOVA – analysis of variance

CHPG - 2-chloro-5-hydroxyphenylglycine

CNS - central nervous system

DAPI – 4′,6-Diamidine-2′-phenylindole dihydrochloride

dlPFC – dorsolateral prefrontal cortex

DRt – dorsal reticular nucleus

GFAP – glial fibrillary acidic protein

GS – goat serum

IASP – international association for the study of pain

iGluR(s) – ionotropic glutamate receptor (s)

i.p. – intraperitoneal injection

IL – infralimbic cortex

K/C - kaolin/carrageenan

LαAA – L-α-amnoadipate

mGluR(s) – metabotropic glutamate receptor (s)

MTEP – 3-((2-methyl-1,3-thiazol-4-yl) ethynyl) pyridine hydrochloride

MPEP – 2-methyl-6[phenylethynyl]-pyridine

mPFC – medial prefrontal cortex

NMDA - N-methyl-D-aspartate

NON-N – non-nociceptive neurones

NS – nociceptive specific neurones

OA - osteoarthritis

PAG – periaquedutal grey matter

PBS – phosphate buffer saline

PAM - pressure application measurement

PFA – paraformaldehyde

PFC – prefrontal cortex

PL – prelimbic cortex

PW – paw withdrawal

PWL – paw-withdrawal latency

RVM – rostral ventromedial medulla

SHAM – control animals

SEM – standard error of the mean

TBS – tris-buffer saline

TF – tail-flick VEH – vehicle

WDR – wide-dynamic range neurons

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" by the International Association for the Study of Pain (IASP)[1]. Acute pain is essential in our life, since it functions as an alert mechanism for potential damaging stimuli and promotes protective behaviours in order to avoid or prevent further injury. However, when prolonged in time, pain loses its biological value and becomes a disease. Usually, pain is described as chronic if it lasts for more than 3 months[2].

Chronic pain is the most problematic form of pain and consequently, in the last decades, the number of studies in this field increased as the peripheral and central mechanisms behind this clinical problem are still not completely understood, mostly due to different etiologies. Chronic pain can be divided in visceral, inflammatory, neuropathic and/or mixed pain. Visceral pain arises from internal organs, and its diffuse nature makes it more difficult for the patient to pinpoint. Inflammatory pain arises as a response of the immune system that in turn activates the nociceptors of the damaged tissue. In contrast, neuropathic pain arises from an injury of the central or peripheral somatosensory nervous system. Finally, mixed pain arises when different types of pain overlap in the same person. It is important to notice that chronic pain can lead to a high number of comorbidities, such as, emotional, affective and cognitive impairments[3,4]. For the purpose of this thesis we will focus particularly on chronic inflammatory pain, more precisely, on experimental monoarthritis.

Osteoarthritis (OA), a major public health problem, results from progressive degradation of single or multiple joints caused by an imbalance in the dynamic equilibrium between the breakdown and repair mechanisms of joint tissues[5]. Interestingly, pain is the major cause for patients to seek medical care. This chronic inflammatory disease is the most common joint disease, affecting about 10% of the world's population and 6% of the Portuguese population[6]. OA is mostly an age-related disease, affecting more frequently people over 60 years[7], but obesity is an important risk factor[5]. The mechanisms underlying the development of OA are not completely understood and current treatments do not prevent or cure this disease[8].

In order to understand the mechanisms behind chronic pain, animal models are continuously being improved in order to mimic acute and chronic pain states for a more focused and direct study of chronic pain mechanisms[9]. Different models can be used to study specific types of chronic pain, either due to peripheral or central nerve injury, such as neuropathic pain, or to prolonged activation of the nociceptors, such as nociceptive pain[2].

1.1. Nociception

Pain sensitization is initiated by the activation of nociceptors. These specific receptors are free nerve endings of first-order afferent neurons and are widely distributed throughout the body. Noxious stimuli activate the nociceptors which relay information to ascending spinal pathways and, finally, to supraspinal regions. Nociceptors can be divided into groups according to their response to different types of noxious stimulation, such as mechanical, chemical or thermal stimulation[10,11].

Additionally, first-order afferent fibers can also be classified into three types of fibers: (i) C-fibers, small unmyelinated fibers with slow conduction velocity (0.4 - 1.4 m/s); (ii) Aδ-fibers, medium sized myelinated fibers with intermediate conduction velocity (5 - 30 m/s) and (iii) Aβ-fibers, large myelinated fibers with higher conduction velocity[12]. All fibers transmit non-nociceptive (NON-N) information, but, in normal conditions, only C- and Aδ-fibers transmit nociceptive information[12,13]. Aδ-fibers respond mainly to only one type of stimuli and are responsible for the rapid and first phase of pain, evoking protective reflexes, whereas C-fibers are polimodal and evoke the second wave of pain, a more diffuse and longer-lasting sensation[14].

After acute tissue damage or in chronic pain conditions, an increase in the sensitivity of the affected and surrounding areas may arise, a phenomenon designated as hyperalgesia. This effect has been correlated with increased excitability of peripheral nerve fibers. In addition, intense activation of neurons in the dorsal horn can increase their excitability and lead to central sensitization. In this case, innocuous stimulation can produce a painful sensation commonly known as allodynia.

1.1.1. Nociceptive processing

The transmission of peripheral inputs to the CNS occurs when primary afferents synapse with second-order neurons in the dorsal horn of the spinal cord (Fig. 1). Primary afferent fibers penetrate the dorsal horn gray matter, ramify and connect with many neurons located in the grey matter of spinal cord. The grey matter can be histologically divided into ten layers, a system known as Rexed´s laminae. The second-order neurons in the dorsal horn are classified according to the specific type of information they receive: (i) non-nociceptive neurons (NON-N) receive inputs mainly from Aβ-fibers and thus only respond to innocuous stimulation and are localized in lamina I, II and VI; (ii) nociceptive-specific (NS) neurons receive inputs exclusively from Aδ and C fibers and are mainly localized in lamina I, II, V and VI; and (iii) wide-dynamic rage (WDR) neurons receive inputs from all fiber types and respond to both innocuous and noxious stimulation, and are mainly localized in lamina I, II, VI-VI and X[15,16].

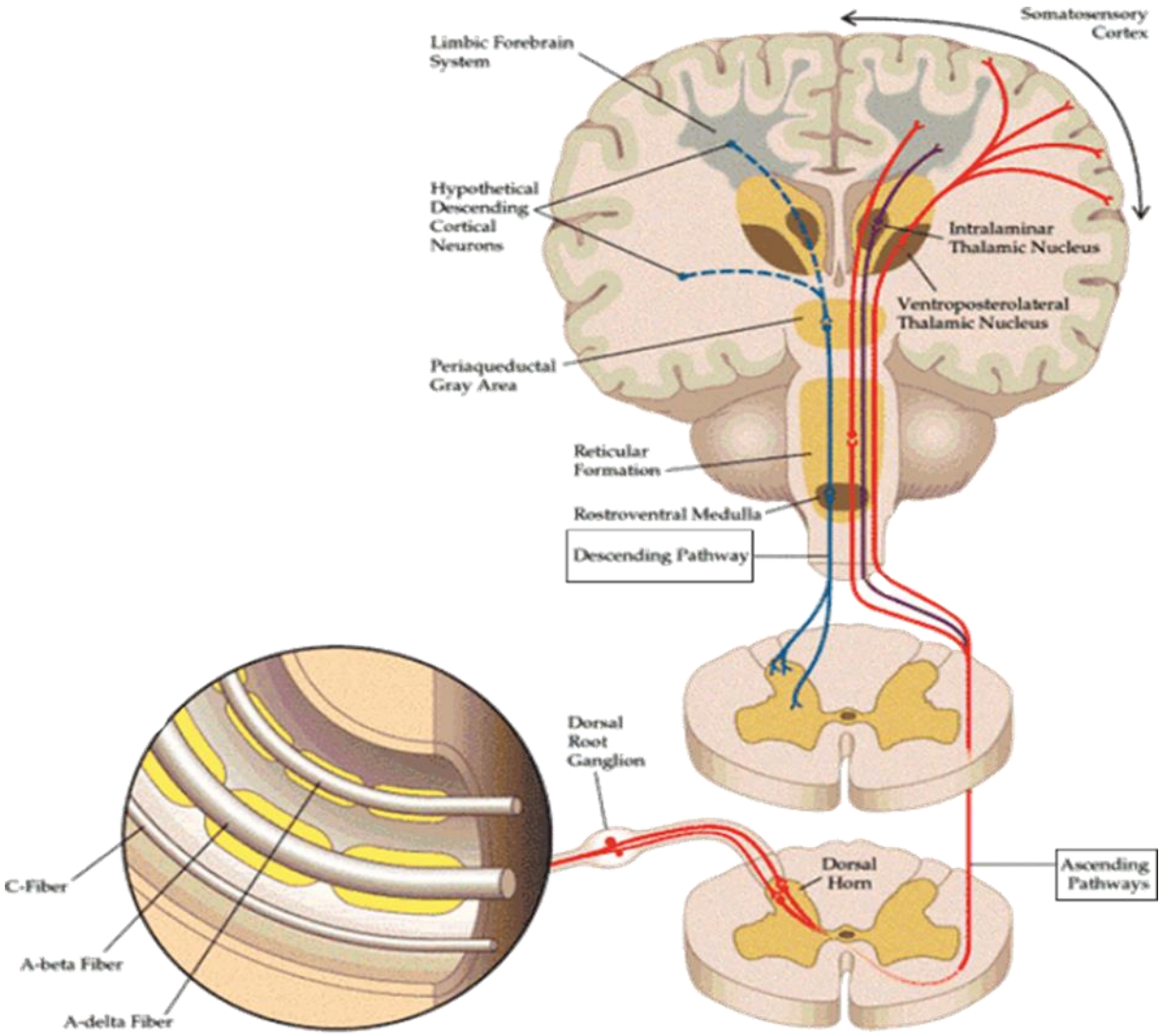


Figure 1. Schematic representation of the neuronal networks involved in nociception processing. Ascending pathways – red lines; Descending pathways – blue lines (Adaptated from Medscape http://www.medscape.org/viewarticle/446350_3).

Second-order neurons are responsible for transmitting information to the brain, the axons of these neurons decussate and ascend along the anterior lateral and posterior tracts. The anterior lateral system is the most important for somatic pain and is composed of five main ascending tracts: the spinothalamic, spinoreticular, spinomesencephalic, spinohypothalamic and spinoreticular-thalamic tracts, the first three are the most studied ascending tracts. These pathways distribute nociceptive inputs to several brain regions that will process the sensory, emotional and cognitive components of pain as well as modulate the autonomic responses to pain[17].

The spinothalamic tract, especially important for pain, ascends mainly in the contralateral side and projects to the lateral thalamus. Nociceptive information it then forwarded to the limbic and cognitive higher centers[18], such as (i) the somatosensory cortices I and II, involved in the processing of the sensory-discriminative components of pain; (ii) the insular cortex, also involved in sensory-discriminative

components of pain, more precisely in temperature sensation and; (iii) the prefrontal cortex[14,19] (PFC), involved in the motivational-affective processing of pain[20].

Neurons in the spinoreticular tract ascend to precerebellar nuclei in the brainstem and reticular formation that in turn project to the thalamus, this tract is considered an indirect pathway of the anterolateral system[20]. Finally, the spinomesencephalic tract projects to the mesencephalic reticular formation, the lateral part of the periaqueductal grey and other sites in the midbrain[20]. Despite the fact the main ascending pathways project to specific supraspinal regions, these regions are not independent in processing the nociceptive information, in fact, these brain areas have collateral interconnection to each other and, generally, the nociceptive inputs are not processed by a single and isolate brain region.

1.1.2. Descending modulation of nociception

After the supraspinal processing of nociception, descending pain modulatory pathways are activated. Several supraspinal brain regions are involved in the descending control of nociception and play a critical role in both acute and chronic pain[21–25]. This supraspinal modulation is a dynamic system where nuclei either send direct projections to the spinal cord or, alternatively, relay this information to other nuclei that project to the spinal cord. In addition, spinal projecting nociceptive modulatory regions can also either facilitate or inhibit the transmission of peripheral nociceptive inputs at the superficial horn level[26–28] (Fig. 2).

One example is the periaqueductal gray (PAG), an area receiving inputs from many cortico-limbic nuclei, that relays information to the rostral ventromedial medulla (RVM) as it does not project directly to the spinal cord. The RVM is considered the output region of the midline pain modulation system. This nucleus plays an important role in both the descending inhibition and facilitation of nociception that results from a balance between the activation of its ON- and OFF-cells[27]. ON-cells are described to be pronociceptive, as they increase their firing activity immediately before the withdrawal reflex elicited by a noxious stimulus applied to the periphery while OFF-cells are considered antinociceptive, as their activity decreases immediately before the withdrawal reflex is observed[28,30].

Inhibition of nociceptive transmission at the spinal cord level is achieved by (i) interactions with the terminals of NS primary afferents, either directly or through the activation of spinal inhibitory interneurons, or by (ii) inhibiting spinal excitatory interneurons[29].

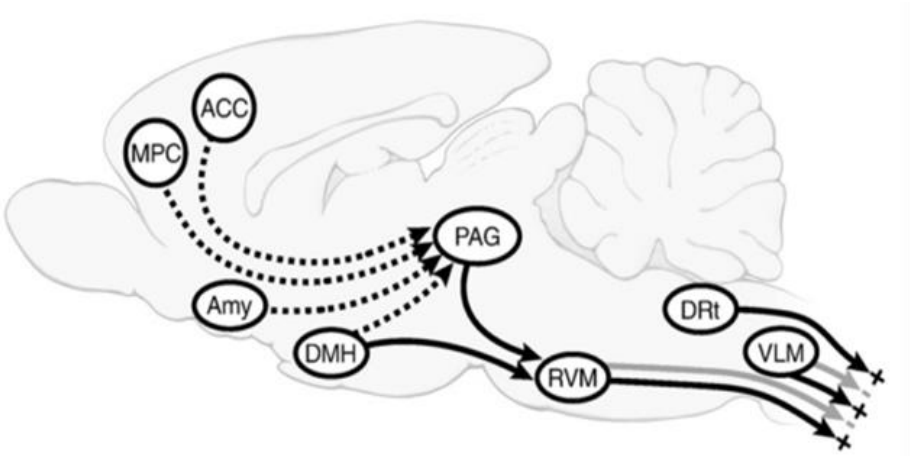


Figure 2. Schematic representation of the descendings pathways of nociception in rats brain. Midline PAG-RVM system, which exerts bidirectional control over dorsal horn nociceptive processing, and the DRt and VLM in the caudal medulla. DRt is thought to be facilitating, and VLM primarily inhibitory, although it may, like the RVM, have both an inhibitory and facilitatory influence. The PAG especially, but also the RVM, DRt and VLM (not shown) receive important direct and indirect inputs from limbic forebrain areas including ACC, AMY, dorsomedial nucleus of the DMH, and MPC. ACC – Anterior cingulate cortex; AMY – amygdala; DMH – dorsomedial nucleus of the hypothalamus; MPC – medial prefrontal cortex; DRt – medullary dorsal reticular nucleus; PAG – periaqueductal gray; RVM – rostral ventromedial medulla (Heinricher, et al., 2009).

1.2. The prefrontal cortex in pain

Recent advances in techniques such as neuroimaging and animal pain models allowed to study subtle alterations in the activation of specific brain areas in chronic pain conditions[31,32]. The PFC, somatosensory cortical areas 1 and 2, the insular cortex, the thalamus and the anterior cingulate cortex (ACC) have been identified as the brain regions most commonly activated during pain[33]. In fact, several studies show increased PFC activity during the application of different types of noxious peripheral stimulation[34–36]. The PFC, thoroughly studied in the last few years, increases its activity in patients with chronic pain[37] and during the anticipation of pain[33]. Additionally, Apkarian and colleagues showed lesions in this area altered pain perception[38] and the PFC undergoes major neurodegeneration in chronic pain patients[39].

In humans the PFC can be divided in different subregions: the medial prefrontal cortex (mPFC), the ACC, the dorsolateral cortex (dlPFC) and the insular cortex, each region is known for playing different roles in pain[35,40]. The mPFC and the ACC have been described as pain facilitatory, or pronociceptive, and important for the processing of the affective component of pain[41,42]. In fact, ACC deep brain stimulation improved pain in patients refractory to chronic pain treatments[43]. The dlPFC has been associated with pain perception[44,45]. The insular cortex is involved in anticipation[34], intensity coding and the construction of a signature of the pain experience[46,47].

The human PFC is significantly different in size and organization when compared with the PFC of other mamalians[48]. In rodents, the PFC is divided in three different areas, (i) the mPFC, a more medially area in the cortical region; (ii) the orbital prefrontal cortex, a ventrally located cortical region and; (iii) the lateral or sulcal PFC also referred as the agranular insular cortex, a more laterally located cortical region. The mPFC in rats can be also divided into at least four more distinct subareas: the medial precentral area (PrCm) or area Fr2, the AC area, the prelimbic area (PL), and the infralimbic area (IL)[49].

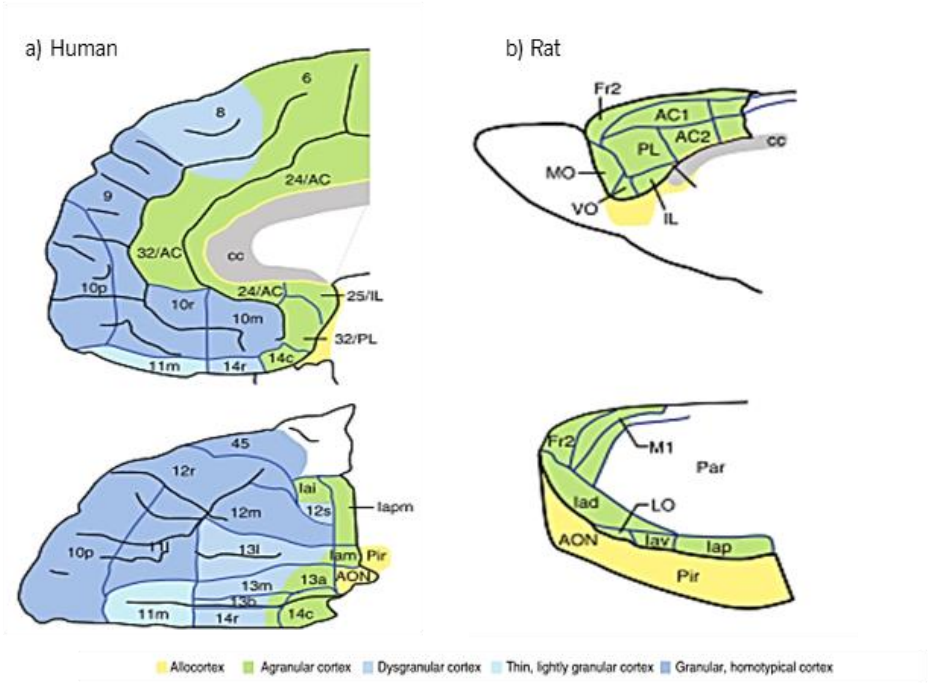


Figure 3. Schematic representation of the prefrontal cortices in a) humans and in b) rats. Homologous areas between the rat and man are coloured in green and yellow while blue areas correspond to areas existing in humans alone. (AC – anterior cingulate area; AON – anterior olfactory nucleus; c – caudal; cc – corpus callosum; Fr2 – second frontal area; I – insula; i – inferior; la – agranular infralimbic cortex; IL – infralimbic cortex; l – lateral; LO – lateral orbital area; m – medial; M1 – primary motor area; MO – medial orbital area; o – orbital; p – posterior; Par – parietal cortex; Pir – Piriform cortex; PL – prelimbic cortex; r – rostral; s – sulcal; v – ventral; VO – ventral orbital area) (adaptated from Wallis 2011).

Only some areas of the rodent PFC have been shown to be homologue to PFC areas in humans, namely the IL, PL and ACC[50] (Fig.3).

Although the involvement of the PL and IL in nociception has been demonstrated[51] their role is not well understood and the literature available is scarce. Regarding the PL, some studies showed it is involved in the aversive dimension of pain[52] and projects to other areas involved in nociception processing, such as the ACC, the amygdala, PAG and RVM[53]. Studies suggested the PL, but not the IL, is involved in the encoding of pain-emotion[52]. Ji and Neubaguer demonstrated an inverse interaction

between the PL and IL, as the activation of the IL inhibits PL activity[54]. The IL is the least studied subregion of the mPFC and was recent described has having a critical involvement in the descending modulation of nociception[51].

1.2.1. The infralimbic cortex

The human IL is considered the architectonically least developed of all prefrontal cortical areas[55]. Similarly, in rats, it is considered a poorly laminated region where several neurons from layer II spread into the marginal layer[56]. This area projects mostly to central autonomic nuclei[53] and to several other limbic structures[57]. Additionally, the IL projects to brain areas such as the hypothalamus, the PAG and the superficial dorsal horn[58]. Interestingly, the IL is interconnected with the PL, the ACC and the insular cortex.

The IL (Fig. 4) plays an important role in visceromotor functions, blood pressure, heart rate, respiration, and gastro-intestinal activity[57]. The IL has also been described as a critical brain area in behavior flexibility, learning[59,60] and suppressing aversive behaviours[61]. However, in the context of nociception the role of the IL remains unclear. David-Pereira and colleagues[51] recently showed the IL facilitated behavioural hyperalgesia in arthritic animals after the activation of its type-5 metabotropic glutamate receptors.

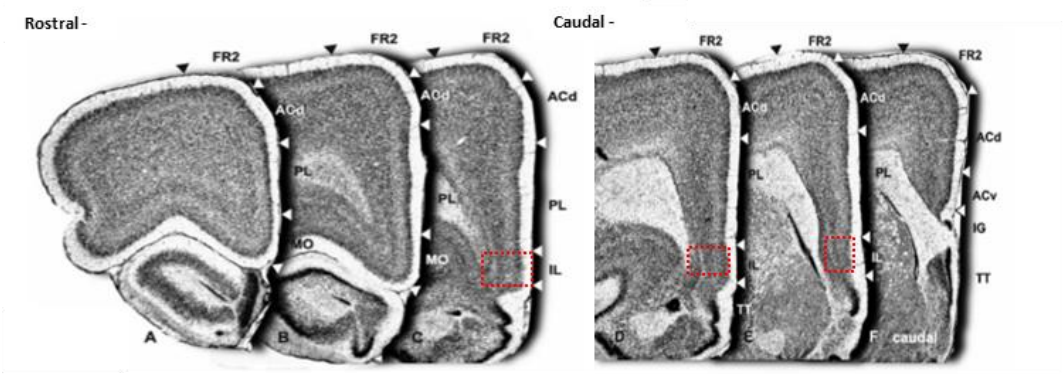


Figure 4. Schematic representation of the medial prefrontal cortex in stained sections through the frontal pole of the rat brain. ACd, dorsal anterior cingulate area; AC – vventral anterior cingulate area; FR2 – frontal cortex area; IG – indusium griseum; IL – infralimbic area (dashed red square); MO – medial orbital area; PL – prelimbic area; TT – tenia tecta. (Heidbreder and Groenewegen. 2003)

1.3. Glutamate receptors in pain

Several studies already demonstrated glutamate receptors play a crucial role in pain pathways. Understanding the mechanisms behind the effect of these receptors will contribute significantly for the development of new therapeutic tools for the treatment of persistent pain conditions[62].

There are three different families of glutamate receptors[63]. Those activated by the glutamate analogue N-methyl-D-aspartate (NMDA) and referred to as NMDA-receptors (NR -1, -2A, -2B, -2C and -2D). Those activated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA; GluR1-4) and kainate (GluR5-9, KA1 and KA2) with lower affinities but easily desensitized, known as AMPA. AMPA receptors are ion channels that conduct Na^+ or both Na^+ and Ca^{2+} and are called ionotropic glutamate receptors (iGluRs). Finally, there is a third family of G-protein coupled receptors designated metabotropic receptors (mGluRs). These can also be subdivided in 3 groups: (i) group I (mGluR1 and mGluR5) receptors that are coupled to phospholipase C and thereby to inositol triphosphate and diacylglycerol production; (ii) group II (mGluR2 and mGluR3) and (iii) group III (mGluR4, mGluR6, mGluR7 and mGluR8), that are negatively coupled to adenylate cyclase.

1.3.1. Metabotropic glutamate receptors

In contrast with iGluRs that yield fast postsynaptic responses, metabotropic receptors produce slower postsynaptic responses with a long-lasting effects[64]. Regarding pain treatment, iGluR are not considered good pharmacological targets, except for some receptor antagonists and in a specific dose, as its activation results in huge side effects including serious amnesia. Interestingly, mGluR receptors are involved in several functions, such as the regulation of the stability of neuronal synapses, but more importantly they play an important role in the modulation of nociception in acute and chronic condition[65].

These receptors can also influence chronic pain at several levels of the nervous system. The involvement of these receptors at the spinal cord level is already well established and studies regarding the role of mGluRs in nociception at the brain level have been increasing in the last years[66]. The expression of each type of mGluRs have been characterized to have a lamina-specific pattern at spinal cord level with mGluR1α in Lamina V, mGluR5 in Laminae I-II and mGluR2/3 in lamina II (inner part), this fact suggests each receptor subtype plays different roles[67]. Depending on the receptor's subtype and localization, mGluR can either increase or decrease cell excitability[64].

The release of glutamate in the dorsal horn of the spinal cord after injury contributes to the development of post-injury pain hypersensitivity[68]. In neuropathic pain conditions, studies show mGluR antagonists partly reverse mechanical allodynia and thermal hyperalgesia[69]. Indeed, Bhave and colleagues[70] demonstrated that intrathecal administration of group I mGluR agonists promotes

hyperalgesia and intrathecal administration of group I mGluR antagonists reduce inflammatory and neuropathic pain. Group I mGluRs can modulate nociceptive processing at different supraspinal levels, such as in the amygdala (AMY), where its activation facilitates nociception or the PAG where it is antinociceptive[64,69]. By contrast, group II mGluR are considered antinociceptive in chronic pain disorders as they contribute to inhibit the release of neurotransmitters from peripheral terminals. The effect of group III is not so well described, different subtypes of receptors in these group have opposite effects upon pain perception[64].

The use of selective antagonists of mGluR5 confirmed its analgesic properties in somatic pain[71] in several animal models of inflammatory and neuropathic somatic pain[72]. Regarding inflammation, Walker and colleagues showed mGluR5 activation promote hyperalgesia while administration of a selective antagonist inhibited hyperalgesia. mGluR5 agonists increased the frequency and duration of firing of WDR neuron in the dorsal horn[73]. In the IL, mGluR5 are described to control negative emotions, specifically the consolidation of extinction behaviours in anxiety and fear disorders[74]. The activation of mGluR5 in this particular brain area promotes fear extinction, as blocking of this receptor leads to impairments in the recall of extinction[74]. However, more studies are required regarding the connection between extinction mechanisms and pain modulation in the IL.

mGluRs are expressed presynaptically and postsynaptically in neurons and the subtype mGluR6 is only expressed in the retina[64]. mGluR1 and mGluR5 (group I) are expressed in neurons and astrocytes and were reported to contribute to the establishment of chronic pain states[65]. Nowadays, both cells types are recognized as playing a critical role in pain modulation and as possible targets for the development of new chronic pain therapies.

1.4. Astrocytes

Over the past decades, astrocytes have not only been considered as central nervous system (CNS) house-keeping cells but as responsible for releasing important molecules such as glutamate, adenosine triphosphate, cytokines and chemokines. These cells also play a vital role in processing information in the brain[75,76]. The “tripartite synapse” hypothesis is based on the cross-talk between astrocytes and neurons, suggesting brain physiology is coordinated by both cell types[75].

More recently, astrocytes have been proposed to play a critical role in the development, maintenance and perception of chronic pain[77]. These cells can respond to both peripheral and CNS tissue damage by changing their morphology and proliferation rate. Studies show an increased

astrocytic activity in both inflammatory and neuropathic pain conditions[78,79]. In fact, astrogliosis and microgliosis have been reported in chronic inflammatory pain conditions, such as arthritis[79]. While the selectively inhibition of astrocytes and microglia metabolism inhibits neuropathic pain, the selective inhibition of microglia only inhibits the development of neuropathic pain states but does not reduce the already established pain condition[80].

Active astrocytes are able to release proinflammatory cytokines and chemokines[81] in the spinal cord to enhance and prolong chronic pain[80]. Pharmacological inhibition of some of these substances, per example of interleukin-1β, in the spinal cord were shown to attenuate pain[81]. Byrnes and colleagues showed mGluR5 are involved in the reduction of the post-injury inflammatory response by limiting microglial activation.

At the spinal cord level, the influence of mGluR5 and astrocytes in chronic pain conditions was already demonstrated[79,82–84]. Additionally, astrocyte activation can also occur in supraspinal areas, such as the RVM[83], but their influence at this level is poorly understood.

CHAPTER 2

OBJECTIVES

2. OBJECTIVES

Advances in neuroimaging and the development of numerous animal models allowed acquiring new insights about alterations in specific brain areas in chronic pain. Recently, it was reported the IL, a subarea of the medial prefrontal cortex plays a facilitatory role in the descending modulation of nociception through its mGluR5.

Taking the above into account, the present project, through the use of behavioural and electrophysiological approaches, aimed at:

1. Identifying whether neurons, astrocytes or both are mediating descending facilitation after activation of IL mGluR5. The selective ablation of astrocytes in the IL was achieved through the microinjection of a gliotoxin (L-αaminoadipate) in the IL of healthy and monoarthritic animals;

2. Evaluating whether the RVM, a nucleus considered to be the main spinal projecting effector of supraspinal pain modulatory pathways, is a potential relay of IL mGluR5 induced pronociception. Involvement of the RVM was assessed through the analysis of its cell activity after activation/inhibition of IL mGluR5 in healthy and monoarthritic animals.

CHAPTER 3

MATERIALS AND METHODS

3.1. Animals and ethical considerations

For this study, 75 adult male Wistar Han rats (Charles Rivers, Barcelona, Spain) were used. Animals were housed two per cage with food and water available *ad libitum* except after the implantation of the chronic intracerebral cannulae where rats were then housed individually and the food was placed in the bed. Animals were maintained in an environmentally controlled room, 22±2°C of temperature and under a 12 h light-dark cycle with lights-on between 8:00am and 8:00pm. All procedures were approved by the ICVS committee and followed the Decreto-Lei 129/92 for the safe use of animals in laboratory experimentation. The experiments were designed in order to minimize potential suffering and the total number of animals used.

3.2. Anesthesia and euthanasia

During induction of monoarthritis, cannula implantation and ablation of astrocytes animals were anaesthetized using a mixture of ketamine (1.5 mg/kg; Imalgene®, Merial, Lisbon, Portugal) and medetomidine (1.0 mg/kg; Dorbene®, ESTEVE, Carnaxide, Portugal) administrated intraperitoneally (i.p.). After the surgical procedures the anesthesia was reversed through the administration of atipamezole i.p. (0.1 mL/kg; Antisedan®, Pfizer, Seixal, Portugal). The animals were monitored until fully recover (eating and grooming)[85].

During the electrophysiological recordings anaesthesia was induced with pentobarbitone i.p. (50 mg/kg, Eutasil®, CEVA, Algés, Portugal) and maintained by infusing pentobarbitone (15–20 mg/kg/h). The level of anesthesia was frequently confirned by evaluating muscular tonus and pupil dilation. In addition, and to avoid eventual pain during the surgery for the placement of the recording electrode, a local anesthetic, lidocaine (2%, Braun, subcutaneously), was administrated to the area of the incision in the scalp. Body temperature was maintained within physiological range with the help of a warming blanket.

After the electrophysiological sessions, animals received a lethal dose of pentobarbitone i.p. (80 mg/kg) and the brains were excised for histological confirmation of cannula placement and electrode recording sites[85,86].

Rats with ablation of astrocytes were euthanized at the end of the behavioural period (7 days after the drug or vehicle injection) with a lethal dose of pentobarbitone i.p. (80 mg/kg) followed by

intracardial perfusion with 200 mL of 4% paraformaldehyde (PFA; Panreac, Barcelona, Spain) in 0.1M phosphate buffer saline (PBS; pH=7.4)[87]. Brains were carefully excised and kept in the same fixative (PFA 4%) for at least 48 hours and then stored in optimal cutting temperature compound (Tissue-Tek O.C.T. compound, Sakura Finetek Europe, Netherlands) for cryoprotection and subsequently frozen in nitrogen[88].

3.3. Induction of experimental monoarthritis

The induction of experimental monoarthritis was performed four weeks before the beginning of the behavioural sessions[85,89]. Animals of the monoarthritic group (ARTH) were injected with 0.1 mL of a solution of 3% kaolin (Sigma-Aldrich, St.Louis, MO, USA) and 3% carrageenan (Sigma-Aldrich, St.Louis, MO, USA) dissolved in 0.9% sodium chloride into the synovial capsule of the right knee joint, while animals in the control group (SHAM) were injected with 0.1 mL of saline. After de injection, the joint was subsequently manipulated for about 1 min by flexion and extension of the limb.

This experimental model of monoarthritis leads to the development of mechanical hyperalgesia resulting from an inflammatory reaction caused by the injection of the irritant carrageenan and additional mechanical damage to the knee joint structures caused by kaolin. The development of experimental monoarthritis was assessed prior to behavioral sessions, and only animals that vocalized every time after five flexion–extension movements of the knee joint were considered to have developed ARTH[89,90].

3.4. Procedures for intracerebral microinjections

For intracerebral microinjections, in the electrophysiological study and behaviour assessments, a guide cannula was placed 1 mm above the target injection site in the IL (+2,76 mm posterior to interaural; - 0.6 mm lateral from the midline; -4.9 mm below the surface of the skull) according to the coordinates of the rat brain atlas[91]. Cannulae were fixed in the skull) using two anchoring screws (Plastics One, Düsseldorf) and acrylic dental cement (Lang Dental Manufactures, USA). Finally, the skin was sutured and a dummy cannula (Plastics One) inserted into the guide cannula in order to prevent contamination[85,92]. Animals were allowed to recover from the surgery for at least one week.

3.5. Ablation of astrocytes in IL

After monoarthritis induction and cannula implantation in the IL, SHAM and ARTH animals received a single microinjection of a selective astrocytic toxin L-α-aminoadipate (LαAA) (SHAM-LAA and ARTH-

LAA groups) or of the vehicle (PBS; SHAM and ARTH groups) [88,92]. Two 2 μL of LαAA or vehicle were administrated with a rate of 1000/s, after which the injection cannula left in place for 4 minutes and then slowly removed. Twenty-four hours later, animals started the behavioural assessments.

3.5.1. Histological confirmation – Immunofluorescence staining

Frozen brains were sectioned coronally into 50 μm thick sections in a cryostat (Leica CM1900). Sections were washed with Tris-buffer in saline (TBS) and permeabilized for 10 min in 0.2% TBS-triton X-100 (TBS-T). After three washes in TBS (5 minutes each) followed by an antigen retrieval step where section bathed in citrate buffer (10mM, pH=6.0) were microwaved (100W) for 20 min.

After cooling, the sections were washed thrice in TBS and incubated in TBS-T with 10% goat serum (GS; Thermofisher, USA) during 30 min to block unspecific reactivity. Finally, sections were incubated overnight with a primary antibody (anti-glial fibrillary acidic protein (GFAP) marker; mouse, 1:800; Sigma-Aldrich, St. Louis, MO, USA) in TBS-T with 4% GS.

The following day and after a new series of washes in TBS (3 x 5 minutes), the sections were incubated with the secondary antibody (anti-mouse; 1:1000; Sigma-Aldrich, St. Louis, MO, USA) in TBS-T with 4% GS for 2 hours at room temperature. After being washed thrice in TBS, sections were incubated with DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride, 1:1000; Sigma-Aldrich) for 10 minutes and again washed thrice with TBS. Slides were finally coverslip using Permofluor mounting media (Thermo Scientific Shandon).

3.6. Drugs

CHPG (100 nmol/μL; 2-chloro-5-hydroxyphenylglycine; Tocris, Bristol, United Kingdom), an agonist of mGluR5, was prepared with sterilized saline (0.9%NaCl, Brown, Bracarena, Portugal) and MTEP (100 nmol/μL; 3-((2-methyl-1,3-thiazol-4-yl) ethynyl) pyridine hydrochloride; Tocris), an antagonist of mGluR5, was dissolved in PBS.

LαAA (25 μg/μL; L-α-aminoadipate, 2 μL[88], Sigma-Aldrich) was prepared with PBS.

3.7. Behavioural assessment of nociception

To assess nociception in free moving animals the paw withdrawal latency (PWL) test was used as described in detail by Hargreaves and colleagues[93].

3.7.1. Paw-withdrawal latency test

PWL allows assessing thermal hyperalgesia by measuring the latency to evoke paw withdrawal following the application of a radiant heat stimulus to the plantar surface of the hind paw of a freely moving animal. During the habituation period, animals were placed on the test apparatus (Plantar Test Device Model 37370, Ugo Basile, Comerio, Italy) for at least 30 min every day of the week preceding the start of the behavioural sessions. During the evaluation period, animals were also allowed a 5 minutes' period prior to assessing baseline PWL value. PWL was measured prior to drug administration and at various intervals (10, 20, 30, 40 and 50 minutes) following the intracerebral injection of a drug. In each trial, the measurements were repeated twice at an interval of 1 minute and the mean was used for further calculations. A cut-off time of 15 s was used to prevent any tissue damage in the hind paws[86].

3.8. Electrophysiological recordings in the rostroventromedial medulla

Once deeply anesthetized the animals was transferred from the animal house the electrophysiological room and placed on the stereotaxic apparatus (KOPF instruments, Bilaney, Düsseldorf, Germany). The animal's eyes were covered with an ophthalmic cream to avoid dehydration and body temperature was maintained using a homoeothermic blanket.

An anterior to posterior incision on the scalp, from in-between eyes to below the ears of the animal, was made in order to expose the *Bregma* and *Lambda* sutures to allow to determine the coordinates of the IL (2.76 mm rostral to the interaural line, ML: 0.5 mm lateral from the midline, and DV: 4.2 mm below the surface of the skull) and the RVM (1.92 mm rostral to the interaural line, ML: 0.0 to 0.4 mm lateral from the midline, and 8.5 mm below the surface of the skull). In each coordinate, a hole was drilled to allow the placement of the guide cannula in the IL and the recording electrode in the RVM.

Single neurone activity was recorded extracellularly using a tungsten microelectrode (75 mm; impedance 5.8–70 MΩ at 1 kHz, 10 nA, 1 mm). Data sampling was performed using a CED Micro 1401 interface and Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Recording of RVM. During the recordings, the animal was under light anesthesia; 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 cells were classified according changes in their discharge rate in response to peripheral thermal noxious stimulation. Cells were considered ON-cells when an increase in their spontaneous activity was observed during noxious stimulation whereas OFF-cells decreased their spontaneous activity

after stimulation. Some RVM cells did not alter their activity during noxious stimulation and where classified as NEUTRAL-cells. This last groups was not further studied in this work[86] (Fig. 5).

Additionally, RVM ON- and OFF-cells were further categorized into nociceptive specific cells if responding exclusively to noxious stimuli (NS-ON- and NS-OFF-cells) or as wide dynamic range cells if responding simultaneously to noxious and innocuous stimuli (WDR-ON- and WDR-OFF-cells)[30].

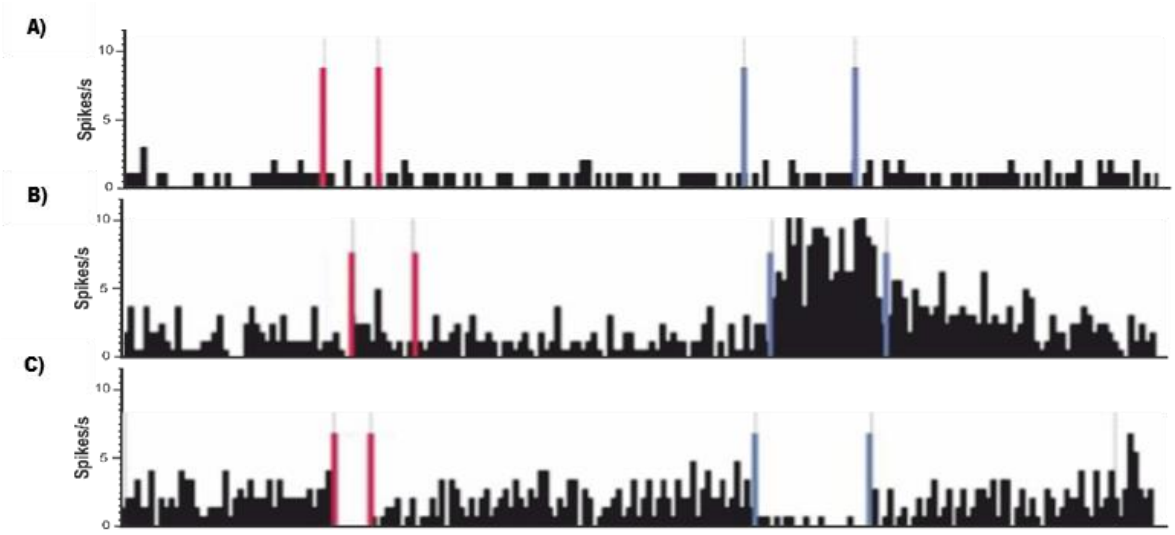


Figure 5. Example of an output from software Spike 2 . A) recording of a neutral cell, this cell does not display any changes in its firing rate during either innocuous or noxious stimulation; B) recording of an ON-NS-like cell, this cell is activated only by peripheral noxious stimulation; C) recording of an OFF-WDR-like cell, this cell responds during the innocuous and noxious peripheral stimulation. Red lines – application of innocuous mechanical stimulation; blue lines – application of noxious thermal stimulation.

Noxious thermal peripheral stimulation consisted of applying a heat source to the tail of the animals for 10 s while innocuous stimulation was achieved by brushing the animals' back with a soft brush in ten slow strikes of 1 s each along the direction of the hairs.

After the administration of the lethal dose of sodium pentobarbital a biphasic stimulus (5 s, 0.7 mA for RVM) was delivered to the electrode and a dye was injected in IL in order to mark the local of recording and activation. Afterwards, the brain was carefully removed and emerged in paraformaldehyde (PFA) 4% for tissue fixation. At least 48 hours later the rat brains were emerged in an agarose block (3% in PBS 1X) and sectioned (50 μm) in a vibratome (Leica Biosystems, Carnaxide, Portugal) apparatus to identify recording site. The brain slices containing the regions of interest were then processed with Cresyl Violet staining.

3.9. Experimental design

3.9.1. Course of Behavioural study

Upon reception animals stayed for a week in quarantine before being transferred to the animal room where they were housed in pairs. After one week of handling and habituation to the testing room and apparatus, half of the animals were administered a solution of carrageenan/kaolin to the synovial cap of the right knee joint to develop ARTH group and the other half was injected with saline and served as SHAM group. Three weeks later, an intracerebral cannula was placed in the right IL of all animals; they were then house individually and allowed to recover for a week. On the day that preceded the beginning of the behavioural sessions, the development/absence of ARTH was confirmed in all animals by registering vocalizations during 5 consecutive flexion/extensions of the right hind leg. After confirmation of their status half of the SHAM and ARTH animals were injected with LAA. In every behavioural session, baseline PWL was assessed prior to drug/vehicle microinjection and then again 10, 20, 30, 40 and 50 minutes after. Drug administration consisted in the administration of either saline and CHPG or saline and MTEP. Each animal was injected each drug twice in a random order. At the end of each behavioural session, the animals were returned to their home cage and the animal room. At the end of the experimental period, all animals received a lethal dose of anaesthetic and their brains were excised for confirmation of cannula placement.

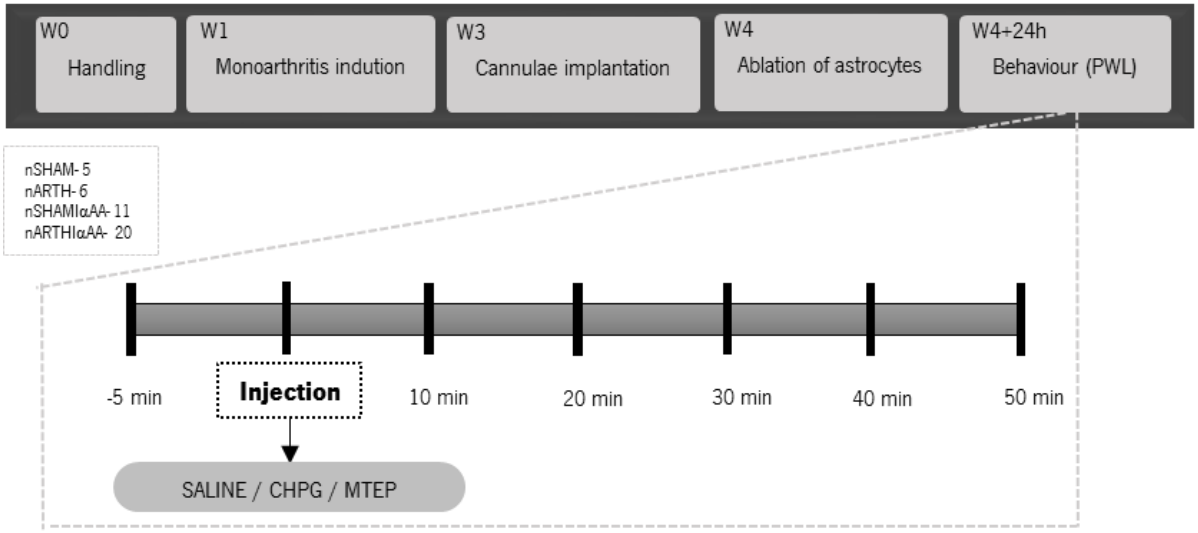


Figure 6. Schematic representation of the behaviour study. Rats used to evaluate the mGluR5/IL-mediating descending modulation of nociception were accustomed to the room and the researcher. Three weeks after the induction of monoarthritis animals were implanted with a chronic cannula in the IL. Animals included in the behavioural study were trained in the behavioural apparatus for one week. Pharmacological tests were performed at the same time-points for both the behavioural study and adjusted to the time of action of each drug, the IL was injected with saline, CHPG or MTEP. (-5 min-pre-injection; IL – Infralimbic cortex; CHPG – 2-chloro-5-hydroxyphenylglycine; MTEP – 3-((2-methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride).

3.9.2. Course of electrophysiological study

Upon reception animals stayed for a week in the quarantine facilities before being transferred to the animal room where they were housed in pairs. One-week later experimental monoarthritis was induced in half of the animals (ARTH group) and the other half was injected with saline and served as controls (SHAM group). Experimental monoarthritis was allowed to evolve during four weeks. On the day that preceded the electrophysiological sessions, the development/absence of ARTH was confirmed in all animals as described in section 3.3.

On the day of the electrophysiological recordings, the animal was anaesthetized in a room in the animal house before being transferred to the electrophysiology room. The animal was placed in a stereotaxic frame and an electrode was placed in the RVM and an intracerebral cannula in the IL. Before performing baseline activity recordings, RVM cells were classified as ON-, OFF- or NEUTRAL-cells taking into account their response to noxious peripheral stimulation and further as NS- or WDR-cells according to their response to noxious and innocuous peripheral stimulation. Baseline activity for each cell type was then recorded prior to drug/vehicle microinjection and then again 10, 20, 30, 40 and 50 minutes after. Drug administration consisted in the administration of either saline or CHPG. Each animal was injected each drug once in a random order.

Before and after the administration of CHPG or SAL in the IL, the activity of RVM neurons was recorded taking into account (i) basal activity of neurons without any peripheral stimulation, (ii) activity of neurons during thermal peripheral noxious stimulation and (iii) activity of RVM neurons during peripheral noxious stimulation after the drug administration in the IL[86]. For thermal stimulation was applied heat stimulation on the tail during 10 s, using a heat source (Plantar Test Device Model 37370, Ugo Basile, Comerio-VA, Italy). At the end of the electrophysiological session, the animals were injected with a lethal dose of anesthetic and brains were excised for later confirmation of injection and recording sites.

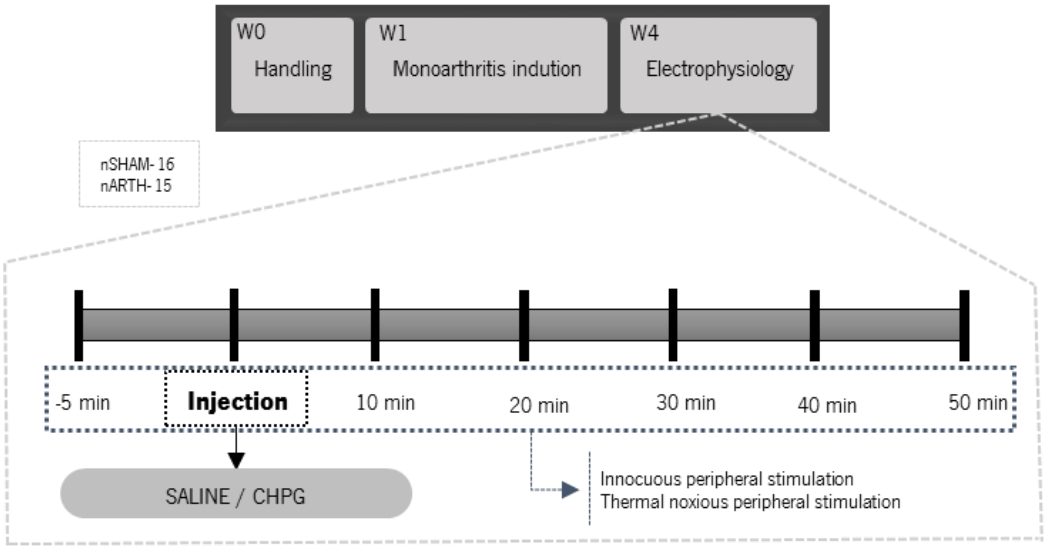


Figure 7. Schematic representation of the electrophysiology study. Rats used to evaluate the mGluR5/IL-mediating descending modulation of nociception were accustomed to the room and the researcher. Four weeks after the induction of monoarthritis animals were submitted to electrophysiological analyze. The cell activity was recorded during a innocuous and thermal noxious peripheral stimulation before and after administration of saline or CHPG in the IL. (-5 min- pre-injection; IL – Infralimbic cortex; CHPG – 2-chloro-5-hydroxyphenylglycine; MTEP – 3-((2-methyl-1,3-thiazol-4-yl) ethynyl)pyridine hydrochloride).

3.10. Data analysis

GraphPad Prism 6 (GraphPad Software, La Jolla California, USA) and IBM SPSS Statistics 22 (IBM Corp, Armonk, NY, USA) were used to perform the statistical analysis. Normality of data was confirmed using the Kolmogorov-Smirmov and Shapiro-Wilk´s tests. Results from the behaviour and electrophysiological studies were then analyzed using an analysis of variance (ANOVA) repeated-measures test with time as within-subjects factor and drug as between-groups factor. The different time points, in both evaluations, were also analyzed using a Two-away ANOVA in order to detect differences within specific time-points. A two-tailed independent-samples t-test was also performed to evaluate differences in basal levels of RVM cell activity between SHAM and ARTH animals.

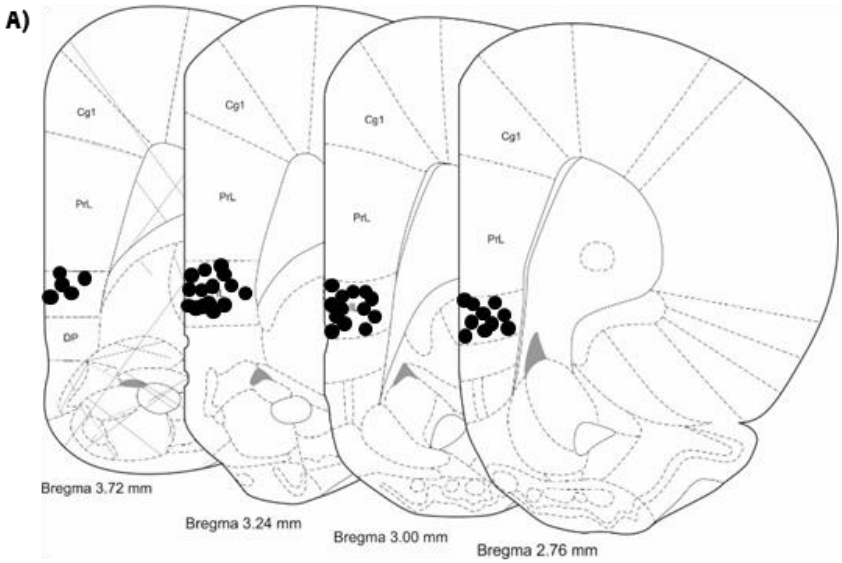
Levene's test was used to verify the equality of variances and Mauchly´s test to evaluate Sphericity. When sphericity was violated, Greenhouse-Geisser or Huynh-Feldt tests were used to correct the differences in variances. Post-hoc Bonferroni correction for multiple comparisons was performed in all the statistical tests. $P<0.05$ was considered to represent a significant difference and all results were expressed as mean±standard error (SEM). Effect size was calculated using $\eta 2$.

CHAPTER 4

RESULTS

4.1. Histological confirmation of cannula placement/injection efficacy

The localization of the guide cannulae used for the injection of drugs was confirmed for all animals and only evaluation performed in animals whose cannula was in the target site, the IL, were considered in this work (Fig. 8A). An example of the efficiency of LαAA in ablating GFAP-positive cells in the IL is shown in figure 8B. Figure 8C shows an example of the effect of injecting PBS in the IL, as a control for



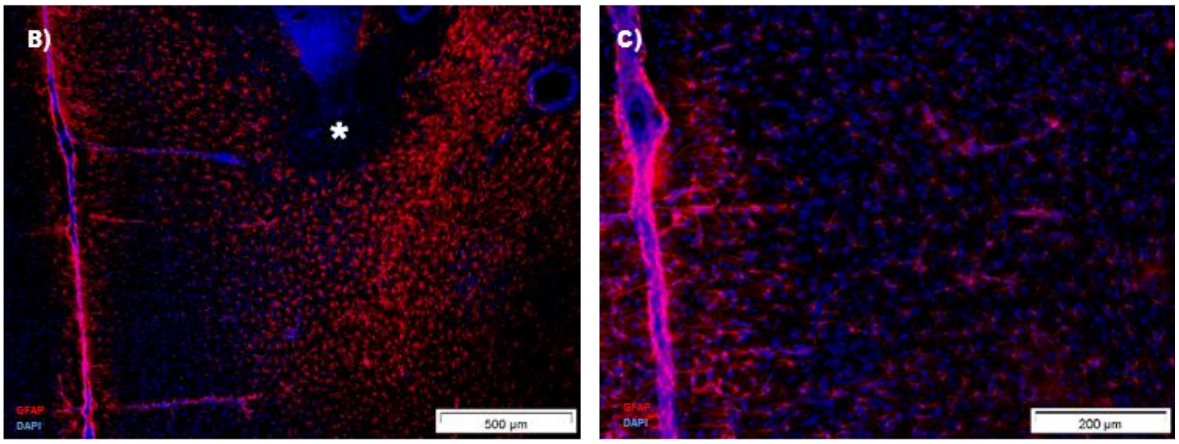


Figure 8. Confirmation of the cannula placement/Lαaa injection efficacy in the IL. A) Schematic representation of the cannula placement in the IL in consecutive brains slices; B) Confirmation of astrocytic ablation, * indicates the area without astrocytes below the cannula scar; C) Details of an animal with intact astrocytes (PBS). IL – Infralimbic cortex; GFAP – anti-glial fibrillary acidic protein (in red); DAPI – 4′,6-Diamidine-2′-phenylindole dihydrochloride (in blue).

Lαaa administration.

The correct placement of the guide cannulae in the IL and respective recording sites in the RVM was confirmed for all animals and is shown in figure 9A. A microphotograph of an example of cannula

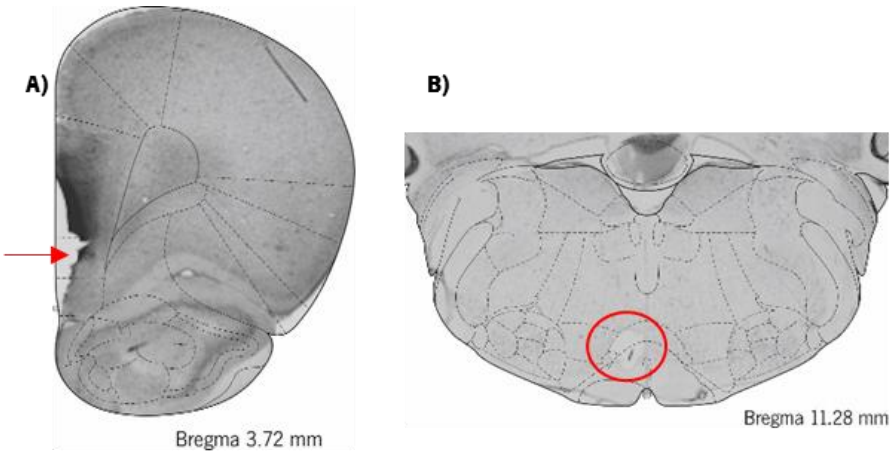


Figure 9. Schematic representation of an example of injections site in the IL and of a recording site. A) injection site in the IL (RC to the bregma: +3,72mm) and B) recording site (RC to the bregma: -11.28mm) according to the coordinates of the rat brain atlas. IL – infralimbic cortex; RC – rostro-caudal; RVM – rostral ventromedial medulla.

placement in the IL and of electrode recording site in the RVM is shown in figure 8B.

4.2. Effect of IL astrocytic-mGluR5 activation/inhibition upon nociceptive behaviour in SHAM and ARTH rats

The ablation of astrocytes in the IL did not alter baseline PWL between SHAM ($t_{(181)}$=0.101) and ARTH ($t_{(181)}$=0.364) (Fig. 10).

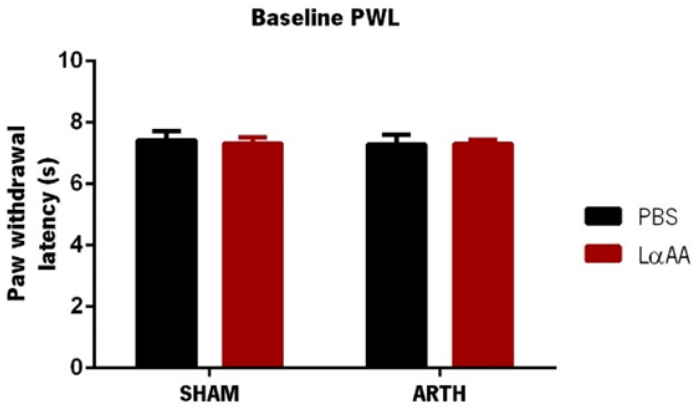


Figure 10. Effect of ablation of astrocytes upon paw withdrawal latencies (PWL) of control (SHAM) and monoarthritic (ARTH) animals in the infralimbic cortex (IL). Data is presented as mean±SEM. (nSHAM=9, nARTH=13, nSHAM Lαaa=15, nARTH Lαaa=17)

4.2.1. Saline microinjection

The injection procedure by itself did not alter the PWL of SHAM and ARTH animals throughout time after saline injection in the IL (ANOVA repeated measures - interaction: $F_{(15,270)}$=0.513, P=0.93; η^2=0.03) (Fig. 11). No differences were observed between groups regarding the different time points after SAL administration (ANOVA repeated measures - time: $F_{(5,270)}$=1.515, P=0.33; ANOVA repeated measures - drug $F_{(3,54)}$=0.245, P=0.86; η^2=0.02). Post-hoc analysis showed no differences between groups (Table I).

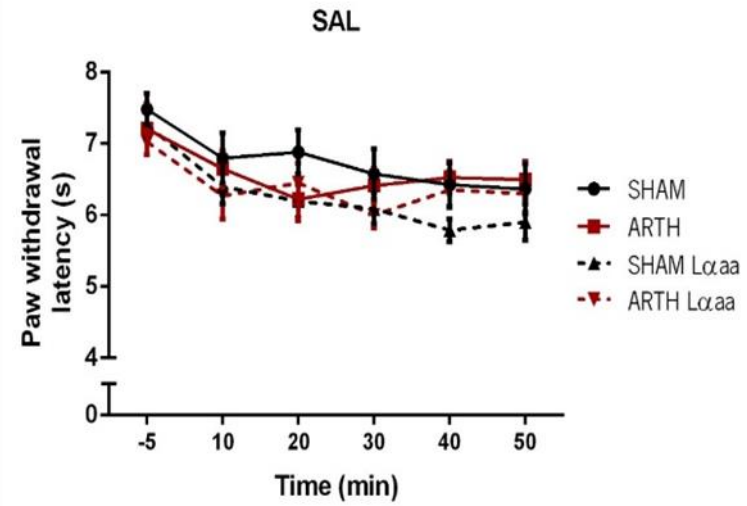


Figure 11. Effect of saline (SAL) administration upon paw withdrawal latencies (PWL). Control (SHAM; black lines) and monoarthritic (ARTH; red lines) animals with intact (SHAM and ARTH; full lines) or selective ablation of astrocytes (SHAM Lαaa and ARTH Lαaa; dashed lines) in the infralimbic cortex (IL). Data is presented as mean±SEM. (nSHAM=10, nARTH=10, nSHAM Lαaa=19, nARTH Lαaa=21)

Table I – Summary of the statistical analysis for the paw-withdrawal test in SHAM and ARTH animals that received vehicle or L-α-aminoadipate in the Infralimbic cortex. Comparasion between the different time points (-5 min (pre-injection) and 10 min, 20 min, 30 min, 40 min and 50 min after the drug administration) taking into account both SHAM and ARTH groups with ablation of astrocytes in the IL (L-α-aminoadipate) or PBS obtained by a repeated measures ANOVA. PBS vs L-α-aminoadipate – comparasion between animals that received the gliotoxin or the vehicle in the IL; SHAM vs ARTH – comparasion between control and monoarthritic animals. (ARTH – monoarthric animals; SHAM – control animals; CHPG – 2-chloro-5-hydroxyphenylglycine; MTEP – 3-((2-methyl-1,3-thiazol-4-yl) ethynyl)pyridine hydrochloride); SAL – saline; IL – infralimbic cortex).

			PBS			L-α-AMINOADIPATE			PBS vs L-α-AMINOADIPATE
			SAL	CHPG	MTEP	SAL	CHPG	MTEP	
SHAM	SAL	-5min	-	0.674	0.343	-	1.000	1.000	0.998
		10min	1.000	1.000	1.000	0.013*	0.265	1.000	1.000
		20 min	1.000	0.001*	0.599	0.043*	0.700	0.441	0.265
		30 min	1.000	0.009*	1.000	0.000*	0.681	1.000	0.771
		40 min	1.000	0.822	0.456	0.000*	0.658	0.504	0.992
		50 min	1.000	1.000	0.455	0.009*	0.855	1.000	0.914
	CHPG	-5min		-	1.000		-	1.000	1.000
		10min		1.000	1.000		0.080	0.308	0.378
		20 min		0.022*	0.032*		0.013*	0.036*	1.000
		30 min		0.003*	0.001*		0.027*	0.164	1.000
		40 min		0.176	0.042*		0.059	1.000	0.505
		50 min		0.789	0.081		0.371	0.672	1.000
	MTEP	-5min			-			-	1.000
		10min			1.000			1.000	1.000
		20 min			1.000			1.000	1.000
		30 min			1.000			1.000	1.000
		40 min			1.000			1.000	1.000
		50 min			1.000			0.180	0.086
ARTH	SAL	-5min	-	1.000	0.232	-	0.753	1.000	1.000
		10min	1.000	1.000	1.000	1.000	0.057	0.856	1.000
		20 min	0.201	0.765	1.000	1.000	0.017*	1.000	1.000
		30 min	1.000	0.261	0.002*	0.161	0.260	1.000	1.000
		40 min	0.623	1.000	0.059	0.390	1.000	1.000	1.000
		50 min	0.881	1.000	1.000	0.294	1.000	1.000	1.000
	CHPG	-5min		-	0.131		-	1.000	1.000
		10min		1.000	1.000		1.000	0.476	0.058
		20 min		0.019*	0.492		0.035*	0.001*	1.000
		30 min		0.023*	< 0,0001*		0.098	0.012*	1.000
		40 min		0.493	0.012*		0.328	1.000	1.000
		50 min		0.141	0.668		0.440	1.000	1.000
	MTEP	-5min			-			-	1.000
		10min			1.000			0.255	1.000
		20 min			1.000			1.000	1.000
		30 min			0.043*			1.000	0.003*
		40 min			0.147			0.147	0.006*
		50 min			1.000			0.258	1.000
SHAM vs ARTH		-5min	1.000	1.000	1.000	1.000	1.000	1.000	
		10min	1.000	1.000	1.000	1.000	1.000	1.000	
		20 min	0.588	1.000	1.000	1.000	0.916	1.000	
		30 min	1.000	1.000	0.181	1.000	1.000	1.000	
		40 min	1.000	1.000	0.521	1.000	1.000	1.000	
		50 min	1.000	1.000	1.000	1.000	1.000	1.000	

4.2.2. Effect of the selective activation of IL mGluR5 upon PWL

Overall, the microinjection of CHPG, an mGluR5 agonist, in the IL decreased PWL (ANOVA repeated measures - time: $F_{(5,225)}$=19.36, P<0.0001). PWL decrease was not different amongst experimental groups (ANOVA repeated measures - interaction: $F_{(15,225)}$=1.129, P=0.33) and was not significantly altered when comparing treatments (ANOVA repeated measures - drug: $F_{(3,45)}$=0.914, P=0.44) (Fig. 12). *Post-hoc* tests showed CHPG decreased PWL in all groups 20 and 30 min after drug administration (table I)

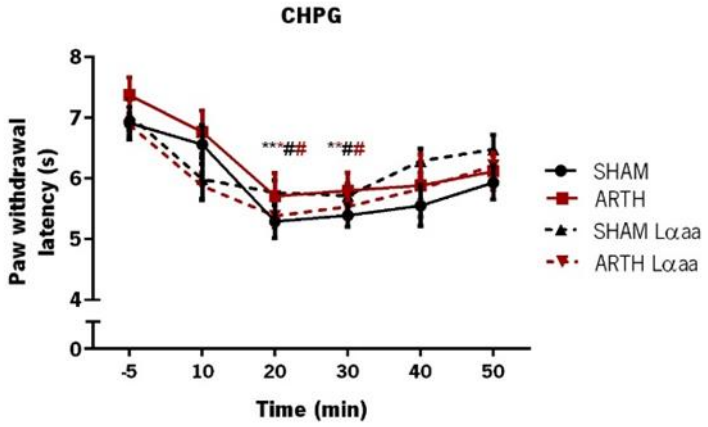


Figure 12. Effect of mGluR5 agonist (CHPG) administration upon paw withdrawal latencies (PWL). Control (SHAM; black lines) and monoarthritic (ARTH; red lines) animals with intact (SHAM and ARTH; full lines) or selective ablation of astrocytes (SHAM Lαaa and ARTH Lαaa; dashed lines) in the infralimbic cortex (IL). Data is presented as mean±SEM. (nSHAM=10, nARTH=12, nSHAM Lαaa=19, nARTH Lαaa=27) */# p<0.05; ** p<0.01. */** represent the comparison of injection results with pre-injection (-5min) value of groups with intact astrocytes. # represents the comparison of injection results with pre-injection (-5min) value of groups with selective ablation of astrocytes.

4.2.3. Effect of the selective inhibition of IL mGluR5 upon PWL

PWL is significantly altered by MTEP, an mGluR5 antagonist, microinjection into the IL (ANOVA repeated measures - interaction: $F_{(15,250)}$=2.590, P=0.001; η^2=0.013). Overall, the microinjection of MTEP did not alter overall PWL differently after treatment (ANOVA repeated measures - drug: $F_{(3,50)}$=0.891, P=0.45; η^2=0.05), in contrast to time (ANOVA repeated measures - time: $F_{(3,250)}$=2.735, P=0.02). Additionally, *post-hoc* tests demonstrated an increase in PWL of ARTH animals 30 minutes after MTEP administration (Fig. 13) (table I).

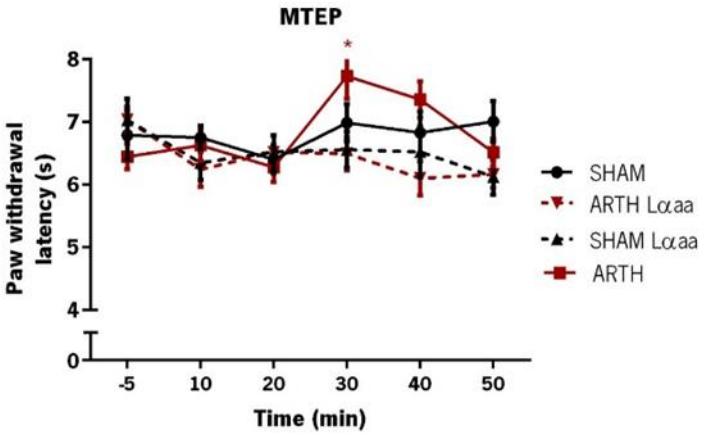


Figure 13. Effect of mGluR5 antagonist (MTEP) administration upon paw withdrawal latencies (PWL). Control (SHAM; black lines) and monoarthritic (ARTH; red lines) animals with intact (SHAM and ARTH; full lines) or selective ablation of astrocytes (SHAM Lαaa and ARTH Lαaa; dashed lines) in the infralimbic cortex (IL). Data is presented as mean±SEM. (nSHAM=13, nARTH=16, nSHAM Lαaa=17, nARTH Lαaa=20) * $p<0.05$. * represents the comparison of injection results with pre-injection (-5min) value.

4.3. RVM ON- and OFF-like cell activity in SHAM and ARTH animals

The total number of cells recorded in the rostral ventromedial medulla (RVM) in SHAM and ARTH animals is represented in table II and III. Only cells classified as ON- and OFF-like were analyzed in this work.

There was no difference between the spontaneous activity of both NS- ($t_{(50)}$=0.97; *P*=0.34) and WDR- ($t_{(105)}$=1.86; *P*=0.07) ON-like cells between SHAM and ARTH animals. The same was observed in NS- ($t_{(8)}$=0.20; *P*=0.84) and WDR- ($t_{(41)}$=0.56; *P*=0.58) OFF-like cells. Regarding the evoked noxious activity, again no differences were observed in NS- ($t_{(50)}$=0.70; *P*=0.49) and WDR- ($t_{(103)}$=0.98; *P*=0.33) ON-like cells between SHAM and ARTH animals. The same was observed for NS- ($t_{(8)}$=0.19; *P*=0.85) and WDR- ($t_{(42)}$=1.05; *P*=0.30) OFF-like cells (Fig. 14).

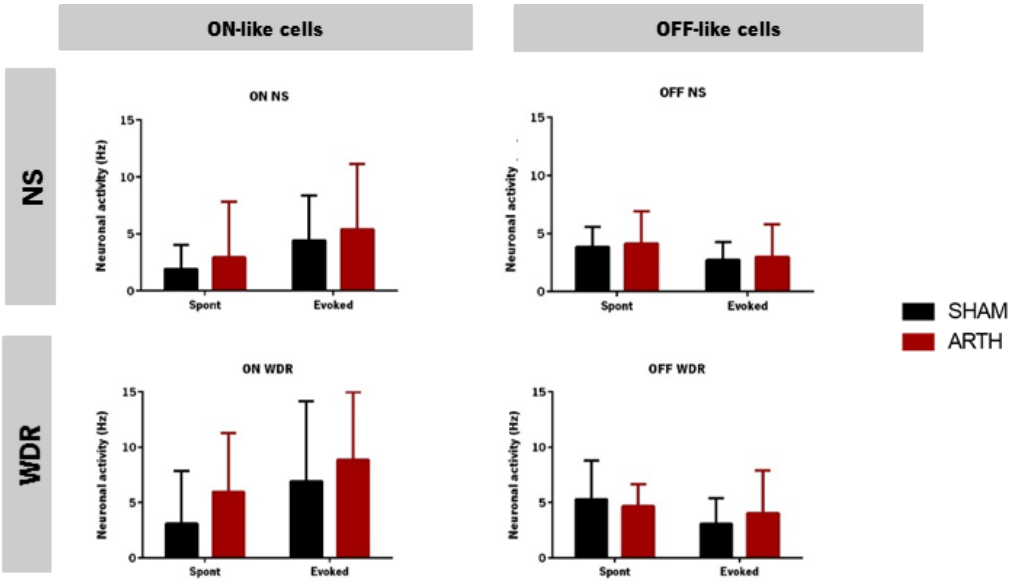


Figure 14. Basal activity of RVM ON-like and OFF-like cells in control (SHAM) and arthritic (ARTH) animals. Spontaneous (Spont) activity and Evoked noxious (Evoked) activity (NS – nociceptive specific; WDR – wide-dynamic range). Data is presented as mean±SEM.

4.3.1. Influence of IL/mGluR5 activation on RVM ON-like cell activity

For the evaluation of the impact of IL-mGluR5 activation/inhibition upon RVM cell activity, the differences between spontaneous and evoked activity prior and after drug administration in each time point were quantified and analyzed. As explained previously (section 3.8.) RVM ON- and OFF-like cells were further classified in Nociceptive Specific (NS) and Wide Dynamic Range (WDR) according to their response to exclusively noxious or both noxious and innocuous peripheral stimuli, respectively. The number of RVM cells recorded is presented in table II.

Table II. Number of RVM pronociceptive ON- and antinociceptive OFF-cells recorded in electrophysiological study. Cells were recorded before and after the microinjection of saline (SAL) and CHPG, a specific antagonist of mGluR5, in the infralimbic cortex in control (SHAM) and monoarthritic (ARTH) animals.

		ON-like cells		OFF-like cells	
		NS	WDR	NS	WDR
SHAM	SAL	8	29	3	13
	CHPG	15	39	3	18
ARTH	SAL	15	41	1	12
	CHPG	15	35	3	3

4.3.1.1. Evaluation of RVM ON-NS-like cells

Overall, the spontaneous activity of RVM ON-NS-like cells in SHAM animals was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction: $F_{(1,13)}$=5.470; P=0.04; η^2=0.30). The analysis of the potential effect of the administration of SAL and CHPG confirmed the previous results, as no changes in cell activity were observed (ANOVA repeated measures – main factor drug: $F_{(1,13)}$=3.891; P=0.07; η^2=0.231). Identically, the comparison of cell activity after the administration of SAL and CHPG in each time point also confirmed no changes (ANOVA repeated measures – main factor time: $F_{(2.24,29.06)}$=1.822, P=0.18; η^2=0.123) (Fig. 15A).

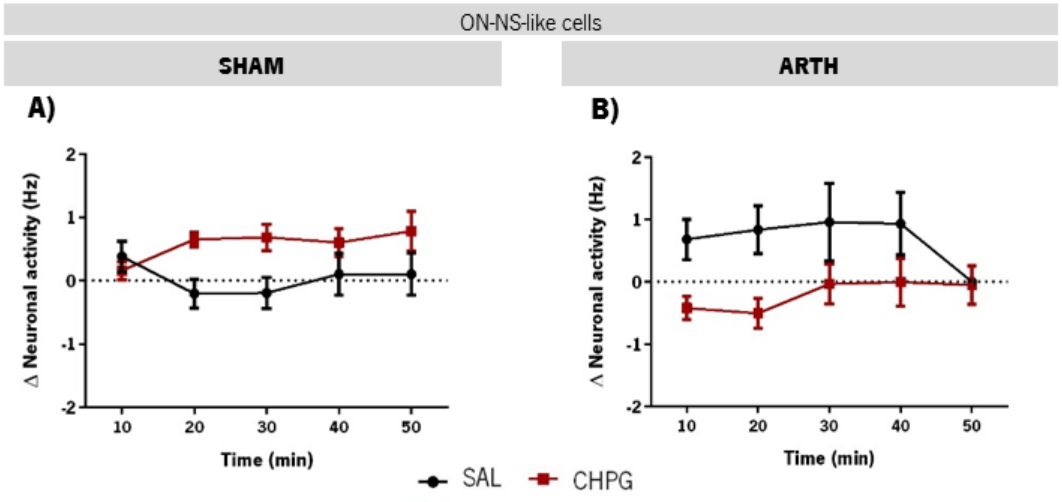


Figure 15. Spontaneous activity of RVM ON-NS-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-NS-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-NS-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ - (activity at a specific time point minus baseline activity of the cell). Data is presented as mean ± SEM.

In ARTH animals the overall spontaneous activity of RVM ON-NS-like cells was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction: $F_{(1,29)}$=2.537; P=0.12; η^2=0.08). Nonetheless, RVM ON-NS-like cell activity was significantly altered when comparing treatments (ANOVA repeated measures – main effect drug: $F_{(1,29)}$=6.302; P=0.02; η^2=0.18) and time points (ANOVA repeated measures – main factor time: $F_{(1.84,62.81)}$=0.520; P=0.61; η^2=0.02) (Fig. 15B). *Post-hoc* analysis show difference between SAL and CHPG 30 and 50 minutes after the drug administration (table III).

Table III. Summary of the ANOVA repeated measures post-hoc results for the electrophysiological study. Comparison of the differences between baseline RVM cell activity at different time points (10 min, 20 min, 30 min, 40 min and 50 min) after drug administration in the IL in SHAM and ARTH animals. (ARTH – monoarthritic animals; SHAM – control animals; CHPG – agonist of mGluR5; SAL – saline; ON-cells – RVM pronociceptive cells; OFF-cells – RVM antinociceptive cells; NS – RVM ON- and OFF nociceptive specific cells; WDR – Wide dynamic range cells).

SPONTANEOUS ACTIVITY														
				SAL					CHPG					SAL vs CHPG
				10min	20min	30min	40min	50min	10min	20min	30min	40min	50min	
SHAM	ON-cells	NS	10 min	-					-					1.000
			20 min	0.089	-				1.000	-				0.127
			30 min	0.282	1.000	-			0.594	1.000	-			0.128
			40 min	1.000	0.245	0.438	-		1.000	1.000	1.000	-		0.410
			50 min	1.000	0.905	0.922	1.000	-	1.000	1.000	1.000	1.000	-	0.396
		WDR	10 min	-					-					1.000
			20 min	1.000	-				1.000	-				1.000
			30 min	1.000	1.000	-			1.000	1.000	-			1.000
			40 min	1.000	1.000	1.000	-		0.520	0.278	0.473	-		1.000
			50 min	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	0.872	-	1.000
	OFF-cells	NS	10 min	-					-					1.000
			20 min	1.000	-				1.000	-				1.000
			30 min	1.000	1.000	-			1.000	0.170	-			1.000
			40 min	1.000	1.000	1.000	-		1.000	1.000	1.000	-		1.000
			50 min	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	-	1.000
		WDR	10 min	-					-					1.000
			20 min	1.000	-				1.000	-				1.000
			30 min	1.000	1.000	-			1.000	1.000	-			1.000
			40 min	1.000	1.000	1.000	-		1.000	1.000	1.000	-		1.000
			50 min	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	-	1.000
ARTH	ON-cells	NS	10 min	-					-					0.169
			20 min	1.000	-				1.000	-				0.169
			30 min	1.000	1.000	-			0.603	0.783	-			0.567
			40 min	1.000	1.000	1.000	-		1.000	0.379	1.000	-		0.390
			50 min	1.000	1.000	1.000	1.000	-	1.000	0.125	1.000	1.000	-	0.379
		WDR	10 min	-					-					0.997
			20 min	0.176	-				1.000	-				1.000
			30 min	0.352	1.000	-			1.000	1.000	-			1.000
			40 min	0.284	1.000	1.000	-		0.467	0.065	0.076	-		0.650
			50 min	1.000	0.060	0.054	0.168	-	0.113	0.025*	0.122	1.000	-	0.009*
	OFF-cells	NS	10 min	-	-	-	-	-	-					1.000
			20 min	-	-	-	-	-	1.000	-				0.652
			30 min	-	-	-	-	-	1.000	1.000	-			0.017*
			40 min	-	-	-	-	-	1.000	1.000	1.000	-		0.058
			50 min	-	-	-	-	-	1.000	1.000	0.171	1.000	-	0.018*
		WDR	10 min	-					-					1.000
			20 min	1.000	-				1.000	-				1.000
			30 min	1.000	1.000	-			1.000	1.000	-			1.000
			40 min	1.000	1.000	1.000	-		1.000	1.000	1.000	-		1.000
			50 min	1.000	1.000	0.920	1.000	-	1.000	1.000	1.000	1.000	-	1.000

Table IV – Summary of the ANOVA repeated measures post-hoc results for the electrophysiological study. Comparison of the differences between baseline RVM cell activity at different time points (10 min, 20 min, 30 min, 40 min and 50 min) after drug administration in the IL in SHAM and ARTH animals. (ARTH – monoarthritic animals; SHAM – control animals; CHPG – agonist of mGluR5; SAL – saline; ON-cells – RVM pronociceptive cells; OFF-cells – RVM antinociceptive cells; NS – RVM ON- and OFF nociceptive specific cells; WDR – Wide dynamic range cells).

EVOKED ACTIVITY														
				SAL					CHPG					SAL vs CHPG
				10min	20min	30min	40min	50min	10min	20min	30min	40min	50min	
SHAM	ON-cells	NS	10 min	-					-					1.000
			20 min	1.000	-				1.000	-				1.000
			30 min	1.000	1.000	-			1.000	1.000	-			1.000
			40 min	1.000	1.000	1.000	-		1.000	1.000	1.000	-		1.000
			50 min	1.000	1.000	1.000	1.000	-	0.084	0.083	0.084	0.085	-	1.000
		WDR	10 min	-					-					1.000
			20 min	0.147	-				0.413	-				1.000
			30 min	1.000	0.012*	-			0.904	1.000	-			1.000
			40 min	0.280	1.000	0.073	-		1.000	1.000	1.000	-		1.000
			50 min	1.000	1.000	1.000	1.000	-	1.000	1.000	1.000	1.000	-	0.625
	OFF-cells	NS	10 min	-					-					0.648
			20 min	1.000	-				1.000	-				0.595
			30 min	1.000	1.000	-			1.000	1.000	-			1.000
			40 min	1.000	1.000	1.000	-		1.000	1.000	1.000	-		1.000
			50 min	1.000	1.000	0.903	1.000	-	1.000	1.000	1.000	1.000	-	1.000
		WDR	10 min	-					-					1.000
			20 min	0.693	-				0.608	-				0.993
			30 min	0.433	1.000	-			0.360	1.000	-			0.998
			40 min	0.481	1.000	1.000	-		1.000	1.000	1.000	-		1.000
			50 min	0.467	1.000	1.000	1.000	-	1.000	0.416	0.723	1.000	-	0.407
ARTH	ON-cells	NS	10 min	-					-					0.482
			20 min	1.000	-				1.000	-				0.318
			30 min	1.000	1.000	-			1.000	1.000	-			0.755
			40 min	1.000	1.000	1.000	-		1.000	0.013	1.000	-		1.000
			50 min	1.000	1.000	1.000	1.000	-	1.000	0.268	1.000	1.000	-	1.000
		WDR	10 min	-					-					1.000
			20 min	1.000	-				1.000	-				1.000
			30 min	1.000	1.000	-			1.000	1.000	-			1.000
			40 min	1.000	1.000	1.000	-		1.000	0.239	0.101	-		0.565
			50 min	1.000	0.470	0.293	0.668	-	1.000	0.150	0.167	1.000	-	0.014*
	OFF-cells	NS	10 min	-	-	-	-	-	-					0.392
			20 min	-	-	-	-	-	1.000	-				0.068
			30 min	-	-	-	-	-	1.000	1.000	-			0.008*
			40 min	-	-	-	-	-	1.000	1.000	1.000	-		0.004*
			50 min	-	-	-	-	-	1.000	1.000	1.000	1.000	-	0.002*
		WDR	10 min	-					-					0.555
			20 min	1.000	-				1.000	-				0.832
			30 min	1.000	1.000	-			1.000	1.000	-			0.646
			40 min	1.000	1.000	1.000	-		1.000	1.000	1.000	-		0.997
			50 min	1.000	1.000	0.777	0.545	-	0.997	1.000	0.371	1.000	-	0.006*

Overall, RVM ON-NS-like cells evoked activity of SHAM animals was not altered by drug administration throughout time effect (ANOVA repeated measures – interaction between drug and time: $F_{(1,12)}$=0.631; P=0.44; η^2=0.05). This results was further confirmed by analysis of changes in cell activity after drug administration to the IL (ANOVA repeated measures – main factor drug: $F_{(1,12)}$=3.910; P=0.07; η^2=0.246) and at each time point (ANOVA repeated measures – main factor time: $F_{(1.64,21.98)}$=3.383; P=0.06; η^2=0.02) (Fig. 16A).

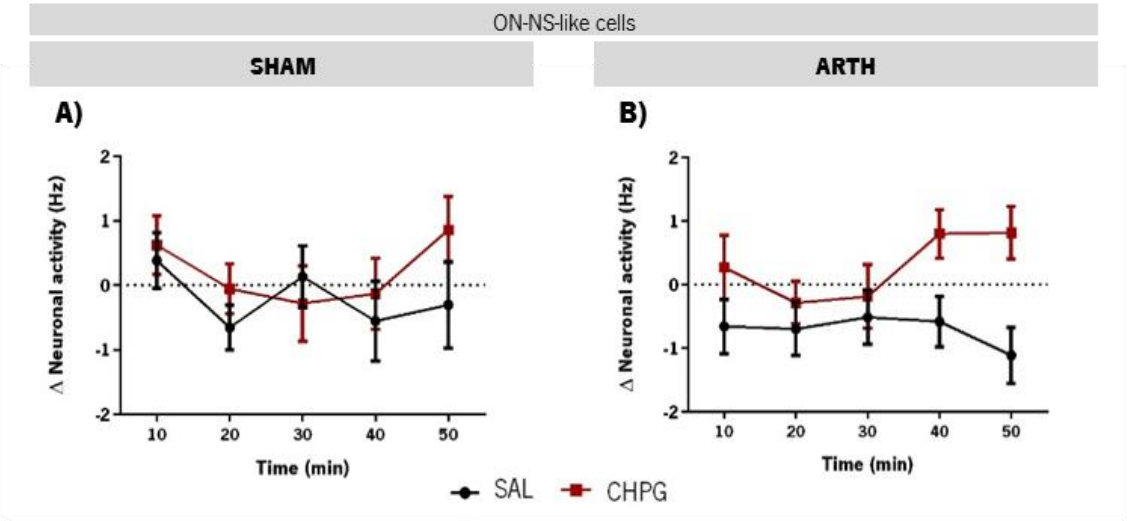


Figure 16. Noxious-evoked activity of RVM ON-NS-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-NS-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-NS-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ – (activity at a specific time point minus baseline activity of the cell). Data is presented as mean ± SEM.

Similarly, in ARTH animals no changes in the evoked activity of RVM ON-NS-like cells throughout time was observed (ANOVA repeated measures – interaction between drug and time: $F_{(1,11)}$=1.334; P=0.259; η^2=0.05). Both the analysis of changes in cell activity after drug administration (ANOVA repeated measures – main factor drug: $F_{(1,25)}$=1.126; P=0.30; η^2=0.04) and at each time point (ANOVA repeated measures – main factor time: $F_{(2.86,71.38)}$=0.368; P=0.77; η^2=0.02) confirmed no effect was observed (Fig. 16B).

4.3.1.2. Evaluation of RVM ON-WDR-like cells

The spontaneous activity of RVM ON-WDR-like cells in SHAM animals was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction between drug and time: $F_{(1,47)}$=1.307; P=0.23; η^2=0.03). The analysis of the potential effect of the administration of SAL and CHPG confirmed the previous results, as no changes in cell activity were observed (ANOVA repeated measures – main factor drug: $F_{(1,47)}$=0.246; P=0.62; η^2=0.005). Identically, the comparison of cell activity after the administration of SAL and CHPG in each time point also confirmed no changes (ANOVA repeated measures – main factor time: $F_{(3.15,147.97)}$=1.138; P=0.34; η^2=0.024) (Fig. 17A).

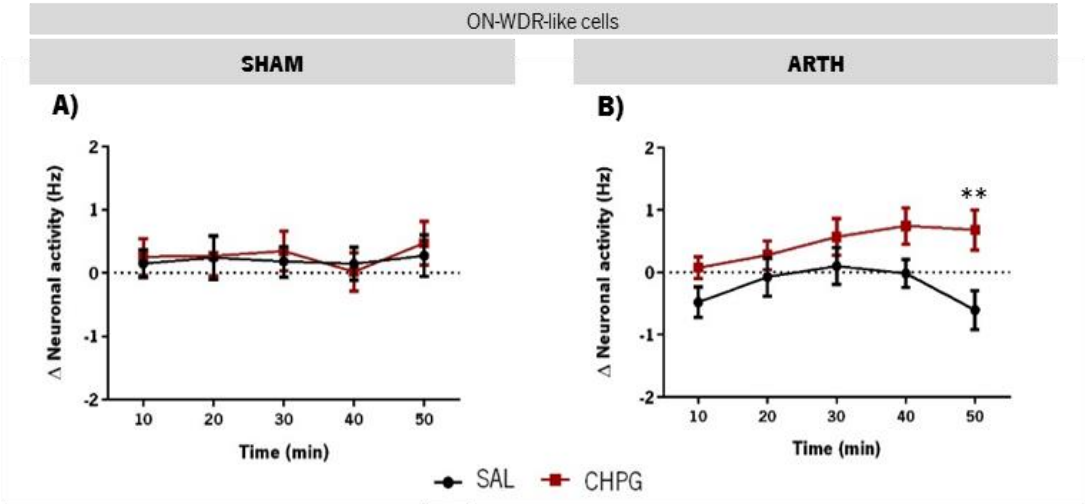


Figure 17. Spontaneous activity of RVM ON-WDR-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-WDR-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-WDR-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ – (activity at a specific time point minus baseline activity of the cell). ** represent the comparison between SAL and CHPG administration. ** P<0.01 (t-test with Bonferroni correction for multiple comparison). Data is presented as mean ± SEM.

In ARTH animals the overall spontaneous activity of RVM ON-WDR-like cells was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction: $F_{(1,11)}$=1.790; P=0.38; η^2=0.01). Yet, the comparison of RVM ON-WDR-like cell activity is significantly altered when comparing treatments (ANOVA repeated measures – main effect drug: $F_{(1,66)}$=9.04; P=0.004; η^2=0.120) and throughout time (ANOVA repeated measures – main factor time: $F_{(2.88,189.92)}$=3.114; P=0.03; η^2=0.029) (Fig. 17B). Post-hoc analysis showed differences between SAL and CHPG 50 minutes after the drug administration (table III).

Overall, RVM ON-WDR-like cells evoked activity of SHAM animals did not vary with drug

administration throughout time effect (ANOVA repeated measures – interaction between drug and time: $F_{(1,41)}$=0.567; P=0.46; η^2=0.01). This result was further confirmed by analysis of changes in cell activity after drug administration to the IL (ANOVA repeated measures – main factor drug: $F_{(1,41)}$=1.562; P=0.219; η^2=0.04). Yet, significant changes were found between time points (ANOVA repeated measures – main factor time: $F_{(2.10,122.98)}$=2.891; P=0.04; η^2=0.07; Fig. 18A) although *post-hoc* test did not show changes at a specific time point (table III).

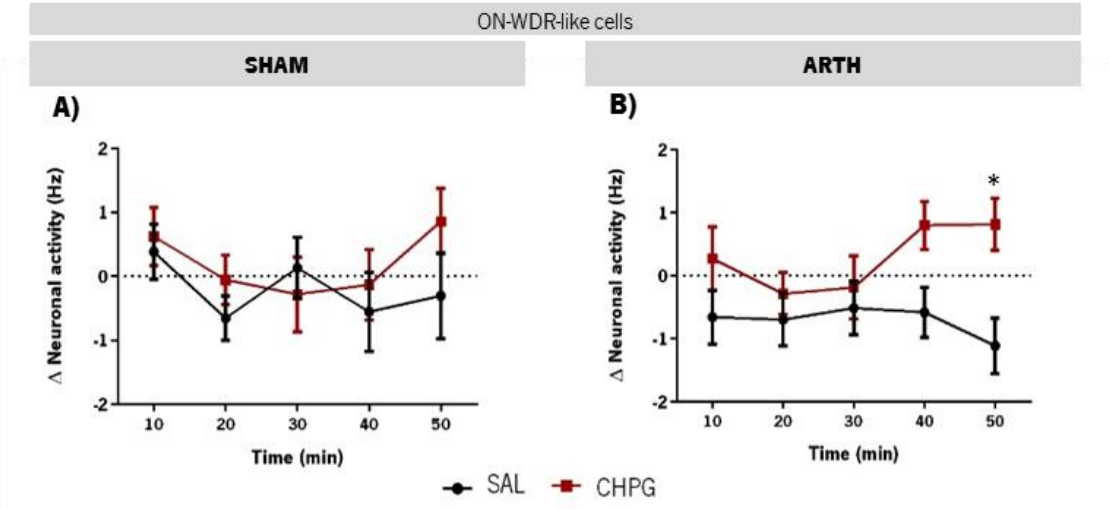


Figure 18. Noxious-evoked activity of RVM ON-WDR-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-WDR-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM ON-WDR-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ – (activity at a specific time point minus baseline activity of the cell). * represent the comparison between SAL and CHPG administration. *P<0.05 (t-test with Bonferroni correction for multiple comparison). Data is presented as mean ± SEM.

In ARTH animals no changes in the evoked activity of RVM ON-WDR-like cells was observed (ANOVA repeated measures – interaction between drug and time: $F_{(1,11)}$=1.141; P=0.29; η^2=0.02). Both the analysis of changes in cell activity after drug administration (ANOVA repeated measures – main factor drug: $F_{(1,57)}$=1.254; P=0.27; η^2=0.02) and at each time point (ANOVA repeated measures – main factor time: $F_{(4, 228)}$=0.569; P=0.69; η^2=0.01) confirmed no effect was observed (Fig. 18B).

4.3.2. Influence of IL/mGluR5 activation upon RVM OFF-like cell activity

4.3.2.1. Evaluation of RVM NS OFF-like cells

Overall, the spontaneous activity of RVM NS OFF-like cells in SHAM animals was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction: $F_{(1,4)}$=0.271; P=0.63; η^2=0.06). The analysis of the potential effect of the administration of SAL and CHPG confirmed the previous results, as no changes in cell activity were observed (ANOVA repeated measures – main factor drug: $F_{(1,4)}$=0.596; P=0.48; η^2=0.130). Identically, the comparison of cell activity after the administration of SAL and CHPG in each time point also confirmed no changes (ANOVA repeated measures – main factor time: $F_{(4,16)}$=0.934; P=0.47; η^2=0.19) (Fig. 19A).

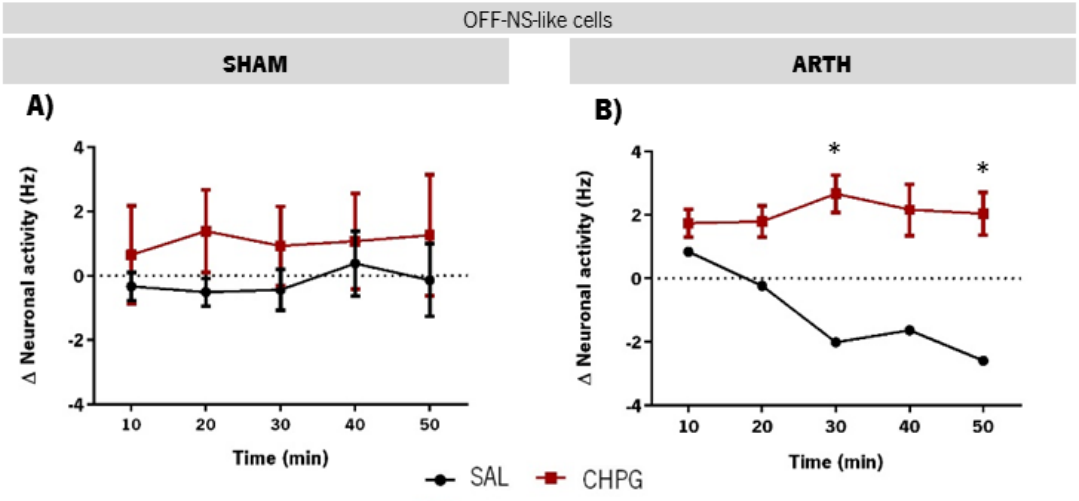


Figure 19. Spontaneous activity of RVM OFF-NS-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-NS-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-NS-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ – (activity at a specific time point minus baseline activity of the cell). * represent the comparison between SAL and CHPG administration. *P<0.05 (t-test with Bonferroni correction for multiple comparison). Data is presented as mean ± SEM.

In ARTH animals the overall spontaneous activity of RVM NS OFF-like cells was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction: $F_{(1,2)}$=0.703; P=0.49; η^2=0.26). Nonetheless, RVM NS OFF-like cell activity was significantly altered when comparing treatments (ANOVA repeated measures – main effect drug: $F_{(1,2)}$=7.859; P=0.11; η^2=0.80) and time points (ANOVA repeated measures – main factor time: $F_{(4.00,8.00)}$=5.523; P=0.11; η^2=0.73) (Fig. 19B).

Overall, RVM OFF-NS-like cells evoked activity of SHAM animals was not altered by drug

administration throughout time effect (ANOVA repeated measures – interaction between drug and time: $F_{(1,4)}$=1.598; P=0.39; η^2=0.19). This result was further confirmed by analysis of changes in cell activity after drug administration to the IL (ANOVA repeated measures – main factor drug: $F_{(1,4)}$=1.598; P=0.28; η^2=0.29) and at each time point (ANOVA repeated measures – main factor time: $F_{(4,16)}$=1.280; P=0.32; η^2=0.319) (Fig. 20A).

Similarly, in ARTH animals no changes in the evoked activity of RVM OFF-NS-like cells was observed (ANOVA repeated measures – interaction between drug and time: $F_{(1,2)}$=0.035; P=0.87; η^2=0.02). The analysis of changes in cell activity after drug administration (ANOVA repeated measures – main factor drug: $F_{(1,2)}$=20.572; P=0.05; η^2=0.91) confirmed no effect was observed.

In contrast, the analysis throughout time showed significant differences (ANOVA repeated measures – main factor time: $F_{(4,8)}$=5.540; P=0.02; η^2=0.74) (Fig. 20B). *Post-hoc* analysis shown differences between SAL and CHPG administration in the 30, 40 and 50 min time points (table IV).

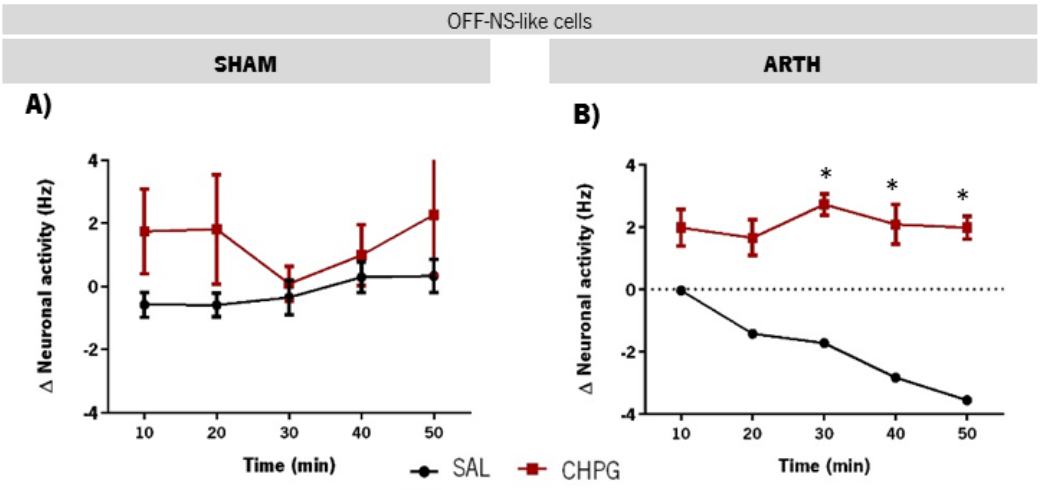


Figure 20. Noxious-evoked activity of RVM OFF-NS-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-NS-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-NS-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ – (activity at a specific time point minus baseline activity of the cell). * represent the comparison between SAL and CHPG administration. * P<0.05 (t-test with Bonferroni correction for multiple comparison). Data is presented as mean ± SEM.

4.3.2.2. Evaluation of RVM OFF-WDR-like cells

Overall, the spontaneous activity of RVM OFF-WDR-like cells in SHAM animals was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction: $F_{(1,28)}$=0.838; P=0.37; η^2=0.03). The analysis of the potential effect of the administration of SAL and CHPG confirmed the previous results, as no changes in cell activity were observed (ANOVA repeated measures – main factor drug: $F_{(1,28)}$=0.357; P=0.56; η^2=0.01). Identically, the comparison of cell activity after the administration of SAL and CHPG in each time point also confirmed no changes (ANOVA repeated measures – main factor time: $F_{(3.11,86.95)}$=0.472; P=0.71; η^2=0.02) (Fig. 21A).

In ARTH animals the overall spontaneous activity of RVM OFF-WDR-like cells was not altered throughout time after drug injection in the IL (ANOVA repeated measures – interaction: $F_{(1,13)}$=0.210; P=0.65; η^2=0.02). Nonetheless, RVM OFF-WDR-like cell activity was significantly altered when comparing treatments (ANOVA repeated measures – main effect drug: $F_{(1,13)}$=0.245; P=0.63; η^2=0.02) and time points (ANOVA repeated measures – main factor time: $F_{(1.08,14.09)}$=0.236; P=0.65; η^2=0.02) (Fig. 21B).

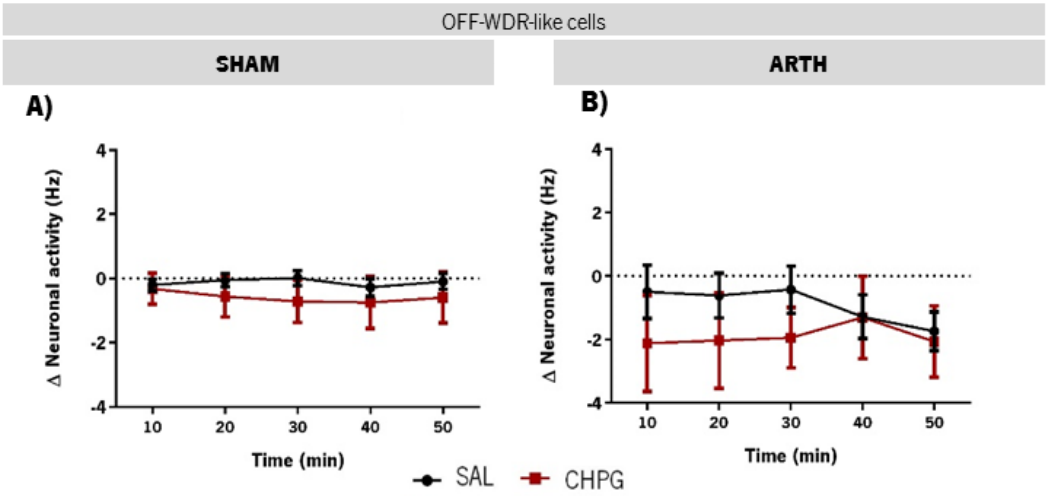


Figure 21. Spontaneous activity of RVM OFF-WDR-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-WDR-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-WDR-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ – (activity at a specific time point minus baseline activity of the cell). Data is presented as mean ± SEM.

Overall, RVM OFF-WDR-like cells evoked activity of SHAM animals was not altered by drug administration throughout time effect (ANOVA repeated measures – interaction between drug and time: $F_{(1,22)}$=0.144; P=0.71; η^2=0.01). This result was further confirmed by analysis of changes in cell activity after drug administration to the IL (ANOVA repeated measures – main factor drug: $F_{(1,22)}$=0.157; P=0.69; η^2=0.01) and at each time point (ANOVA repeated measures – main factor time: $F_{(3.29,72.52)}$=1.705; P=0.17; η^2=0.07) (Fig. 22B).

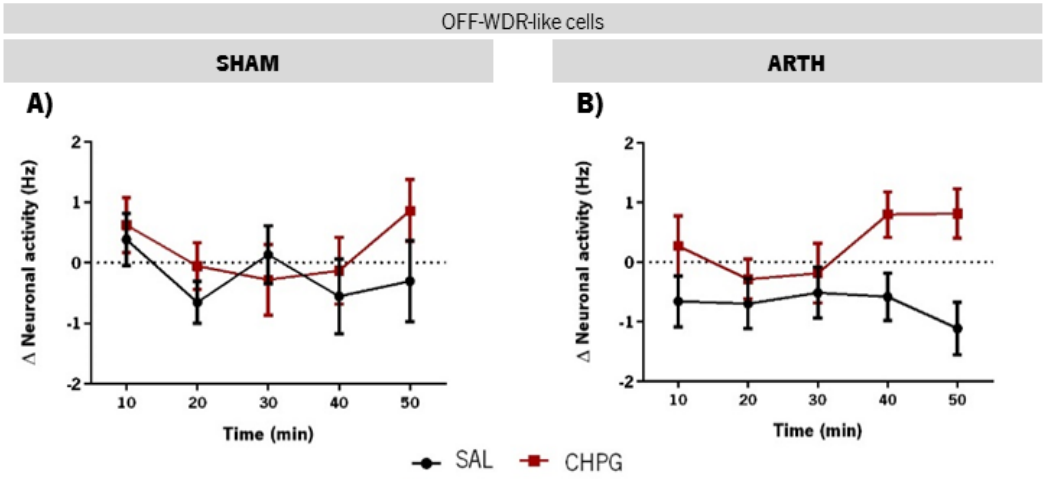


Figure 22. Noxious-evoked activity of RVM OFF-WDR-like cells in control (SHAM) and monoarthritic (ARTH) animals after the administration of saline or CHPG in the Infralimbic cortex (IL). A) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-WDR-like cells in SHAM animals. B) Effect of the administration of saline and CHPG in the IL upon the spontaneous activity of RVM OFF-WDR-like cells in ARTH animals. NS – nociceptive specific; WDR – wide-dynamic range; CHPG – 2-chloro-5-hydroxyphenylglycine; SAL – saline; Δ - (activity at a specific time point minus baseline activity of the cell). Data is presented as mean ± SEM.

Similarly, in ARTH animals no changes in the evoked activity of RVM OFF-WDR-like cells was observed (ANOVA repeated measures – interaction between drug and time: $F_{(1,11)}$=0.668; P=0.43; η^2=0.06). The analysis of changes in cell activity showed no effect after drug administration (ANOVA repeated measures – main factor drug: $F_{(1,11)}$=3.578; P=0.09; η^2=0.25). In contrast, analysis of effect throughout time showed significant differences (ANOVA repeated measures – main factor time: $F_{(4,44)}$=6.008; P=0.001; η^2=0.353) (Fig. 22B). *Post-hoc* analyzes did not show specific differences between time points (table IV).

CHAPTER 5

DISCUSSION

5. DISCUSSION

Our work showed for the first time, astrocytic IL/mGluR5 are involved in behavioural facilitation of nociception in animals with monoarthritis but not in healthy controls. Unexpectedly, RVM ON- and OFF-like cells do not mediate IL-mGluR5 descending facilitation of nociception although a potential role of RVM NEUTRAL-like cells could not be excluded.

5.1. Technical considerations

5.1.1. Animal model

In this study, the rat as an animal model was chosen to evaluate the role of astrocytes in the IL/mGluR5 pronociceptive effect and to evaluate if the RVM is a potential downstream spinal-projecting effector of IL/mGluR5-mediated pronociception in both control and arthritic animals. The main question was whether the model was the most suitable for the study. The rat model is frequently used to investigate human-related diseases and provides an opportunity to translate basic research findings to the clinics, as well in the field of chronic pain research[94] . Concerning this work, some studies have already demonstrated the existence of homologies between some brain regions of human and rats and, more specifically, PFC homologies in rat and primate brains[95]. Taking into account, all of these questions this animal model suitable in our study.

5.1.2. Experimental model of monoarthritis

In this work, the experimental model of monoarthritis used, to evaluate the IL/mGluR5 pronociceptive effect in healthy controls and in animals with chronic pain, was the kaolin/carrageenan (K/C) model[96]. The K/C model was previously defined as a model of acute monoarthritis (4h to 1 week), where injection of carrageenan induces an inflammatory reaction that is complemented by mechanical damage to intra-joint structures caused by the injection of kaolin. However, this model can also be used to study the effects of chronic monoarthritis, as it was also shown to cause mechanical hyperalgesia that lasts for at least 8 weeks, as well as depressive-like comorbid behaviour 4 weeks after induction[85,90].

5.1.3. Anesthesia

A mixture of ketamine and medetomidine was used in short duration procedures such as monoarthritis induction, cannula implantation and ablation of astrocytes. Medetomidine is a potent and selective agonist of α-2adrenergeic receptors usually used as an analgesic in animals. The combination of this drug with ketamine, an antagonist of NMDA receptors, is frequently used to anesthetize animals during surgical procedures[97]. The biggest advantage is that after the surgical procedures, the sedative effect can be reversed with the administration of atipamezole, an antagonist of α-2adrenergeic receptors. Additionally, this mixture does not have a significant effect in the respiratory rate of the animals[97].

Pentobarbitone was used in the electrophysiological studies. This anesthetic acts upon pathways dependent on ligand-gated ion channels. While pentobarbitone can lead to cardiorespiratory deficits[98], this side effect that can be easily managed through the administration of low doses and regular monitorization. Another disadvantage of this drug is the need to periodically reinforce the anaesthesia, which *per se* could influence the firing activity of the recorded cells. Performing control injections and assessing baseline cell activity were, therefore, important to exclude the potential effects of the anesthesia from those resulting from drug injection and experimental monoarthritis.

5.1.4. Behavioral assessment

The assessment of nociceptive behaviour in animals is a difficult task. As no tools to directly quantify the spontaneous nociception exist, in this work we measured only responses to acute painful stimulation[99].

The test chosen to evaluate nociception was the Hargreaves test where noxious heat-evoked paw-withdrawal latencies are quantified. One important advantage of using this test is the fact that animals not being restrained by the experimenter which in addition to habituation to the apparatus and the testing room allows to maintain stress levels to a minimum. Interestingly, baseline paw-withdrawal latencies of SHAM and ARTH animals were similar. This result can be explained by the fact noxious stimuli were not applied directly to the affected area, the knee joint, but rather to the tail. Nonetheless, the Hargreaves test allows to assess the effect of experimental monoarthritis upon synaptic remodeling of descending nociceptive pathways. As an alternative, the pressure application measurement (PAM)[100] could have been used. The PAM test allows to evaluate mechanical hypersensitivity in rodent joints by

measuring the force necessary to evoke a nociceptive response. However, in opposition to the Hargreaves test, the PAM test requires the animals to be restrained during the application of the stimulus, a procedure that could, by itself, bias the results. Additionally, the duration and frequency of the stimulation protocol could also bias to our observations as in our pharmacological studies the animals were placed in the apparatus for long periods of time (>50min) and stimulated twice every 10min. The continued application of a noxious mechanical stimulus could damage and sensitize the tested area, while the application of radiant heat does not have this effect, as verified through the evaluation of control injections.

5.1.5. Drugs

5.1.5.1. L-α-aminoadipate

Astrocytic function can be impaired by the administration of several types of drugs, such as gliotoxin L-α-aminoadipate (LαAA) and fluorocitrate. In this work, LαAA was administered intracerebrally to disrupt astrocytic function in the IL to allow the evaluation of the role these cells in IL/mGluR5-mediated pronociceptive effect. LαAA is a natural product of lysine metabolism in the brain and has been described as a potent and selective toxin for cultured astrocytes as it inhibits Na^+-independent and -dependent glutamate transport. LαAA is a structural analogous of glutamine that blocks glutamine synthetase and prevents the uptake of synaptic vesicles, leading to abnormal changes in nuclear morphology followed by progressive swelling of the cell body and membrane blebbing, that ultimately leads to lysis of astrocytes[101,102,103,104].

Several studies have already shown the efficacy of LαAA in ablating astrocytes. This toxin is selective for astrocytes and does not seem to directly affect the morphology and density of neurons[88,105]. Other cells of the CNS, such as oligodendroglia, microglia and endothelial cells are also not affect by low doses of this toxin[105,106]; however, an inappropriate concentration of any glial inhibitor can also affect other cell types[88,107,108]. Taking previous studies into account, the concentration (25μL/mg) and volume (2μL) injected in our study were suitable for the IL.

Importantly, disruption of the homeostasis guaranteed by astrocytes[75] can indirectly influence the function of other cell types. In fact, previous studies have demonstrated that neuronal morphology can be affected as a consequence of astrocytic ablation[108]. Hence, all our pharmacological evaluations were performed within a 7 days post astrocyte ablation time frame.

5.1.5.2. Pharmacological manipulation of mGluR5

In this work two different drugs were used to activate/inhibit IL mGluR5, CHPG, a specific agonist, and MTEP, a specific antagonist of these receptors. These drugs were reported to have high affinity for mGluR5[66,109]. CHPG and DHPG are both key agonist to mGluR5, but only CHPG is specific to type-5 of mGluRs, DHPG can also interact with type-1[66]. In prior studies we also used MPEP, another antagonist of mGluR5, however, some studies showed MTEP has a greater selectivity and less non-specific effects for other mGluRs[110]. MTEP was reported for maintaing >75% mGluR5 occupancy for 2 h, in the rat brain[110].

5.1.6. Electrophysiological study

Single-cell extracellular recordings were performed in the RVM to evaluate the possible involvement of its nociceptive cells in IL/mGluR5-mediated pronociception. This technique allows to measure the discharge rate of single cells in specific brain areas *in vivo*[86]. Alternatively, we could have performed whole-field recordings, however, single-cell recordings have the advantage of allowing to distinguish between the specific activity of different cell types (RVM ON-, OFF- and NEUTRAL-like cells) instead of whole areas.

Cell type categorization was based on their specificity to response only to a noxious stimulation (NS) or also to innocuous stimulation (WDR), these two types of cells are also subdivided based on changes in cell discharge rate (10% more or less in relation to spontaneous discharge rate[86]) after innocuous and noxious peripheral stimulation. One of the disadvantages of this method of classification is that in cells with a low spontaneous firing rates, a very small change is enough to classify the cell as responding to stimulation. For this reason, we considered a minimum variation of 0.45Hz to classify a cell as responding. Additionally, traditional RVM cell classification is based on an increase or decrease of basal discharge rate associated to a motor withdrawal reflex of the stimulated limb (in this case, the tail). In our study, however, the use of pentobarbitone does not allow animals to maintain the motor reflex. For this reason, we classified our cells as ON- and OFF-like based on changes in discharge rate alone.

5.2. Influence of mGluR5/IL upon nociceptive behaviour

5.2.1. mGluR5/IL-mediated nociceptive behaviour

Our results show mGluR5 in the IL seem to play an important role in the descending modulation of

nociception with the activation of this pathway leading to behavioural hyperalgesia. The administration of CHPG, an agonist of these receptors, increased pain sensitivity 20 to 30 minutes after the receptor activation in both healthy and monoarthritic animals, an effect in accordance with previous studies[51,73]. In fact, mGluR5 were previously described to play an important role in chronic pain[51], as the activation of these receptors can modulate descending nociception[69]. Ji and Neugebauer[111], showed mGluR5 was inhibited in prolonged arthritis which lead o decreased mPFC basal activity, an effect reversed through the administration of mGluR5 agonists.

Conversely, the microinjection of MTEP in the IL reduced behavioural hyperalgesia, an effect also in accordance with the literature. Some studies showed systemic administration of MTEP and MPEP decreased hyperalgesia and mechanical allodynia after acute inflammation (formalin) in mice and spinal nerve ligation in rats, respectively[112].

Overall, our results indicate mGluR5 might be tonically active in chronic inflammatory conditions. In line with our hypothesis, Nicholson and Winkelstein[113] reported the levels of mGluR5 in the spinal cord are increased after peripheral injury. However, further studies are needed to not only understand the physiological/funtional impact of greater mGluR5 availability.

5.2.2. Impact of astrocytes upon IL/mGluR5 facilitation of nociception

In our study, we showed ablating astrocytic function does not alter mGluR5/IL-mediated pronociception. Yet, the analgesic effect of MTEP in the IL of ARTH animals was lost in animals with impaired astrocytic function, indicating chronic inflammatory pain can lead to tonic activation or increased expression of mGluR5 in astrocytes. Interestingly, there is evidence of the importance of astrocytes and glial cells in the development and maintenance of chronic pain conditions[77]. Immediately after peripheral damage, microglia is transiently activated, leading to the release of pro-inflammatory factors[79,114], that, in turn, activate astrocytes[79,114,115]. In line with this hypothesis, increased levels of astrocyte marker GFAP have been observed in the spinal cord of rats after peripheral nerve damage[113] and in a model of peripheral inflammation injury[116].

Astrocytes are important partners of neurons in the regulation of synaptic transmission[75]. Their role in regulating synaptic transmission and neuronal excitability has been highly studied. Astrocytes detect and regulate the release of some neurotransmitter, such as glutamate, in the synaptic cleft through a variety of glial receptors[75]. Of these receptors, mGluR5 is important in the detection and regulation of glutamatergic transmission[117].

The activation of glial mGluR5 can regulate several neuronal and synaptic mechanisms and, in fact,

astrocytic mGluR5 are involved in the detection and regulation of synaptic signals[118]. Cell culture studies using healthy and mGluR5 knockout animals showed the activation of mGluR5 can significantly reduce microglial activation and promote anti-inflammatory effects[82]. In a model of bone cancer with increased spinal astrocyte activation, intrathecal delivery of mGluR5 antagonist MTEP decreased mechanical hypersensitivity[84].

Most studies on the role of astrocytes in chronic pain focus on events occurring at the spinal cord level[79,84,116,119]. However, a study in the RVM showed an identical progression of events as seen in the spinal cord where microglial activation is followed by astrocytic activation in a model of neuropathic pain[83]. Additionally, in a recent study Kim and colleagues reported neuropathic pain leads to astrocyte activation and to a reemergence of immature mGluR5 spines in the somatosensory cortex I[120]. Remarkably, these molecular alterations were correlated to the development of mechanical allodynia, a behaviour blocked by suppression of the astrocytic mGluR5-signaling pathway. These findings support our hypothesis and provide a possible mechanism through which the administration of MTEP is only effective in animals with chronic pain and intact astrocytic function. Furthermore, they provide a clue as to possible targets for future chronic pain therapies.

5.2.3. Influence of mGluR5 activation in the IL upon RVM cell activity

In this study, we evaluated whether the RVM was mediating IL/mGluR5-induced facilitation of nociception, taking into account, in rats, the IL directly projects to the RVM[57] and this area is considered the main effector of supraspinal descending modulatory pain drives. Importantly, the RVM is involved in the nociceptive deregulation of chronic pain disorders through changes in the balance between the activity of RVM pronociceptive ON- and antinociceptive OFF-cells[121].

Our results show ON- and OFF-like cell activity was not altered after mGluR5 activation in the IL suggesting this nucleus does not play a critical role in the descending modulation of nociception after IL/mGluR5 activation. However, this hypothesis cannot be completely excluded, since the number of OFF-like cells recorded was low.

Additionally, recent studies point to a possible role of RVM NEUTRAL-cells in nociceptive modulation despite their lack of response to acute peripheral noxious stimulation[122,123]. In fact, a portion of NEUTRAL-cells express 5-HT receptors[124], a pathway known to be involved in facilitatory pain mechanisms (especially through spinal 5-HT_3 receptors)[122]. Interestingly, this pathway connects brain regions important in fear, aversion, anxiety and depression, all common comorbidities of chronic pain[83].

Another hypothesis is that NEUTRAL RVM cells are a subtype of ON- or OFF-cells[123]. Ellrich and

colleagues analyzed the responses of cells classified as NEUTRAL to noxious heating of the tail and have found they respond to stimulation in other body parts, such as the ears or nose, or to other types of stimulation, such as pinching of the tail and the hind and fore paws[123]. Hence, it is possible a cell we classified as NEUTRAL might respond to other modalities of stimuli.

Nevertheless, the IL has projects to other brain areas that are described to play an important role in nociceptive processing and modulation, such as the anterior cingulate cortex, amygdala, hypothalamus, periaqueductal gray and the medullary dorsal reticular nucleus (DRt)[53,57,125]. The latter has been thoroughly implicated in the descending facilitation of nociception in acute and chronic pain models[126]. In fact, the DRt was identified as a downstream mediator of pronociception of the anterior cingulate cortex, another subregion of the mPFC[42]. Additionally, reciprocate projections from the IL to the DRt have already been reported[21], making it an interesting target to study in future studies.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Our results strongly suggest the expression of mGluR5 is altered in experimental monoarthritis. Nonetheless, our findings raise several questions concerning the mechanisms underlying behavioural differences between arthritic animals with or without astrocyte ablation. Future studies should include quantitative real time polymerase chain reaction (rt-PCR) of mGluR5 and of the astrocytic activation marker, GFAP, in the IL of healthy and monoarthritic animals to assess the impact of experimental monoarthritis on their mRNA expression. This technique could be coupled to immunofluorescence to confirm protein expression. Additionally, to confirm the role of astrocytes in mGluR5/IL pronociceptive effect, an optogenetic approach could also be performed to transiently silence these cells in IL, in order to exclude possible side effects of L-α-aminoadipate injection upon other cell types, such as neurons or microglia.

As our evaluation of the role of the RVM as the potential downstream supraspinal effector of IL/mGluR5-mediated pronociception was not conclusive, future studies should aim at increasing the number of RVM OFF-like cells as well as evaluating the activity of NEUTRAL-like cells.

Finally, a broader approach can be adopted to search for other potential downstream mediators of IL/mGluR5-induced pronociception by analyzing differences in c-Fos expression in brainstem areas between control and monoarthritic animals.

CHAPTER 7

REFERENCES

7. REFERENCES

1. Merksey, H. & Bogduk, N. *Classification of chronic pain*. (IASP Press, 1994).
2. Apkarian, A. V., Baliki, M. N. & Geha, P. Y. Towards a theory of chronic pain. *Prog. Neurobiol.* **87,** 81–97 (2009).
3. Attal, N. *et al.* Does cognitive functioning predict chronic pain? Results from a prospective surgical cohort. *Brain* **137,** 904–917 (2014).
4. Moriarty, O., McGuire, B. E. & Finn, D. P. The effect of pain on cognitive function: A review of clinical and preclinical research. *Prog. Neurobiol.* **93,** 385–404 (2011).
5. Hunter, D. J. Osteoarthritis. *Best Pract. Res. Clin. Rheumatol.* **25,** 801–814 (2011).
6. Monjardino, T., Lucas, R. & Barros, H. Frecuency of Rheumatic diseases in Portugal: a sistematic review. *Acta Reum. Port* **36,** 336–363 (2011).
7. Adães, S. *et al.* Intra-articular injection of collagenase in the knee of rats as an alternative model to study nociception associated with osteoarthritis. *Arthritis Res. Ther.* **16,** R10 (2014).
8. Buckwalter, J. A. & Martin, J. A. Osteoarthritis. *Adv. Drug Deliv. Rev.* **58,** 150–167 (2006).
9. Farmer, M. A., Baliki, M. N. & Apkarian, A. V. A dynamic network perspective of chronic pain. *Neurosci. Lett.* **520,** 197–203 (2012).
10. Lewin, G. R. & Moshourab, R. Mechanosensation and pain. *J. Neurobiol.* **61,** 30–44 (2004).
11. Dubin, A. E. & Patapoutian, A. Nociceptors: The sensors of the pain pathway. *J. Clin. Invest.* **120,** 3760–3772 (2010).
12. Millan, M. J. The Induction of Pain: an Integrative Review. *Prog. Neurobiol.* **57,** 1–164 (1999).
13. Devor, M. Ectopic discharge in Abeta afferents as a source of neuropathic pain. *Exp. Brain Res.* **196,** 115–128 (2009).
14. Craig, A. D. Pain Mechanisms: Labeled Lines Versus Convergence in Central Processing. *Annu. Rev. Neurosci.* **26,** 1–30 (2003).
15. Han, Z.-S., Zhang, E.-T. & Craig, A. D. Nociceptive and thermoreceptive lamina I neurons are anatomically distinct. *Nat. Neurosci.* **1,** 218–225 (1998).
16. D'Mello, R. & Dickenson, A. H. Spinal cord mechanisms of pain. *Br. J. Anaesth.* **101,** 8–16 (2008).
17. Melzack, R. From the gate to the neuromatrix. *Pain Suppl.* **6,** 121–126 (1999).
18. Sikandar, S. & Dickenson, A. H. Europe PMC Funders Group Visceral Pain – the Ins and Outs , the Ups and Downs. **6,** 17–26 (2012).
19. Treede, R.-D., Kenshalo, D. R., Gracely, R. H. & Jones, A. K. P. The cortical representation of pain. *Pain* **79,** 105–111 (1999).
20. Willis, W. D. Nociceptive patways: anatomy and physiology of nociceptive ascending pathways. *Phil. Trans. R. Soc. L.* **308,** 253–268 (1985).
21. Lima, D. & Almeida, A. The medullary dorsal reticular nucleus as a pronociceptive centre of the pain control system. *Prog. Neurobiol.* **66,** 81–108 (2002).

22. Mayer, E. A. *et al.* Differences in brain responses to visceral pain between patients with irritable bowel syndrome and ulcerative colitis. *Pain* **115,** 398–409 (2005).

23. Pinto-Ribeiro, F., Ansah, O. B., Almeida, A. & Pertovaara, A. Response properties of nociceptive neurons in the caudal ventrolateral medulla (CVLM) in monoarthritic and healthy control rats: Modulation of responses by the paraventricular nucleus of the hypothalamus (PVN). *Brain Res. Bull.* **86,** 82–90 (2011).

24. Pinto-Ribeiro, F., Ansah, O. B., Almeida, A. & Pertovaara, A. Influence of arthritis on descending modulation of nociception from the paraventricular nucleus of the hypothalamus. *Brain Res.* **1197,** 63–75 (2008).

25. Price, D. D. Psychological and neural mechanisms of the affective dimension of pain. *Science* **288,** 1769–1772 (2000).

26. Pertovaara, A. & Almeida, A. in *Handbook of clinical neurology* **81,** 179–92 (2006).

27. Heinricher, M. M., Tavares, I., Leith, J. L. & Lumb, B. M. Descending control of nociception: Specificity, recruitment and plasticity. *Brain Res. Rev.* **60,** 214–25 (2009).

28. Gebhart, G. F. Descending modulation of pain. *Neurosci. Biobehav. Rev.* **27,** 729–37 (2004).

29. Millan, M. J. Descending control of pain. *Prog. Neurobiol.* **66,** 355–474 (2002).

30. Fields, H. L. & Heinricher, M. M. Anatomy and physiology of a nociceptive modulatory system. *Phil. Trans. R. Soc. L.* **308,** 361–374 (1985).

31. Apkarian, A. V., Bushnell, M. C., Treede, R. D. & Zubieta, J. K. Human brain mechanisms of pain perception and regulation in health and disease. *Eur. J. Pain* **9,** 463–484 (2005).

32. Brooks, J. & Tracey, I. From nociception to pain perception: Imaging the spinal and supraspinal pathways. *J. Anat.* **207,** 19–33 (2005).

33. Tracey, I. & Mantyh, P. W. The Cerebral Signature for Pain Perception and Its Modulation. *Neuron* **55,** 377–391 (2007).

34. Porro, C. A. *et al.* Does anticipation of pain affect cortical nociceptive systems? *J. Neurosci.* **22,** 3206–3214 (2002).

35. Zhang, R., Tomida, M., Katayama, Y. & Kawakami, Y. Response durations encode nociceptive stimulus intensity in the rat medial prefrontal cortex. *Neuroscience* **125,** 777–785 (2004).

36. Tanaka, E. *et al.* A transition from unimodal to multimodal activations in four sensory modalities in humans: an electrophysiological study. *BMC Neurosci.* **9,** 116 (2008).

37. Apkarian, A. V., Grachev, I. D., Krauss, B. R. & Szeverenyi, N. M. Imaging Brain Pathophysiology of Chronic CRPS Pain. *Complex Reg. Pain Syndr.* **22,** (2001).

38. Apkarian, A. V. *et al.* Chronic pain patients are impaired on an emotional decision-making task. *Pain* **108,** 129–136 (2004).

39. Apkarian, A. V. *et al.* Chronic Back Pain Is Associated with Decreased Prefrontal and Thalamic Gray Matter Density. *Group* **24,** 10410 –10415 (2004).

40. Matre, D. A., Hernandez-Garcia, L., Tran, T. D. & Casey, K. L. 'First pain' in humans: convergent and specific forebrain responses. *Mol. Pain* **6,** 81 (2010).

41. Fuchs, P. N., Peng, Y. B., Boyette-Davis, J. A. & Uhelski, M. L. The anterior cingulate cortex and pain processing. *Front. Integr. Neurosci.* **8,** 35 (2014).

42. Zhang, L., Zhang, Y. & Zhao, Z.-Q. Anterior cingulate cortex contributes to the descending facilitatory modulation of pain via dorsal reticular nucleus. *Eur. J. Neurosci.* **22,** 1141–8 (2005).

43. Boccard, S. G. J. *et al.* Targeting the affective component of chronic pain: a case series of deep brain stimulation of the anterior cingulate cortex. *Neurosurgery* **74,** 628–35; discussion 635–7 (2014).

44. Medalla, M. & Barbas, H. Synapses with inhibitory neurons differentiate anterior cingulate from dorsolateral prefrontal pathways associated with cognitive control. *Neuron* **61,** 609–620 (2009).

45. Lorenz, J., Minoshima, S. & Casey, K. L. Keeping pain out of mind: The role of the dorsolateral prefrontal cortex in pain modulation. *Brain* **126,** 1079–1091 (2003).

46. Starr, C. J. *et al.* Roles of the Insular Cortex in the Modulation of Pain: Insights from Brain Lesions. **29,** 2684–2694 (2009).

47. Craig, A. D., Chen, K., Bandy, D. & Reiman, E. M. Thermosensory activation of insular cortex. *Nat. Neurosci.* **3,** 184–190 (2000).

48. Teffer, K. & Semendeferi, K. *Human prefrontal cortex. Evolution, development, and pathology. Progress in Brain Research* **195,** (Elsevier B.V., 2012).

49. Heidbreder, C. A. & Groenewegen, H. J. The medial prefrontal cortex in the rat: Evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. *Neurosci. Biobehav. Rev.* **27,** 555–579 (2003).

50. Wise, S. P. Forward frontal fields: phylogeny and fundamental function. *Trends Neurosci.* **31,** 599–608 (2008).

51. David-Pereira, A. *et al.* Metabotropic glutamate 5 receptor in the infralimbic cortex contributes to descending pain facilitation in healthy and arthritic animals. *Neuroscience* **312,** 108–119 (2016).

52. Jiang, Z. C. *et al.* Inactivation of the prelimbic rather than infralimbic cortex impairs acquisition and expression of formalin-induced conditioned place avoidance. *Neurosci. Lett.* **569,** 89–93 (2014).

53. Sesack, S. R., Deutch, A. Y., Roth, R. H. & Bunney, B. S. Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: An anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *J. Comp. Neurol.* **290,** 213–242 (1989).

54. Ji, G. & Neugebauer, V. Modulation of medial prefrontal cortical activity using in vivo recordings and optogenetics. *Mol. Brain* **5,** 36 (2012).

55. Pandya, D. N. & Yeterian, E. H. Prefrontal cortex in relation to other cortical areas in rhesus monkey: architecture and connections. *Prog. Brain Res.* **85,** 63–94 (1990).

56. Krettek, J. E. & Price, J. L. The cortical projections of the mediodorsal nucleus and adjacent thalamic nuclei in the rat. *J. Comp. Neurol.* **171,** 157–191 (1977).

57. Vertes, R. P. Differential Projections of the Infralimbic and Prelimbic Cortex in the Rat. *Synapse* **51,** 32–58 (2004).

58. Hurley, K. M., Herbert, H., Moga, M. M. & Saper, C. B. Efferent projections of the infralimbic cortex of the rat. *J. Comp. Neurol.* **308,** 249–276 (1991).

59. Herry, C. *et al.* Neuronal circuits of fear extinction. *Eur. J. Neurosci.* **31,** 599–612 (2010).

60. Barker, J. M., Taylor, J. R. & Chandler, L. J. A unifying model of the role of the infralimbic cortex in extinction and habits. *Learn. Mem.* **21,** 441–448 (2014).

61. Sotres-bayon, F. & Quirk, G. J. Prefrontal control of fear: more than just extinction. *Curr. Opin. Neurobiol.* **20,** 231–235 (2011).

62. Bleakman, D., Alt, A. & Nisenbaum, E. S. Glutamate receptors and pain. *Semin. Cell Dev. Biol.* **17,** 592–604 (2006).

63. Danbolt, N. C. Glutamate uptake. *Prog. Neurobiol.* **65,** 1–105 (2001).

64. Chiechio, S. & Nicoletti, F. Metabotropic glutamate receptors and the control of chronic pain. *Curr. Opin. Pharmacol.* **12,** 28–34 (2012).

65. Sun, H. & Neugebauer, V. mGluR1, but not mGluR5, activates feed-forward inhibition in the medial prefrontal cortex to impair decision making. *J. Neurophysiol.* **106,** 960–973 (2011).

66. Neugebauer, V. Metabotropic glutamate receptors - important modulators of nociception and pain behavior. *Pain* **98,** 1–8 (2002).

67. Latremoliere, A. & Woolf, C. J. Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity. *J. Pain* **10,** 895–926 (2009).

68. Woolf, C. J. Evidence for a central component of post-injury pain hypersensitivity. *Nature* **306,** 686–688 (1983).

69. Montana Michael C. & Gereau IV Robert W. Metabotropic glutamate receptors as targets for analgesia: antagonism, activation, and allosteric modulation. *Curr Pharm Biotechnol* **12,** 1681–1688 (2011).

70. Bhave, G., Karim, F., Carlton, S. M. & Iv, R. W. G. Peripheral group I metabotropic glutamate receptors modulate nociception in mice. *Group* 417–423 (2001).

71. Swanson, C. J. *et al.* Metabotropic glutamate receptors as novel targets for anxiety and stress disorders. *Nat.Rev.Drug Discov.* **4,** 131–144 (2005).

72. Blackshaw, L. A., Page, A. J. & Young, R. L. Metabotropic glutamate receptors as novel therapeutic targets on visceral sensory pathways. *Front. Neurosci.* **5,** 40 (2011).

73. Walker, K. *et al.* mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurones mediate inflammatory hyperalgesia. *Neuropharmacology* **40,** 10–19 (2000).

74. Fontanez-Nuin, D. E., Santini, E., Quirk, G. J. & Porter, J. T. Memory for fear extinction requires mGluR5-mediated activation of infralimbic neurons. *Cereb. Cortex* **21,** 727–735 (2011).

75. Wang, D. D. & Bordey, A. The astrocyte odyssey. *Prog. Neurobiol.* **86,** 342–67 (2008).

76. Halassa, M. M., Fellin, T. & Haydon, P. G. Tripartite synapses: Roles for astrocytic purines in the control of synaptic physiology and behavior. *Neuropharmacology* **57,** 343–346 (2009).

77. Watkins, L. R. & Maier, S. F. Glia: a novel drug discovery target for clinical pain. *Nat. Rev. Drug Discov.* **2,** 973–985 (2003).

78. Hansen, R. R. & Malcangio, M. Astrocytes - Multitaskers in chronic pain. *Eur. J. Pharmacol.* **716,** 120–128 (2013).

79. Old, E. A., Clark, A. K. & Marzia Malcangio. in *Pain Control, Handbook of Experimental Pharmacology* **227,** 145–170 (2015).

80. Marchand, F., Perretti, M. & McMahon, S. B. Role of the immune system in chronic pain. *Nat Rev Neurosci* **6,** 521–532 (2005).

81. Gao, Y.-J. & Ji, R.-R. Targeting Astrocyte Signalin for Chronic Pain. *October* **7,** 482–493 (2010).

82. Byrnes, K. R. *et al.* mGluR5 activation inhibits microglial associated inflammation and neurotoxicity. *Glia* **57,** 550–560 (2009).

83. Wei, F., Guo, W., Zou, S., Ren, K. & Dubner, R. Supraspinal Glial-Neuronal Interactions Contribute to Descending Pain Facilitation. *J Neurosci* **28,** 10482–10495 (2008).

84. Ren, B. *et al.* Intrathecal Injection of Metabotropic Glutamate Receptor Subtype 3 and 5 Agonist / Antagonist Attenuates Bone Cancer Pain by Inhibition of Spinal Astrocyte Activation. *Anesthesiology* **116,** 122–132 (2012).

85. Amorim, D. *et al.* A Role of Supraspinal Galanin in Behavioural Hyperalgesia in the Rat. **9,** 1–14 (2014).

86. Pinto-Ribeiro, F. *et al.* Pronociception from the dorsomedial nucleus of the hypothalamus is mediated by the rostral ventromedial medulla in healthy controls but is absent in arthritic animals. *Brain Res. Bull.* **99,** 100–8 (2013).

87. Baumans, V., Bertens, A., Hackbarth, H. & Timmermans, A. *Principles of Laboratory Animal Science*. (Elsevier, 2001).

88. Khurgel, M., Koo, A. C. & Ivy, G. O. Selective ablation of astrocytes by intracerebral injections of alpha-aminoadipate. *Glia* **16,** 351–358 (1996).

89. Ansah, O. B. & Pertovaara, A. Peripheral suppression of arthritic pain by intraarticular fadolmidine, an alpha2-adrenoceptor agonist, in the rat. *Anesth. Analg.* **105,** 245–250 (2007).

90. Radhakrishnana, R., Moore, S. A. & Sluka, K. A. Unilateral carrageenan injection into muscle or joint induces chronic bilateral hyperalgesia in rats. *Changes* **29,** 997–1003 (2012).

91. Paxinos, G. & Watson, C. *The Rat Brain: In Stereotaxic Coordinates: Paxinos and Watson*. **6,** (1986).

92. Domin, H., Szewczyk, B., Wo, M., Wawrzak-wleciał, A. & Smiałowska, M. Antidepressant-like effect of the mGluR5 antagonist MTEP in an astroglial degeneration model of depression. *Behav. Brain Res.* **273,** 23–33 (2014).

93. Hargreaves, K. *et al.* A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32,** 11 (1988).

94. Bendele, A. M. Animal models of osteoarthritis. *Rheumatol. Sixth Ed.* **1,** 363–376 (2001).

95. Wallis, J. D. Cross-species studies of orbitofrontal cortex and value-based decision-making. *Nat. Neurosci.* **15,** 13–9 (2012).

96. Teeple, E., Jay, G. D., Elsaid, K. a & Fleming, B. C. Animal models of osteoarthritis: challenges of model selection and analysis. *AAPS J.* **15,** 438–46 (2013).

97. Jang, H. S., Choi, H. S., Lee, S. H., Jang, K. H. & Lee, M. G. Evaluation of t e anaesthetic effects of medetomidine and ketamine in rats and their reversal with atipamezole. *Vet. Anaesth. Analg.* **36,** 319–327 (2009).

98. Field, K. J., White, W. J. & Lang, C. M. Anaesthetic effects of chloral hydrate, pentobarbitone and urethane in adult male rats. *Lab. Anim.* **27,** 258–269 (1993).

99. Sandkühler, J. Models and mechanisms of hyperalgesia and allodynia. *Physiol. Rev.* **89,** 707–58 (2009).

100. Barton, N. J. *et al.* Pressure application measurement (PAM): A novel behavioural technique for measuring hypersensitivity in a rat model of joint pain. *J. Neurosci. Methods* **163,** 67–75 (2007).

101. Bridges, R. J. *et al.* Gliotoxic actions of excitatory amino acids. *Neuropharmacology* **31,** 899–907 (1992).

102. Mcbean, G. J. Inhibition of the glutamate transporter and glial enzymes in rat striatum by the gliotoxin , oxaminoadipate. 536–540 (1994).

103. Guidetti, P. & Schwarcz, R. Determination of α-aminoadipic acid in brain, peripheral tissues, and body fluids using GC/MS with negative chemical ionization. *Mol. Brain Res.* **118,** 132–139 (2003).

104. Pow, D. V. Visualising the activity of the cystine-glutamate antiporter in glial cells using antibodies to aminoadipic acid, a selectively transported substrate. *Glia* **34,** 27–38 (2001).

105. Takada, M. & Hattori, T. Fine structural changes in the rat brain after local injections of gliotoxin, alpha-aminoadipic acid. *Histol. Histopathol.* **1,** 271–275 (1986).

106. Tsai, M. J., Chang, Y. F., Schwarcz, R. & Brookes, N. Characterization of L-alpha-aminoadipic acid transport in cultured rat astrocytes. *Brain Res.* **741,** 166–173 (1996).

107. Hassel, B., Paulsen, R. E., Johnsen, a & Fonnum, F. Selective inhibition of glial cell metabolism in vivo by fluorocitrate. *Brain Res.* **576,** 120–124 (1992).

108. Lima, A. *et al.* Astrocyte pathology in the prefrontal cortex impairs the cognitive function of rats. *Mol. Psychiatry* **19,** 834–41 (2014).

109. Schoepp, D. D., Jane, D. E. & Monn, J. a. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology* **38,** 1431–1476 (1999).

110. Lea IV, P. M. & Faden, A. I. Metabotropic glutamate receptor subtype 5 antagonists MPEP and MTEP. *CNS Drug Rev.* **12,** 149–166 (2006).

111. Ji, G. & Neugebauer, V. CB1 augments mGluR5 function in medial prefrontal cortical neurons to inhibit amygdala hyperactivity in an arthritis pain model. *Eur. J. Neurosci.* **39,** 455–466 (2014).

112. Varty, G. B. *et al.* The antinociceptive and anxiolytic-like effects of the metabotropic glutamate receptor 5 (mGluR5) antagonists, MPEP and MTEP, and the mGluR1 antagonist, LY456236, in rodents: A comparison of efficacy and side-effect profiles. *Psychopharmacology (Berl).* **179,** 207–217 (2005).

113. Nicholson, K. J., Guarino, B. B. & Winkelstein, B. a. Transient nerve root compression load and duration differentially mediate behavioral sensitivity and associated spinal astrocyte activation and mGLuR5 expression. *Neuroscience* **209,** 187–195 (2012).

114. Milligan, E. D. & Watkins, L. R. Pathological and protective roles of glia in chronic pain. *Nat. Rev. Neurosci.* **10,** 23–36 (2009).

115. O'Callaghan, J. P. & Miller, D. B. Spinal glia and chronic pain. *Metabolism.* **59,** S21–S26 (2010).

116. Sweitzer, S. M., Colburn, R. W., Rutkowski, M. & DeLeo, J. A. Acute peripheral inflammation induces moderate glial activation and spinal IL-1β expression that correlates with pain behavior in the rat. *Brain Res.* **829,** 209–221 (1999).

117. Panatier, A. *et al.* Astrocytes are endogenous regulators of basal transmission at central synapses. *Cell* **146,** 785–798 (2011).

118. Panatier, A. & Robitaille, R. Astrocytic mGluR5 and the tripartite synapse. *Neuroscience* **323,** 29–34 (2016).

119. Tenorio, G., Kulkarni, A. & Kerr, B. J. Resident glial cell activation in response to perispinal inflammation leads to acute changes in nociceptive sensitivity: Implications for the generation of neuropathic pain. *Pain* **154,** 71–81 (2013).

120. Kim, S. K. *et al.* Cortical astrocytes rewire somatosensory cortical circuits for peripheral neuropathic pain. *J. Clin. Invest.* **126,** 1983–97 (2016).

121. Heinricher, M. M., Tavares, I., Leith, J. L. & Lumb, B. M. Descending control of nociception: specificity, recruitment and plasticity. *Brain Res.* **60,** 214–225 (2009).

122. Suzuki, R., Rygh, L. J. & Dickenson, A. H. Bad news from the brain: Descending 5-HT pathways that control spinal pain processing. *Trends Pharmacol. Sci.* **25,** 613–617 (2004).

123. Ellrich, J., Ulucan, C. & Schnell, C. Are 'neutral cells' in the rostral ventro-medial medulla subtypes of on- and off-cells? *Neurosci. Res.* **38,** 419–423 (2000).

124. Ellrich, J., Ulucan, C. & Schnell, C. Is the response pattern of on- and off-cells in the rostral ventromedial medulla to noxious stimulation independent of stimulation site? *Exp. Brain Res.* **136,** 394–399 (2001).

125. Almeida, A., Tavares, I. & Lima, D. Reciprocal connections between the medullary dorsal reticular nucleus and the spinal dorsal horn in the rat. *Eur. J. pain* **4,** 373–87 (2000).

126. Dugast, C., Almeida, A. & Lima, D. The medullary dorsal reticular nucleus enhances the responsiveness of spinal nociceptive neurons to peripheral stimulation in the rat. *Eur. J. Neurosci.* **18,** 580–588 (2003).