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REGULATION OF THE ACTIVITY OF THE LOCUS COERULEUS-NORADRENERGIC SYSTEM IN CHRONIC PAIN

FOCUS ON INFLAMMATION AND MOOD DISORDERS

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"Você precisa saber que o coração da gente tem que ser muito grande e caber tudo que a gente gosta."

Meu pé de laranja lima - José Mauro de Vasconcelos.

Abbreviations

(p)ERK1/2	(phosphorylated) Extracellular signal-regulated kinases 1 and 2
A5 and A7	Noradrenergic clusters 5 and 7
ACC	Anterior cingulate cortex
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CRF	Corticotropin-releasing factor
DRG	Dorsal root ganglion
DRN	Dorsal raphe nucleus
EW	Edinger-Westphal nucleus
GABA	γ-aminobutyric acid
HPA	Hypothalamic–pituitary–adrenal axis
IASP	International Association for the Study of Pain
LC	Locus coeruleus
L-DOPA	Dihydroxyphenylalanine
MA	Monoarthritis
MAO	Monoamine oxydase
MAPK	Mitogen-activated protein kinase
NA	Noradrenaline
NSAID	Non-steroidal anti-inflammatory drug
NET	Noradrenaline transporter
PAG	Periaqueductal gray
PFC	Prefrontal cortex
PGi	Paragigantocellularis nucleus
PRH	Prepositus hippoglossi
RA	Rheumatoid arthritis
RVM	Rostral ventromedial medulla
TH	Tyrosine hydroxylase
WDR	Wide dynamic range neurons

1. Abstract/Resumo

1.1. English Version

Both neuropathic and inflammatory chronic pains are associated with the lack of successful treatments and, consequently, are highly detrimental for the patients' quality of life. Depression and anxiety disorders may emerge as a consequence of the long-term exposure to such painful conditions. This will not only have repercussions in the psychological state of the patient but will also potentiate the mechanisms underlying the negative effects of chronic pain. However, nowadays, such assumptions are still mainly hypothetical as the mechanisms underlying the association of pain with the development of mood disorders are still barely known. The Locus coeruleus-noradrenergic system is an important candidate orchestrating the neuronal circuitries lying behind chronic painful conditions, and also has an exceptional role in the regulation of anxiety and depression disorders.

In the first publication of this thesis (**Publication I**), we studied the activation pattern of extracellular signal-regulated kinases (pERK1/2, a marker of activation and plasticity) in the spinal cord and brainstem nuclei including several noradrenergic clusters like the A5, Locus coeruleus (LC) and A7, following seven days after chronic constriction injury (CCI) and/or after noxious mechanical stimulation. Associated with previous behavioral, electrophysiological and molecular data already existent in this pain model, these data led us to hypothesize that the noradrenergic system, and its main supplier nucleus, the LC, could be playing an important role mediated through ERK1/2 signaling. Moreover, the underlying mechanisms could be different for pain types of distinct origins. Thus, in **Publication II**, we also quantified the expression of pERK1/2 in the spinal cord and in the circuitry comprising the LC, the Paragigantocellularis nucleus (PGi, the main excitatory LC afferent), and the Prefrontal Cortex (PFC, the LC's main projection), but now in the monoarthritis (MA) model of chronic joint inflammation, using an early- and a late-phase of the disease development. On the contrary to the neuropathic pain model, where it was detected a decreased pERK1/2 expression in LC upon seven days of CCI, in **Publication II** we described a late-phase activation of ERK1/2 accompanied by the emergence of anxiety

and depressive behaviors. These changes were reversed by topical anti-inflammatory therapy in the affected hind paw, suggesting a cause-effect relation. Thus, in **Publication III**, we also studied the electrophysiological activity of LC neurons in the same MA model at prolonged time-points of the inflammatory condition. The results obtained led us to hypothesize that ERK1/2 increased activation was related with an increased sensory-evoked response of the LC neurons to noxious mechanical stimulation, as well as with pain-related anxiety. This was confirmed by the administration of an ERK1/2 inhibitor (SL327) in the LC of rats submitted to chronic inflammation, which resulted in the reversion of the anxiety-like behavior, reversion of the electrophysiological changes and normalization of the ERK1/2 activation levels in the PFC. Additionally, we also found significant increases in the expression of tyrosine hydroxylase enzyme (a rate-limiting enzyme in the synthesis of noradrenaline) in the LC, in prolonged MA conditions. Finally, as most of the electrophysiological and biochemical characteristics observed in chronic inflammatory conditions were indicative of a stress component in this model, we evaluated the hypothesis of corticotropin-releasing factor (CRF)-mediated neurotransmission, a neuropeptide deeply implicated in the regulation of stress, being a precursor event triggering ERK1/2 activation in the LC. Indeed, using a CRF antagonist, which blocks the activity of the endogenous CRF through CRF receptors, we observed the reversion of both the anxiety-like behavior and pERK1/2 levels in the LC of the animals with prolonged monoarthritis but no effects on the nociceptive behavior, strongly indicating that the CRF neuropeptide is as possible molecule triggering ERK1/2 activation in the LC in response to prolonged joint inflammatory conditions (**Publication IV**).

In conclusion, neuropathic and inflammatory pains have different patterns of ERK1/2 activation in the LC at early time-points of disease duration. Particularly in chronic inflammatory pain, we showed that increased ERK1/2 activation in the LC plays a role in the increased perception to noxious stimuli and it is related with the emergence of pain-related anxiety. The CRF neurotransmission may act as precursor in these ERK1/2-mediated events.

1.2. Portuguese Version

Tanto a dor neuropática como a dor crónica inflamatória estão associadas à falta de tratamentos bem-sucedidos e, consequentemente, são altamente prejudiciais à qualidade de vida dos doentes. A depressão e a ansiedade podem surgir como consequência da exposição, a longo-prazo, a essas condições dolorosas. Tal efeito não terá apenas repercussões no estado psicológico do doente mas também irá potenciar os mecanismos subjacentes aos efeitos negativos da dor crónica. No entanto, atualmente, tais suposições são hipotéticas pois os mecanismos subjacentes à associação da dor com o desenvolvimento de transtornos de humor são, ainda, pouco conhecidos. O sistema noradrenérgico-Locus coeruleus é um importante candidato a orquestrar os circuitos neuronais implicados nas condições dolorosas crónicas, tendo também um papel excecional na regulação da ansiedade e da depressão.

Na primeira publicação desta tese (**Publicação I**), nós estudámos o padrão de ativação das cinases reguladas por sinais extracelulares (pERK1/2, um marcador de atividade e plasticidade) na medula espinhal e em núcleos do tronco cerebral incluindo vários grupos noradrenérgicos como o A5, o Locus coeruleus (LC) e o A7, após sete dias da cirurgia de constrição crónica do nervo ciático (CCI), e/ou após estimulação nóxica mecânica. Associados a dados prévios de comportamento, eletrofisiologia e moleculares para o modelo CCI, os resultados obtidos permitiram-nos supor que o sistema noradrenérgico e a sua maior fonte, o LC, pudessem estar a exercer um papel importante mediado pela sinalização via ERK1/2. Para além disso, o mecanismo subjacente poderia ser diferente para tipos de dor com diferentes origens. Assim, na **Publicação II**, também quantificámos a expressão de pERK1/2 na medula espinhal e no circuito compreendendo o LC, o núcleo Paragigantocelular (PGi, o aferente excitatório do LC mais significativo), e o Córtex Pré-frontal (PFC, a mais representativa projeção do LC), mas agora no modelo de inflamação articular crónica induzida pela monoartrite (MA), usando uma fase inicial e uma fase tardia do desenvolvimento da doença. Ao contrário do que foi verificado no modelo de dor neuropática, onde foi detetada uma diminuição significativa dos níveis de expressão de pERK1/2 no LC após 7 dias de CCI, na **Publicação II** nós descrevemos uma ativação tardia de ERK1/2 que foi acompanhada pelo aparecimento de comportamentos típicos de ansiedade e de depressão. Estas alterações foram revertidas por terapia anti-inflamatória tópica na

pata afetada, sugerindo uma relação causa-efeito. Assim, na **Publicação III,** nós também estudámos a atividade eletrofisiológica dos neurónios do LC no mesmo modelo da MA em tempos prolongados da condição inflamatória. Os resultados obtidos permitiram supor que o aumento de ativação de ERK1/2 estaria relacionado com um aumento da resposta evocada dos neurónios do LC à estimulação nóxica mecânica, bem como com a ansiedade associada com a dor. Isto foi confirmado pela administração de um inibidor de ativação de ERK1/2 (SL327) no LC de ratos submetidos à inflamação crónica, a qual resultou na reversão do comportamento típico de ansiedade, na reversão das alterações eletrofisiológicas e na normalização dos níveis de ativação de ERK1/2 no PFC. Aparte disto, também encontrámos um aumento significativo da expressão da enzima hidrólase da tirosina (a enzima limitante da síntese de noradrenalina) no LC, em condições de monoartrite prolongada. Finalmente, como grande parte dos resultados eletrofisiológicos e bioquímicos observados em condições de inflamação crónica eram indicativos de uma componente de estresse neste modelo, também avaliámos a hipótese da neurotransmissão mediada pelo fator libertador da corticotropina (CRF), um neuropéptido profundamente implicado na regulação da resposta ao estresse, poder ser um evento precursor da ativação de ERK1/2 no LC. De facto, usando um antagonista de CRF, um composto que bloqueia a atividade do CRF endógeno através dos seus recetores, observámos a reversão tanto dos comportamentos típicos de ansiedade como dos níveis de pERK1/2 no LC dos animais com monoartrite prolongada, mas sem alteração do comportamento nociceptivo, claramente indicando que o neuropéptido CRF é possivelmente uma molécula percursora que desencadeia a ativação de ERK1/2 em resposta a condições prolongadas de inflamação articular (**Publicação IV**).

Em conclusão, a dor inflamatória e neuropática apresentam diferentes padrões de ativação de ERK1/2 no LC em tempos iniciais da doença. Particularmente na dor crónica inflamatória, nós mostrámos que um aumento da ativação de ERK1/2 no LC desempenha um papel na perceção aumentada a estímulos nóxicos e está relacionada com a manifestação de ansiedade relacionada com dor. A neurotransmissão pelo CRF parece atuar como percursor nos eventos mediados por ativação de ERK1/2.

Introduction

2. Introduction

In a healthy person, pain sensation is an alert against a threat capable of damaging the body and, therefore, it is undeniable that the pain feeling has an important survival function. However, for reasons not completely understood so far, a painful condition may become permanent, even when there is no apparent threat, lacking its physiological function. In this case, the body turns into a state where the endogenous pain-controlling mechanisms are disrupted. This shift originates a condition that, nowadays, it is recognized as a disease by itself: Chronic Pain (Tracey and Bushnell 2009).

Chronic pain is highly prevalent in developed countries (Breivik et al. 2006; Azevedo et al. 2012). In Portugal, a recent study estimated that about 37% of the Portuguese population suffers from chronic pain (Azevedo et al. 2012). Moreover, 13% of the chronic pain patients were diagnosed with depression and a huge percentage claimed interference with their job performance (Azevedo et al. 2012; Agaliotis et al. 2014). Logically, such unpleasant condition is accompanied by high disability in what regards to the performance of normal daily tasks, affecting the family and social environment, producing not only depressive symptoms but also anxiety and stress disorders (Reid et al. 2011; Gorczyca et al. 2013). The emergence of these disorders deteriorates the clinical diagnosis in chronic pain patients and, thus, it is common the recognition of a negative self-perpetuating pain cycle (Figure 1). From an economic perspective, it represents considerable costs to health-related governmental services (Reid et al. 2011; Breivik et al. 2013). Indeed, in 2011 and just

in general indirect costs related with chronic pain in the lower back and joints, Portugal spent almost 750 million euros (Gouveia and Augusto 2011). More recently, it has been estimated that chronic pain in the Portuguese population is associated to a total of 2,000 million euros per year in direct costs that include visits to physicians and other health care professionals, pharmacologic and non-pharmacologic treatments and medical tests, while the total annual indirect costs, which have been underestimated, amount to 2,600 million euros, mostly related to early retirement due to chronic pain as well as job loss and absenteeism (Azevedo et al. 2014). In order to overcome such a problematic and complex disease, the comprehension of the possible causes and consequences of chronic pain is a justifiable need.

Figure 1: Diagram representing some of the aspects leading to a negative self-perpetuating pain cycle. Pain leads to a guarding posture, which, at long-term, and potentiated by the presence of the painful stimulus, promotes a restricted mobility which leads to muscle weakness and decreased activity. Consequently, depression and anxiety emerge, aggravating pain diagnosis.

2.1. Pain definition

The most accepted and complete definition has been provided by the International Association for the Study of Pain (IASP), which postulates that pain is "*an unpleasant sensation and an emotional experience associated with a real or a potential tissue damage or described in terms of such damage*" (Merskey and Bogduk 1994). This definition indicates that pain is a multifaceted phenomenon, which can be dissociated in various dimensions. Thus, as being an experience, it is implicit that pain is always subjective, with each individual interpreting his pain according to previous motivational feelings and/or other painful experiences. In addition, it is undeniable that the unpleasantness associated to pain sensations prompts emotional distress. Accordingly, it is recognized that pain has well established components: the sensory-discriminative, the cognitive-evaluative and the motivational-affective (Figure 2). As indicated by its designation, the temporal and spatial localization, as well as the physical characteristics and intensity quantification of the stimulus originating pain (noxious stimulus) are conveyed by the sensory-discriminative component. Similarly, the emotional perspective associated to such painful experience and its assimilation is provided by the cognitive and affective components, which are also responsible for the behavioral responses to pain (Melzack 2001; Handwerker 2002; Melzack and Katz 2013).

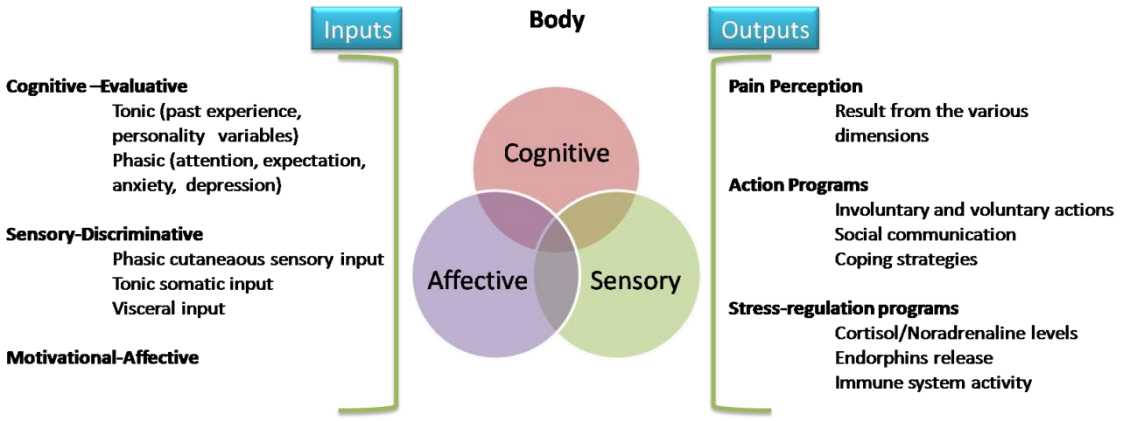

Figure 2: Factors that contribute to the pain experience according to the dimensions of pain (Cognitive-Evaluative, Sensory-Discriminative and Motivational-Affective). As a result, the output consists in pain perception as well as other body-self actions. Adapted from Melzack and Katz (2013).

2.2. Pain classification

Pain can be divided in several types according to its duration, etiology, localization or others, contributing to its complexity. Therefore, regarding the duration, pain can be acute or chronic, although this differentiation is, admittedly, artificial. Acute pain is defined as a short duration, phasic and intense physiological event which normally resolves immediately or a few hours/days after the phenomenon which initially originated pain is healed. It should be noted that several acute stimuli may produce pain, being therefore called noxious (e.g. strong pinch, a deep cut, heat/cold burn), but there are also several stimuli that, in normal conditions, are non-painful (innocuous; e.g. soft touch). Contrarily to acute pain, chronic pain is a long-lasting, tonic, persistent pathological pain that is present even when there is no apparent biological reason. In this case, it is characterized by its spontaneous nature (not elicited

by external stimuli), and by the presence of hyperalgesia and/or allodynia (Sandkuhler 2009) (Figure 3).

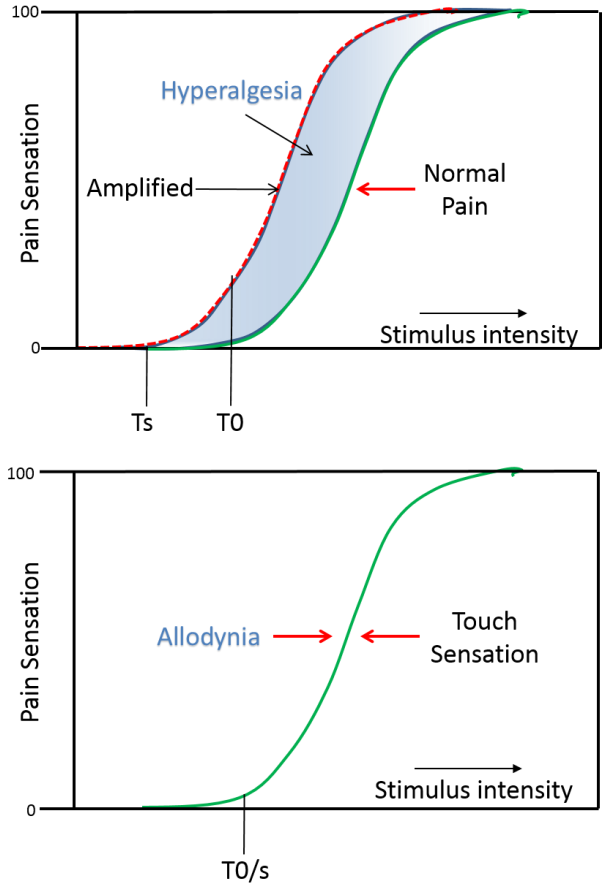

Figure 3: Graphic representation of the concepts of hyperalgesia, defined as an increased pain sensation due to a normal noxious stimulation (top), and allodynia, which is pain sensation following innocuous stimulation (bottom). This representation covers the changes made by IASP in 2008. All forms of pain amplification are now considered as hyperalgesia. Only in the case that pain is produced by activation of low-threshold fibers (e.g. soft touch), the use of the term allodynia is correct. T0 represents the normal threshold for the correspondent sensation and Ts represents the time were the stimulation was performed. Illustration modified from Sandkuhler (2009).

Chronic pain is the most problematic and challenging form of pain and represents a paradigm from the clinical perspective, mostly because there are several types of chronic pain in what respects to their etiology: visceral, inflammatory, neuropathic and/or mixed pain. Visceral pain arises from the viscera and is quite diffuse and difficult to localize, often producing what is called referred pain, where pain sensation is felt away from the site of the originating painful stimulus. Although viscera are not sensitive to cuts or burns, they are highly sensitive to distension,

ischemia and inflammation. Inflammatory pain is a response triggered by the activation of the immune system and it is included in the IASP description of nociceptive pain, which is defined as pain arising from the activation of nociceptors in non-neural damaged tissue. Although visceral and inflammatory pain may become chronic afterwards, they both have an evident acute component. On the contrary, neuropathic pain is caused by an injury of the central or peripheral somatosensory nervous system. Finally, mixed pain is a broad term which includes, for example, cancer pain. Thus, when these several types of pain overlap in the same patient the treatment is difficult due to the complex etiologies. This thesis will focus mainly on the chronic inflammatory pain process but will also address neuropathic pain, as justified in the following sections.

2.3. Chronic inflammatory pain

An inflammatory reaction is a normal response of the immune system to infection and injury. Besides being an unpleasant process, it is normally characterized by the presence of typical signs in the inflamed area, such as redness, heat, swelling and pain. This acute phase of inflammation has a beneficial effect that, hopefully, will lead to problem solving and to repair of the affected tissue. However, the imbalance of this process generates a predisposition to autoimmune disorders, excessive tissue damage, and other pathological conditions (Nathan 2002). Consequently, long-term inflammation triggers adaptive changes in the central nervous system (CNS) that originate exacerbated pain sensation (Ji et al. 2011). As mentioned above, such pain is

defined by IASP as nociceptive pain which categorizes the type of pain arising from the activation of nociceptors in non-neural damaged tissue. Several clinical conditions are associated with chronic inflammation. A representative example is arthritis which includes the highly prevalent autoimmune condition known as rheumatoid arthritis (RA, World Health Organization website: www.who.int/chp/topics/rheumatic/en/).

Arthritis, and particularly RA, is a severe inflammatory condition and a common autoimmune disease characterized by high disability and systemic complications. Its causes are mainly unknown, although it is recognized the involvement of a complex interaction between genetic susceptibility and environmental risks (McInnes and Schett 2011). It produces synovial inflammation and hyperplasia (swelling), cartilage and bone destruction (deformity), which are perpetuated by positive feedback loops (Teramachi et al. 2011) (Figure 4). In RA, pain is the most prevailing symptom demanding medical assistance and immediate treatment (Phillips and Clauw 2013). Although the currently available pharmacological strategies, such as opioids and non-steroid anti-inflammatory drugs (NSAIDs), are effective for pain relief, they are also quite limited due to their well-known adverse side effects (Ong et al. 2007). Additionally, a severe RA condition leads to the development of secondary symptoms such as anxiety, depression and stress (Covic et al. 2012) that not only burden the disease in itself, but that also enhance the negative emotional perspective regarding pain sensation and interpretation, as well as deeply affect the psychosocial environment of the patient (Gafvels et al. 2014). Despite the relevance of the emotional component of pain in RA patients, this has been neglected both in the

clinical practice and pre-clinical research. Indeed, from a molecular and mechanistic point-of-view, how these negative feelings, which are mostly a consequence of the RA (such as depression and anxiety), worsen the diagnosis of the disease is barely understood. One model that mimics the RA condition is the monoarthritis model, described by (Butler et al. 1992), but others are also available (Asquith et al. 2009). Monoarthritis is induced by a single unilateral injection of a complete Freund's adjuvant (CFA) solution in the tibio-tarsal joint. Basically, the CFA solution contains killed/inactivated *Mycobacterium*, usually the *Mycobacterium butyricum*, in a water-in-oil emulsion, and causes a slow release of antigens at the site of inoculation. The *Mycobacterium* is recognized by the immune system and triggers the innate adaptive immune response. This model is widely used mainly because it is easy to induce and has high reproducibility, it allows to predict the efficacy of therapeutic agents in humans, and lastly, because its pathogenesis and pathology are similar to what happens in the human condition (Bendele 2001). Additionally, the monoarthritis model has the great advantage of being restricted to the site of inoculation, therefore avoiding all the effects caused by systemic inflammation, such as in the polyarthritis model, which would, certainly, mask or interfere with the results.

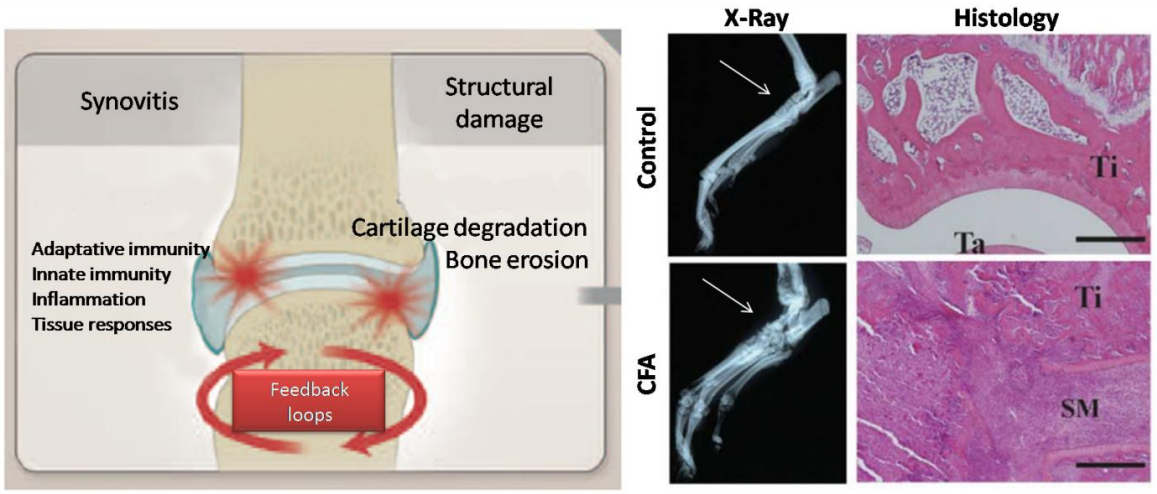

Figure 4: Development and progression of rheumatoid arthritis. Left panel – synovitis is initiated and potentiated by positive feedback loops, promoting systemic responses that pop up the disorder (adapted from McInnes and Schett, 2011). Right panel: X-ray and histological staining of the rat's hind paw in a control and a CFA condition, where inflammation and bone destruction are evident. Ti, tibia; Ta, tarsus; SM, synovial membrane. Scale bar: 200µm. Adapted from Teramachi et al (2011).

2.4. Neuropathic pain

According to IASP, neuropathic pain can be defined as "pain caused by lesion or disease of the somatosensory nervous system" (definition updated from Merskey and Bogduk 1994 – IASP). An example of a lesion or damage that may lead to neuropathic pain is amputation (stump pain; Jackson and Simpson 2004). Likewise, several diseases may be also in the origin of neuropathic pain. Exemplifying, autoimmune (e.g. multiple sclerosis), metabolic (e.g. diabetes), infectious (e.g. herpes zoster) and oncologic diseases are some of the conditions that may lead to deep changes in the nervous system generating neuropathic pain (Woolf and Mannion 1999; Cohen and Mao 2014).

The treatment of neuropathic pain syndromes is commonly based on the use of several pharmacological approaches such as antidepressants, opioids, calcium-channel

alpha 2 delta ligands (gabapentin and pregabalin), topical capsaicin and lidocaine (Jefferies 2010). Although the number of clinical trials has been increasing in the last years, the efficacy of the pharmacological treatments in promoting pain alleviation is quite variable and, for countless patients, it is highly unsatisfactory (Attal et al. 2006; Attal et al. 2010). Similarly to what is observed in chronic inflammatory pain, neuropathic pain deeply affects the quality of life of the patients suffering from this condition. In addition, if it is accompanied by several unsuccessful attempts for getting an acceptable pain relief, the worst consequence is the development of secondary symptoms such as mood disorders (Yalcin et al. 2011; Alba-Delgado et al. 2013; Radat et al. 2013; Yalcin and Barrot 2014).

The number of preclinical studies focusing on neuropathic pain has increased significantly in the last few years. According to Jaggi et al. (2011), the most used rat model of neuropathic pain is the one described by Bennett and Xie (1988). These authors demonstrated that the constriction of the sciatic nerve of the rat, achieved by tying four chromic catgut sutures around the nerve with 1mm apart, leads to persistent pain with significant mechanical and thermal hypersensitivity, as well as recurrent spontaneous pain, mimicking what occurs in human neuropathic conditions. Although the constriction injury is recognizably the main cause of pain in this model, an inflammatory component may not be discarded. The first indications that inflammation might be playing a role in the CCI model were raised by the fact that greater hyperalgesia is produced by the chromic suture than by the silk suture (Maves et al. 1993), suggesting that a chemical irritation produced by this suture is the basis of

pain symptoms development in the CCI model. Indeed, gene expression of inflammatory cytokines was detected in the CCI model of neuropathic pain in rats (Okamoto et al. 2001). Additionally, neuroinflammation was also observed in the sciatic nerve following CCI surgery (Vasudeva et al. 2014). Nevertheless, this inflammatory phenomenon resembles a special case of neuropathy, neuritis, which is defined by IASP as an inflammatory event affecting nerves.

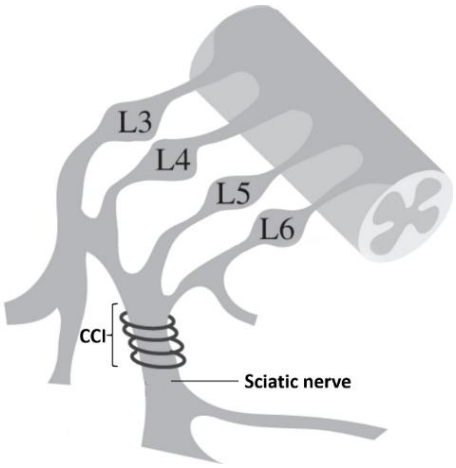

Figure 5: Illustration of the constriction of the sciatic nerve in rodents. The constriction is produced by 4 chromic catgut sutures with 1mm apart. The muscle and the skin are sutured and pain is evident few days after the surgery.

2.5. The processing of pain

Despite their recognized complexity, the mechanisms by which the nociceptive inputs are transmitted from their point of origin to central supraspinal encephalic structures are reasonably well studied. However, nowadays there is an increased interest in other pain-related features that not only interfere with nociception but that may even potentiate it. Thus, for better therapeutic strategies enclosing all the pain-

related components, it is fundamental to understand the neuroanatomical pathways implicated in noxious input sensing, transmission and processing, but it is also important to recognize the structures more susceptible to be involved in all the pain-related spheres (sensorial, emotional, cognitive, etc.).

Pain transmission is initiated by the activation of specific physiological receptors widely distributed throughout the body called nociceptors. These receptors are free nervous endings of the first-order afferent neurons. Usually, the nociceptors have a high activation threshold, which means that only intense stimuli are able to activate them, initiating the transduction and encoding of noxious stimuli into action potentials. Once activated, they display a response that gradually increases with the intensity of the stimulus, which can be mechanical, thermal or chemical (Dubin and Patapoutian 2010). Under certain pathological circumstances, these receptors may become sensitized, resulting in lower activation thresholds and even in their ongoing spontaneous activity (Dubin and Patapoutian 2010).

The cutaneous first-order or primary afferent fibers can be classified into three types: A-Beta, A-Delta and C-fibers. All of these are capable of transmitting sensory information and have their soma localized at the dorsal root ganglion (DRG), but almost only the A-Delta type (small-diameter myelinated; 12-30 m/s) and C type (unmyelinated; 0.5-2.0 m/s) are specialized in noxious stimuli conduction (Treede et al. 1998; Ringkamp 2008; Basbaum et al. 2009). In the skin, these afferents are present in the proportion of 70% and 10%, respectively. When a painful stimulus reaches the skin, A-Delta fibers propagate modality-specific information with marked intensity and

short-latency, promoting a quick pain sensation and triggering withdrawal actions, while C fibers conduct the information slowly. The information regarding the painful stimulus reaches the spinal cord level, particularly the dorsal horn of the spinal cord (Figure 6).

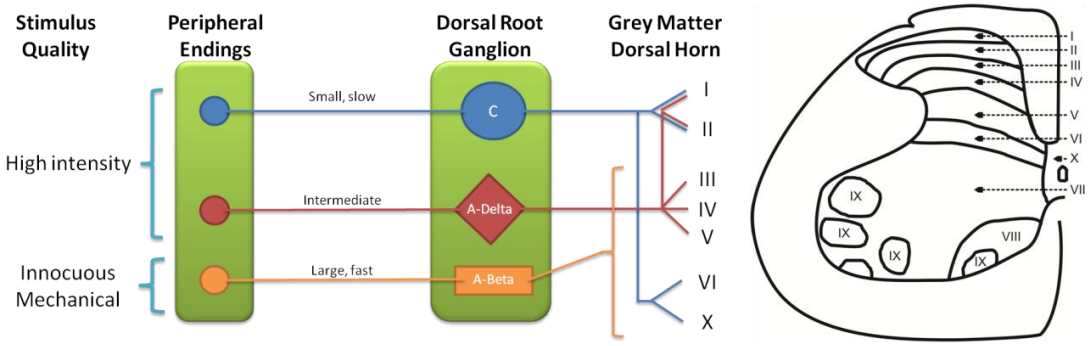

Figure 6: Schematic representation of the primary afferent fibers organization and their specific inputs into the grey matter of the dorsal horn (left, adapted from Millan (1999)). It is important to note that the fibers specifically responsible for the transmission of noxious information overlap their areas of innervation in lamina I and II, being this region of the spinal cord the most common target in pain studies. Lamina VII, VIII and IX (mostly motor neurons) only receive innervations from spinal interneurons or from supraspinal centers. Distribution of Rexed laminae in the L4 segment of the spinal cord (right, adapted from Paxinos and Watson (2009)).

At the spinal cord, the nociceptors establish synapses with second-order neurons distributed along the dorsal horn of the spinal cord. The grey matter of the spinal cord is organized in a ten laminae system known as the Rexed laminae (Molander et al. 1984) (Figure 6). This organization is based on the fact that each spinal cord lamina has its own predominance of neurons with a specific size and shape, and a particular cytological density and characteristics (Rexed 1952; Rexed 1954; Molander et al. 1984). Laminae I to VI constitute the dorsal horn, with lamina VI being only well

differentiated in the cervical and lumbar-sacral enlargements. Lamina VII corresponds to the intermediate zone, lamina VIII and IX compose the medial and lateral ventral horn, respectively, and lamina X comprises the area surrounding the central canal (Rexed 1952; Rexed 1954; Millan 1999). Lamina I (also known as the marginal zone or layer) and lamina II constitute the superficial dorsal horn and receive the nociceptive inputs from the primary afferents. In lamina I, most of the neurons are interneurons classified as fusiform, multipolar and pyramidal (Lima and Coimbra 1990). It contains very few projection neurons (about 5% in segment L4), that reach the brainstem, particularly the lateral parabrachial nucleus (LPB), and the thalamus (Lima and Coimbra 1988). The lamina II neurons own their main classification to Ramón and Cajal's (1909) work (Ramón y Cajal 1909). There are two main cell types: the central cell, which is widespread throughout the lamina, and the limitroph cell, that occurs in an outer band close to the lamina I-II border (lamina II outer; Paxinos 2004). In lamina III, 5 main cell types can be found: islet, central, radial, medial-lateral and vertical (Lu and Perl 2003; Lu and Perl 2005). Again, a small number of neurons in lamina III project to higher centers such as the thalamus, lateral cervical nucleus or pontine-medullary junction (Willis 1978). On the contrary to what is observed in laminae I and II, in lamina III, most of the neurons project diffusely to supraspinal levels (Todd et al. 2000; Todd et al. 2009). In the dorsal horn, the neurons are differentiated according to the type of input they receive, being classified into three groups: the nociceptive neurons, the Wide Dynamic Range (WDR) neurons, and the non-nociceptive neurons (Grant 2004; Heise 2009). The nociceptive specific neurons respond exclusively to stimuli with a painful

nature and are mainly localized in lamina I, II, V and VI. These neurons are implicated in the discrimination of the stimulus' physical quality and localization (Grant 2004; Heise 2009). The WDR neurons receive inputs from the A-Delta and A-Beta fibers, are localized in lamina I, II, IV-VI and X, and have a higher ability to discriminate the stimulus intensity. Finally, the non-nociceptive neurons are activated by innocuous stimuli propagated by A-Delta and A-Beta fibers and can be found in lamina I, II and IV (Grant 2004; Heise 2009). Taking this into consideration, a great part of the spinal neurons that respond to nociceptive inputs from the periphery are localized in the superficial layers/laminae, which are, very often, the subject of study in several pain conditions.

Most of the axons of the second-order dorsal horn neurons decussate to the contralateral side of the body and ascend through the anterior or posterior fascicle, forming bundles that allow the flow of the nociceptive information, which had been integrated in the different laminae of the spinal cord, until it reaches important supraspinal structures implicated in the modulation of the pain response. Afterwards, the nociceptive information is processed in order to trigger a response via the descending pain pathways.

2.5.1. Ascending pain pathways

It has been described that ascending bundles form tracts that can be named according to the most important brain areas where the axons end (Lima 2008). The most studied ascending tracts are the spinothalamic tract, the spinoreticular tract and

the spinomesencephalic tract. The spinothalamic tract neurons ascend majorly by the contralateral side (67-81%). In the lumbar segment of the spinal cord, the spinothalamic neurons are located in the medial part of lamina V and their axons terminate either in the lateral or medial thalamus. Projections to the lateral thalamus (lateral spinothalamic tract) target the ventroposterolateral and ventroposteromedial nuclei, collectively known as the ventrobasal complex of the thalamus, and the posterior complex, all of them involved in the sensory-discriminative aspects of pain (Albe-Fessard et al. 1985). The medial spinothalamic tract projects to the intralaminar nuclei and the central lateral nucleus of the thalamus, and plays an important role in the motivational-affective responses to pain. Collaterals of the spinothalamic tract reach the medullary reticular formation, the periaqueductal gray (PAG) and accumbens nucleus.

The spinoreticular tract neurons originate mainly in laminae I, V, VII and VIII, X and lateral spinal nucleus. They ascend contralaterally from the spinal cord and reach the lateral, dorsal and gigantocellularis reticular nuclei, the oral and caudal pontine reticular nuclei, the dorsal and lateral paragigantocellularis nuclei (PGi), the Locus coeruleus (LC), the raphe magnus nucleus (RMg) and the median raphe nucleus (Kayalioglu 2009). Neurons in this tract respond to innocuous and noxious radiant heat and light tactile stimuli.

The spinomesencephalic tract includes projections from the spinal cord to the midbrain, namely the PAG, dorsal raphe nucleus (DRN), cuneiform nucleus, deep layers of the superior colliculus, the red nucleus, the Edinger-Westphal nucleus (EW), among

others (Willis 2007; Kayalioglu 2009). Neurons in this tract respond to noxious and innocuous stimulation from cutaneous or deep structures such as joints and muscles. The projections to PAG are responsible for the motivational-affective responses to pain and for the descending control of nociception (Paxinos 2004).

Although the main ascending pathways described originate in the spinal cord and have main targets in specific supraspinal regions, it is nowadays, recognized that they are not independent but, instead, they are interconnected with each other as they send to and receive collaterals from other nuclei. Thus, the nociceptive information is not processed in a single and isolated brain structure, and neither that brain structure receives information from only one area but, instead, it appears to be organized in a "pain matrix". As proposed by Ossipov (2010), in this "pain matrix" there is constant interaction between brain structures, assuring that pain encompasses aspects like unpleasantness, anxiety, stress and emotion, contributing, undoubtedly, to the complexity of the pain experience (Ossipov et al. 2010; Tracey and Johns 2010). The "down-top" transmission originated by the painful signal is responsible for the pain perception at the cortical areas and it initiates the "top-down" mechanisms, which will be responsible for the motor-behavior responses.

2.5.2. Descending pain pathways

After the information has been processed by the supraspinal centers of pain modulation, the balanced response to pain is achieved by the activation of descending pain pathways. In this "top-down" transmission, there are several nuclei implicated,

either directly (whose neurons send projections to the spinal cord) or indirectly (whose neurons send projections to other structures that are directly connected with the spinal cord), and some of them also participate in the ascending pain transmission. Included in these descending modulatory pathways are the corticospinal tract (originated in the cerebral cortex) and smaller descending tracts like those originated in the hypothalamus (paraventricular nucleus, PVN), in the midbrain (superior colliculus, PAG, supraoculomotor nucleus, interstitial nucleus of Cajal and cuneiform nucleus), and hindbrain (nucleus gigantocellularis, DRN, solitary tract nucleus, LC, subcoeruleus nuclei) (Paxinos 2004). Some of these structures are particularly interesting as they are part of the "endogenous pain inhibitory system" (Ossipov et al. 2010), which results from the interconnectivity of several nuclei, mostly located in the brainstem. An important contributor to this inhibitory system is the noradrenergic system, through the activation of adrenergic receptors in the spinal cord (Ossipov et al. 1990; Ossipov et al. 1990; Eisenach et al. 1998; Pertovaara 2006). Spinal noradrenaline (NA) is supplied by three main noradrenergic clusters in the brainstem, namely the A5, LC (or A6) and the A7 (the LC-NA system is described in detail in section 2.6). Besides the inhibitory effects, it is also known that the descending pain circuitry may be responsible for some facilitation of pain transmission, which may occur through the activation of the on-cells in the rostral ventromedial medulla (RVM; Kaplan and Fields 1991; Morgan and Fields 1994), among other mechanisms.

At the end, pain is a consequence of a global effect where the facilitatory descending mechanisms exceed the inhibitory mechanisms, while analgesia (absence

of pain to painful stimulation) represents a state where the inhibitory mechanisms triumph.

2.6. The noradrenergic system

The noradrenergic system comprises a vital circuitry based on the action of NA on different and strategically located adrenergic receptors. In this way, NA exerts many important autonomic functions in the CNS, such as in sleep, arousal, vigilance and cognition, among others. Noradrenaline is a neurotransmitter biosynthesized by sequential enzymatic reactions starting with the amino acid tyrosine. The first and the most important step in this cascade is the conversion of tyrosine into dihydroxyphenylalanine (L-DOPA) through the enzymatic action of tyrosine hydroxylase (TH). After this step, the L-DOPA is converted into dopamine through the action of the aromatic L-amino acid descarboxylase. It is only in the noradrenergic neurons that dopamine is converted into noradrenaline by the dopamine β-hydroxylase enzyme (Figure 7). The step catalyzed by TH is known as a rate-limiting step, as it directly interferes with the availability of dopamine and noradrenaline in the body, and the immunodetection of TH expression is often used as marker of dopaminergic/noradrenergic neurons as well as is indicative of these neurotransmitters synthesis demand (Bacopoulos and Bhatnagar 1977). After being synthesized, NA is stored in vesicles in the synaptic terminal of the axon. Following a specific electrical input, these vesicles attach to the neuronal membrane through the vesicular monoamine transporter, and their content is released into the synaptic cleft.

Then, NA binds to post-synaptic adrenergic receptors and, subsequently, activates intracellular signaling cascades that will translate the signal according to the type of adrenergic receptor activated (facilitatory or inhibitory receptor). After exerting their signaling function, the NA molecules can follow two different pathways: either they undergo reuptake by the presynaptic NA transporters and are recycled, or they undergo degradation by enzymes in the synaptic cleft and nerve terminal (Figure 7).

The noradrenergic neurons are organized in seven clusters or groups, classified from A1 to A7 and located in the brainstem (Pertovaara 2006). From these, the A5 and A7 are responsible for the noradrenergic innervations of the spinal cord and the A6 (also known as LC) innervates almost the entire forebrain and projects to the spinal cord as well. Pain regulation is one of the functions played by the noradrenergic system and, in particular, by the LC (Pertovaara 2006; Pertovaara 2013). However, to understand how NA modulates pain, it is important to know how the adrenergic receptors work.

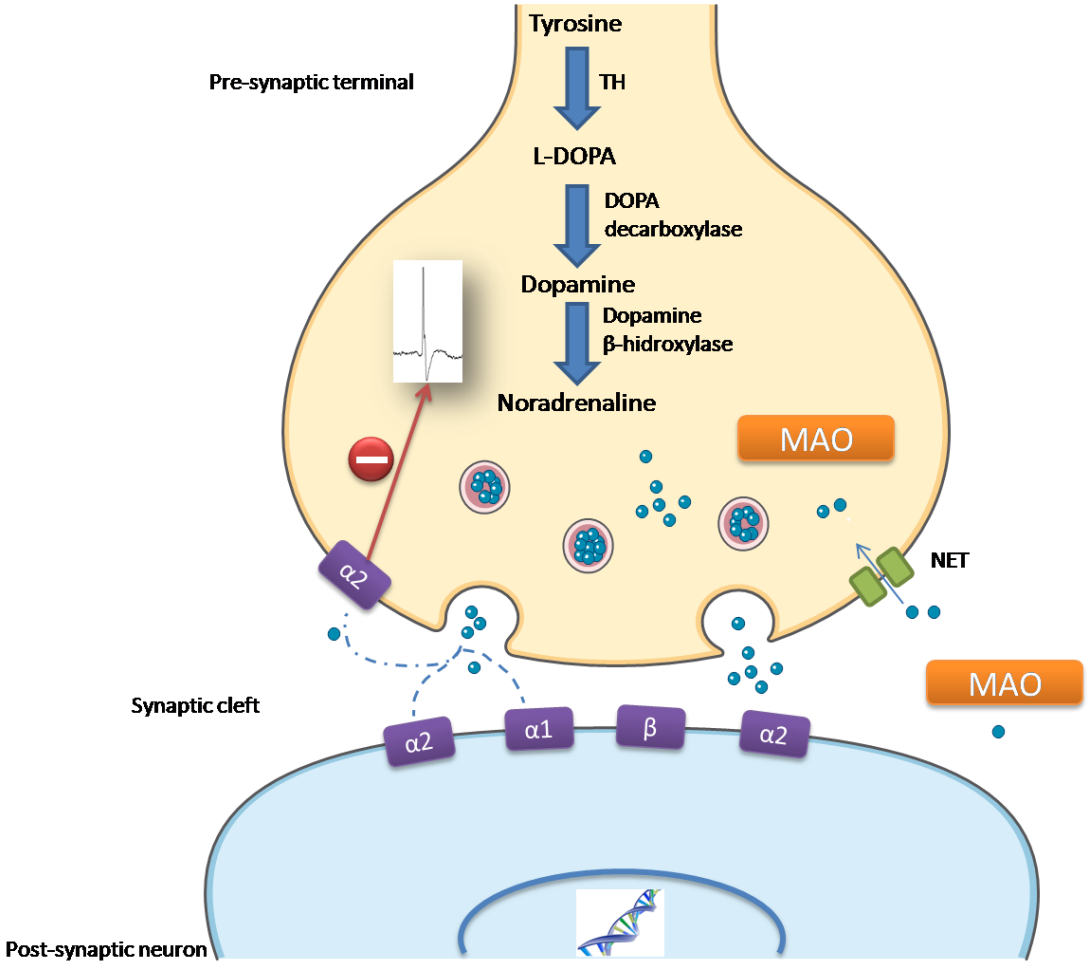

Figure 7: Schematic representation of a noradrenergic synapse. Noradrenergic neurons, here represented as a pre-synaptic neuron, are the source of NA for the entire CNS. NA is synthesized from the aminoacid tyrosine through a cascade of events. After the synthesis, NA is stored in vesicles at the axon terminal. Following specific electrical inputs, these vesicles attach to the membrane and release the NA content into the synaptic cleft. In the extracellular space, NA exerts its function by binding to adrenergic receptors in the post-synaptic membrane. When the flow of NA in the synaptic cleft is high, α2-adrenoceptors in the pre-synaptic membrane (auto-receptors) are also activated and inhibit/cease NA release. At the same time, the extracellular NA content that is not binding to any receptor or already exerted its function may be recycled upon reuptake at the pre-synaptic membrane or may follow degradation. L-DOPA – L-dihydroxyphenylalanine; MAO – Monoamine oxydase; TH – Tyrosine hydroxylase.

2.6.1. Adrenergic receptors

Two main categories divide the adrenergic receptors: type α- and type β-adrenoceptors. Although both categories have been found to be implicated in pain modulation, the α-adrenoceptors play a key role by mediating the pain regulatory effects of noradrenaline. Within each type, several subtypes are recognized. Thus, the α-adrenoceptors can be found as α1A, α1B, α1D, α2A, α2B, α2C and α2D (Ruffolo and Hieble 1994). In general, the action of these receptors is mediated by guanine nucleotide-binding regulatory proteins (G proteins). The α2-adrenoceptors are able to decrease the intracellular adenylcyclase activity through the G_i protein or directly modify the activity of certain ion channels acting, therefore, as inhibitors of signal transmission. In contrast, α1-adrenoceptors are coupled either to phospholipase C through the G_q protein or directly to calcium influx (Summers and McMartin 1993), being, therefore, excitatory to the transmission of the signal.

The activation of α2-adrenoceptors localized in the somatodendritic area of catecholaminergic neurons (auto-receptors) produces inhibition of the nervous impulse discharge and further inhibition of NA release from the adrenergic terminals. However, α2-adrenoceptors are also found in non-adrenergic cells, acting as hetero-receptors and contributing to the regulation of other important functions such as sedation (Gyires et al. 2009).

The α1A, α1B, α2A and α2B-adrenoceptors are, so far, the most studied adrenoceptors. The α1A and α2A-adrenoceptors can be found widely throughout the supraspinal areas, mostly coinciding with the wide distribution of ascending pain

pathways. In contrast, the subtype α2B and α1B are more restricted to the thalamus (Pertovaara 2013) and the cerebral cortex, respectively (Nalepa et al. 2005). In the particular case of the LC, a large proportion of α2A receptors are inhibitory auto-receptors located in the cell bodies (Aghajanian and VanderMaelen 1982), highlighting their role in a negative feedback mechanism involved in the regulation of LC electrical activity.

2.6.2. The Locus coeruleus

The LC, the main structure coordinating the central noradrenergic system, is located in the lateral floor of the fourth ventricle, in the pons of the brainstem. Its neuronal noradrenergic terminations reach almost the entire CNS, constituting an elaborated network. Although NA is the main neurotransmitter synthesized in the LC neurons, it was already shown that other substances are also produced in the nucleus as, for example, vasopressin, neurotensin and galanin, or others that are expressed in smaller amounts (Olpe and Steinmann 1991; Singewald and Philippu 1998).

2.6.2.1 The Locus coeruleus main pathway

The LC is strategically localized and establishes important connections in the CNS, which, in the particular case of chronic pain modulation, allow the coordination of both the sensory, by participating in the ascending and descending pain pathways (Figure 8), and the affective components (Renn and Dorsey 2005; Weiss et al. 2005).

In what regards to the ascending pain pathways, the LC receives and integrates inputs from two major nuclei, the Paragigantocellularis nucleus (PGi) and the Prepositus Hypoglossi (PrH; Aston-Jones et al. 1991), whose projections were demonstrated to be predominantly excitatory (glutamatergic; Ennis et al. 1992) and inhibitory (GABAergic; Ennis and Aston-Jones 1989), respectively. Additionally, the noradrenergic neurons in the LC extensively innervate forebrain structures that are involved in a wide range of functions, as for example the prefrontal cortex (PFC) (Morrison et al. 1979; Porrino and Goldman-Rakic 1982). It is described that the PFC is implicated in the cognitive and emotional/affective aspects of pain (Gusnard et al. 2001; Phelps et al. 2004), and particularly the affective component of pain is modulated by the anterior cingulate cortex (ACC) subarea in humans (Rainville et al. 1997; Wager et al. 2004) and rats (Johansen et al. 2001; Wei and Zhuo 2008; Cao et al. 2009; Dai et al. 2011). Additionally, retrograde and anterograde labeling studies showed reciprocal connections between the LC and PFC (Porrino and Goldman-Rakic 1982; Jodo et al. 1998; Heidbreder and Groenewegen 2003), establishing an interesting and regulatory feedback loop. Another quite important area of projection of LC neurons is the amygdala, one of the centers of excellence for the regulation of emotion (Asan 1998; Davidson et al. 2000). Additionally the LC receives inputs from the Hypothalamus (Reyes et al. 2005), reinforcing the importance of the NA-LC system in the affective/emotional sphere. In fact, is important to highlight the reciprocal connection between the LC and the PVN of the Hypothalamus, which represents the major source of corticotropin-releasing factor (CRF) although PGi neurons also deliver

CRF into the LC at a lesser extent (Valentino et al. 1992). CRF is implicated in stress, depression and pain and exerts its neuromodulatory function partially by targeting the noradrenergic LC pathway (Valentino and Van Bockstaele 2008). Interestingly, pain, particularly inflammatory pain, may act as a "stressor" agent modulated by CRF (Hummel et al. 2010) which, by itself, increases LC neuronal activity (Kubota et al. 2012). Curiously, CRF administration into the LC area promoted c-Fos and pERK1/2 expression in the PFC (Snyder et al. 2012), and glutamate-induced excitation of PVN neurons induced an increased activation of the LC neurons and PVN neurons containing CRF (Kubota et al. 2012). However, it still is rather unknown how chronic pain conditions affect this pathway.

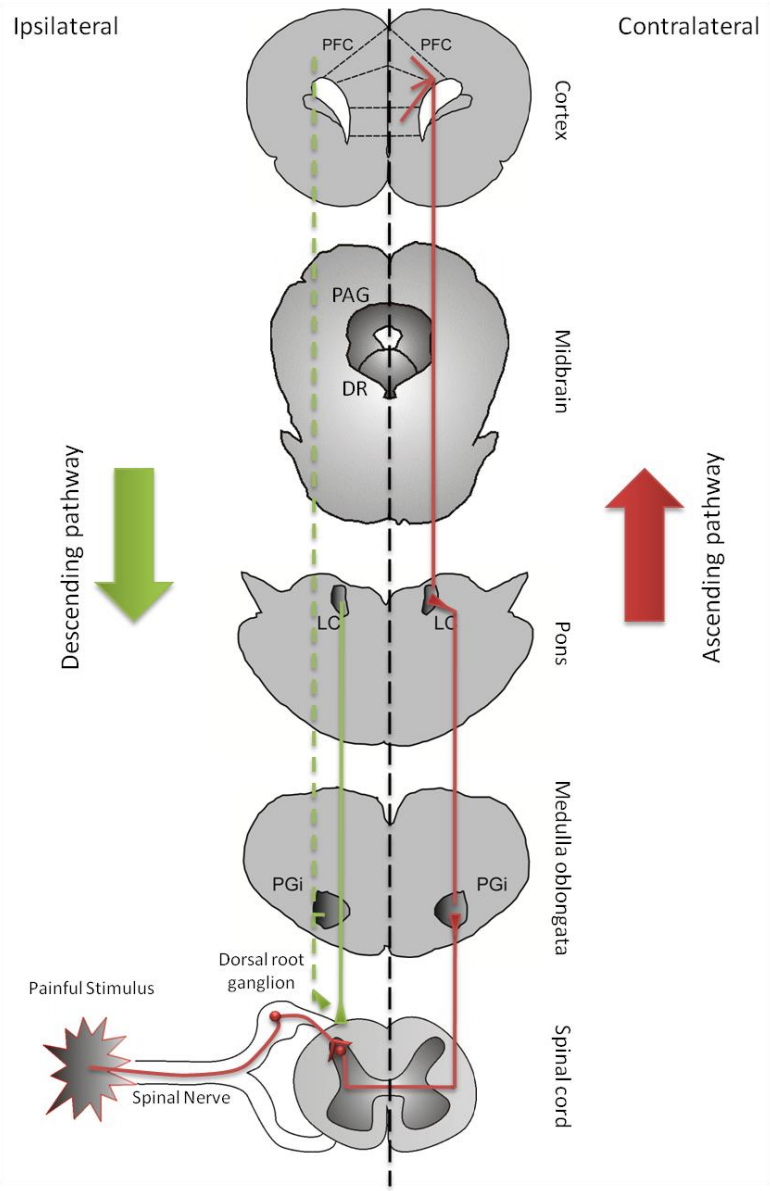

Figure 8: Schematic representation of the most important ascending (red) and descending (green) pain pathways where the LC is a key structure. Briefly, when a painful stimulus is applied at the periphery, the noxious information is transduced and the nociceptive input is transmitted to the spinal cord where it triggers specific neurons within the superficial dorsal horn. The ascending axons decussate and ascend to several supraspinal structures. Those reaching the PGi nucleus and predominantly excitatory will, in turn, activate the LC neurons, and subsequently, the noradrenergic neurotransmission is increased. At the same time, descending pain pathways are triggered. These may be originated at the cortical areas or directly in the LC. Their axonal terminations are predominantly targeting the superficial dorsal horn, and will modulate the quality of the information that will ascend, therefore completing the circuitry.

2.6.2.2 The electrical activity of the Locus coeruleus

As a major modulator of the state of arousal, the activity of LC neurons promotes wakefulness, a behavioral attribute whose relevance is undeniable in either healthy or unhealthy conditions. When a pathological condition affects the normal activity of the NA-LC system, harmful consequences in the normal arousal state may emerge. Chronic pain is one of these pathological conditions that, as a result of its sensory and emotional components, produce several changes in the normal functioning of the NA-LC system (Hirata and Aston-Jones 1994; Pertovaara 2006; Pertovaara 2013).

At least two discharge patterns were identified in LC neurons: the tonic and phasic activity (Aston-Jones and Cohen 2005). The LC spikes are action potentials presenting long-duration (>2 ms) and positive-negative waveforms. The tonic activity corresponds to a spontaneous and regular firing, with a slow rhythm (0.5 and 5 Hz). The phasic activity occurs as a consequence of external relevant stimuli such as intense sounds or painful stimulation (Aston-Jones and Bloom 1981; Valentino and Foote 1988). It is characterized by a rapid burst of 2-3 action potentials followed by an inhibitory or suppression period (Korf et al. 1974). This sensory-evoked response of the LC neurons is correlated with increased arousal and vigilance towards the initiating external stimulus (Aston-Jones and Cohen 2005), and it is accompanied by increased release of noradrenaline in the terminal areas (Florin-Lechner et al. 1996). Mechanistically, it has been shown that noxious hind paw stimulation activates the PGi glutamatergic nucleus which, hypothetically, may be responsible for an increased and

rapid activation of LC neurons upon external stimuli (Ennis and Aston-Jones 1988; Torres-Sanchez et al. 2013). This activation of the LC causes increased release of NA at the spinal cord level. Thus, the ascending noxious information will allow the LC neurons to engage descending feedback systems that regulate the output from the spinal cord. Pharmacological manipulation may act on this sensory-evoked response of the LC neurons as is demonstrated by Torres-Sanchez and partners (2013) in a study where tapentadol, an analgesic compound, inhibited the LC response to acute noxious stimulation (Torres-Sanchez et al. 2013). In normal healthy conditions, innocuous stimulation, such as low level auditory stimuli, does not produce this kind of response in the LC neurons (Grant et al. 1988), suggesting that the phasic activity is intensity-dependent.

As reviewed by Aston-Jones and Cohen, a direct correlation exists between the tonic to phasic ratio of the LC activity and the performance of tasks that require specific levels of attention (Aston-Jones and Cohen 2005). Animals are inattentive and lethargic at very low levels of LC tonic activity, and, on the contrary, they are distractible and overactive at very high levels of LC tonic activity (absence of phasic activity). These two situations reflect a poor behavior performance. An optimal behavior performance is observed when the tonic activity of the LC is moderate and a phasic LC activation occurs following a relevant stimuli related with the goal of the task (Aston-Jones and Cohen 2005). It was already shown that the tonic to phasic ratio was altered in several pathological conditions such as stress, pain and depression (Bravo et al. 2013; George et al. 2013).

2.6.3. The noradrenergic system in the sensorial and emotional pain sphere.

Several years ago, it was described that the antinociceptive effect produced by electrical stimulation of the PAG or the RVM was accompanied by increased levels of noradrenaline in the cerebrospinal fluid and was blocked by the intrathecal administration of adrenergic antagonists (Barbaro et al. 1985; Hammond et al. 1985; Cui et al. 1999). These were the first findings implicating the noradrenergic system in descending pain modulation, particularly at the spinal cord level. In the following years, several studies contributed for the establishment of the spinal adrenergic receptors, particularly α2-adrenoceptors, as strongly involved in pain inhibition, since the use of several agonists, administered mainly at the spinal cord level, resulted in antinociceptive behaviors (Ossipov et al. 2010; Pertovaara 2013). Thus, it is well known that descending noradrenergic projections to the spinal cord, which are not all originated in the LC, result in pain inhibition (Hayashida and Eisenach 2010; Pertovaara 2013).

At supraspinal level, a great part of the structures implicated in pain modulation also express several adrenergic receptors (MacDonald and Scheinin 1995) and consequently, the noradrenergic system acts directly and indirectly in order to deal with a specific painful condition. The existence of this network pops out different modes of action and processing according to the type of stimulus. This explains why the activation of α2-adrenoceptors in the spinal cord is widely accepted as having a potent antinociceptive effect (Hayashida and Eisenach 2010; Pertovaara 2013) while at supraspinal level such relation is not always true. Indeed, administration of an agonist

for α2-adrenoceptors in the LC resulted in spinal antinociception in several pain conditions (Guo et al. 1996) but also produced pain behaviors without interfering with acute pain (Pertovaara and Hamalainen 1994). In contrast, the administration of an antagonist for α2-adrenoceptors in the LC had an anti-allodynic effect in nerve-injured rats which was reversed by the intrathecal administration of the same antagonist (Wei and Pertovaara 2006). Moreover, no significant effects were observed in control non-pathological conditions. Overall, these results clearly suggest that, in neuropathic conditions, spinal and supraspinal α2-adrenoceptors have opposite effects (Wei and Pertovaara 2006). Thus, blocking or antagonizing the supraspinal activity of α2-adrenoceptors may promote the descending noradrenergic transmission which, at spinal cord level, could act, for example, on α2-adrenoceptors present in the primary afferents terminals (presynaptic inhibition, Pan et al. 2002; Kawasaki et al. 2003) or inhibit excitatory interneurons (Olave and Maxwell 2002).

Pharmacological manipulation of the α2-adrenoceptors is a widely used method for studying the role of these receptors in specific experimental pain conditions, and it is an indirect way to test the functionality under specific experimental conditions. On the other hand, protein quantification for the expression of adrenoceptors provides quantitative data that is also quite important. Indeed, in chronic pain conditions it was shown that the α2A adrenoceptor expression in the LC is enhanced while, in the spinal cord, it is decreased in the ipsilateral side (Stone et al. 1999; Alba-Delgado et al. 2013). These results strongly indicate a relevant attenuation of the descending pain control at both spinal and supraspinal levels. Interestingly, in

post-mortem tissue from depressed patients it was found an elevated number of agonist binding sites for α2-adrenoceptors in the LC (Ordway et al. 2003), evidencing a similarity between depression and chronic pain conditions. Quantification of the TH enzyme expression is also often used as an indication of the noradrenaline synthesis demand and several studies addressed it in several experimental conditions. Alba-Delgado et al. (2013) demonstrated that TH expression is increased in neuropathic conditions, when anxiety and depression were also detected. Also, this increase in TH expression was higher in comorbid neuropathic and depressive conditions, induced by using the CCI model and the chronic mild stress (CMS, model of depression) or social stress models simultaneously in the same animals (Bravo et al. 2013; Bravo et al. 2014). Again, similar results were observed in post-mortem tissue from depressed patients (Zhu et al. 1999). Curiously, chronic administration of several antidepressants, known for their analgesic effects as well, resulted in a substantial down-regulation of the TH messenger ribonucleic acid (mRNA) expression in the LC (Nestler et al. 1990).

These techniques are quite useful but they do not allow inferring about the role of a specific structure in specific conditions. One interesting study was performed by Tsuruoka et al. (2012) where the effects of a bilateral lesion in the LC/Subcoerular area on the development of peripheral hyperalgesia were evaluated in rats submitted to inflammatory pain (carrageenan model; Tsuruoka et al. 2012). They observed that, a few hours after induction of the inflammation, the LC-lesioned animals had significantly shorter latencies to heat stimuli than those with no lesion in the LC, indicating that peripheral inflammation activates the inhibitory pathway from the LC to

the spinal cord (the so called coeruleospinal inhibitory pathway, CSIP; Tsuruoka et al. 2012), and reflecting a delay in the development of hyperalgesia in the acute phase of inflammation. In a study focused on the influence of the opioid system, the same authors observed that systemic administration of naloxone, a mu-opioid receptor antagonist, significantly decreased the paw withdrawal latencies in the LC-lesioned rats, in the acute phase of inflammation. This indicates that the opioid pain control mechanisms are active and interact with the CSIP. However, as no significant changes were observed with systemic administration of naloxone in sham non-lesioned rats, the authors concluded that the CSIP is predominant in acute inflammatory conditions (Tsuruoka et al. 2012).

Another work that used a similar methodological approach in a model of neuropathic pain was performed by Brightwell and Taylor in 2009. Briefly, they used a model of neuropathic pain, the spared sural nerve injury and, two weeks after the surgery for the induction of neuropathic pain, they bilaterally administered lidocaine in the LC. This procedure reduced the neuropathic pain behaviors (allodynia and hyperalgesia), suggesting that, in a chronic pain condition, the LC contributes to pain facilitation (Brightwell and Taylor 2009). Additionally, they observed a bilateral increase in the expression of activation markers (c-Fos and phosphorylated CREB, cAMP response element-binding protein) in the LC after the application of an innocuous stimulus to the spared sural innervation territory of the hind paw (Brightwell and Taylor 2009).

Few studies were performed in order to understand the precise role of the NA-LC system in pain-related affective disorders. In the CCI model of neuropathic pain, Alba-Delgado et al. (2013) demonstrated that some electrophysiological parameters of the LC firing, such as the variation coefficient and burst activity, were altered in rats following 28 days of CCI, a time-point that was accompanied by anxiety- and depressive-like behaviors, increased TH expression, increased noradrenaline transporter (NET) expression, and enhanced α2-adrenoreceptor expression and sensitivity (Alba-Delgado et al. 2013). Thus, the authors concluded that a noradrenergic dysfunction was occurring in the long-term CCI condition.

Overall, the scientific community studying the role of the NA-LC system in chronic pain conditions recognizes that a deeper role may be attributed to this system, since inhibition of nociception is not the only function carried out by noradrenergic neurons. In this context, a role regarding the modulation of the development of secondary traits associated with untreated chronic painful conditions, such as anxiety and depression, is one of the hypotheses being tested by pre-clinical research.

2.7. The extracellular signal-regulated kinases 1 and 2 (ERK1/2)

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinases (MAPK) superfamily, ubiquitously expressed throughout the body, either in the activated and/or inactivated state (total proportion of ERK1/2). Because they have high homology in what respects to their protein structure and function, and are often activated together in the Ras-Raf-MEK-ERK signal

transduction cascade (Figure 9), they are commonly referred jointly as ERK1/2 (Ji et al. 2009). Their activation is achieved by phosphorylation and, therefore, the activated ERK1/2 is known as pERK1/2. Then, the main fate of pERK1/2 is the translocation into the nucleus in order to activate a wide range of transcriptional factors (c-fos or CREB) or remain in the cytoplasm exerting other functions (Figure 9). In normal and healthy conditions, the activation of the ERK1/2 cascade upon extracellular stimulation lasts between 20 minutes (transient activation) up to 2 to 3 hours (sustained activation). After exerting its function, the normal tracking of the ERK1/2 signaling cascade is dephosphorylation produced by phosphatases. However, several pathological conditions, including pain syndromes (Ji et al. 2002; Wei and Zhuo 2008), cancer and depressive disorders, deregulate the normal course of the Ras-Raf-MEK-ERK cascade producing either persistent or sustained ERK1/2 activation, by escaping from the phosphatases action, and/or by exacerbated extracellular stimulation (Wortzel and Seger 2011).

Several conditions and factors were shown to induce ERK1/2 phosphorylation but it is the acknowledgement of their activation in several painful conditions that provides an increased interest for this work. Indeed, in the study of chronic pain it is imperative the use of good tools and appropriate targets and the activation of these kinases have emerged as a valuable and promising biomarker, particularly at the spinal cord level (Ji et al. 2009; White et al. 2011). Moreover, another subject where the MAPK cascade seems to be implicated is in the regulation of the affective sphere, such as in depression (Duric et al. 2010) and anxiety (Wang et al. 2010). This is a great

advantage considering that pERK1/2 expression may also be used as an activation marker when studying prolonged painful conditions where the affective component of pain is also triggered.

Another benefit of the use pERK1/2 is the fact that it can be immunodetected in multiple cellular compartments, as neuronal perikarya and dendrites, allowing the delimitation of the cell morphology (Figure 9) (Flood et al. 1998; Koh et al. 2002).

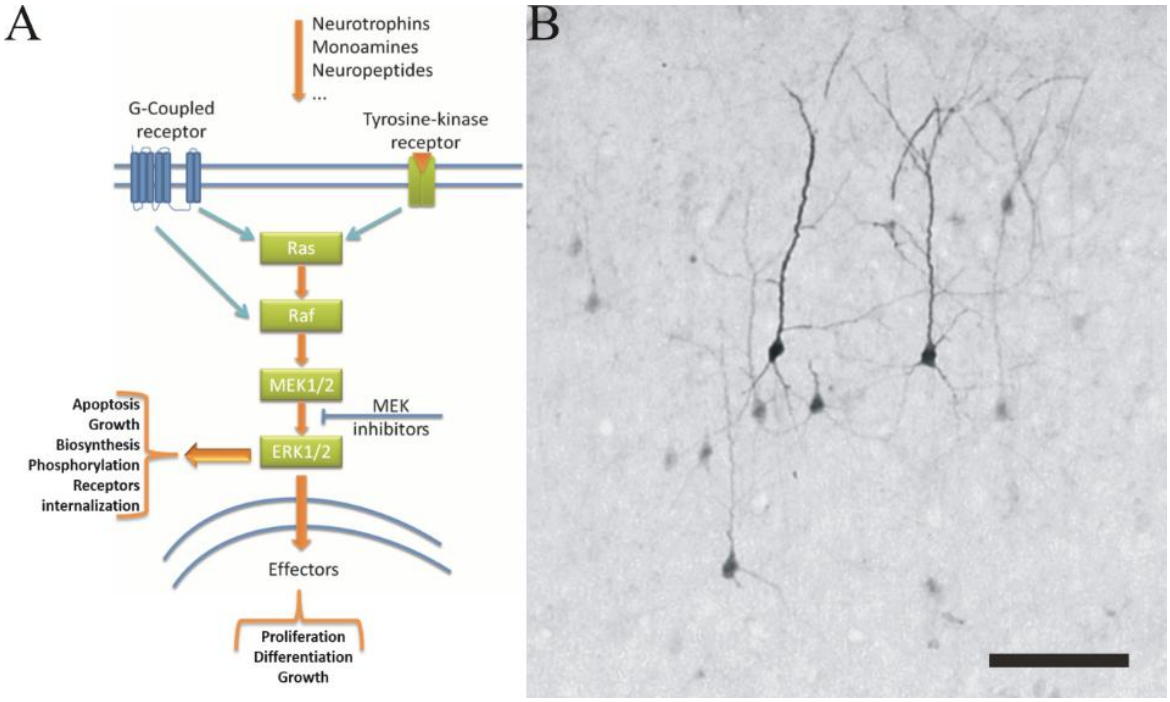

Figure 9: Schematic representation of the intracellular ERK1/2 activation/signaling cascade. A) The activation of Ras promotes the activity of the kinase RAF serine/threonine protein kinases and, subsequently, promotes the phosphorylation of the MEK 1/2 and ERK1/2. Most of the ERK1/2 activation inhibitors, such as SL327, act on this last step. The activated/phosphorylated ERK1/2 translocate into the nucleus and trigger the translation of many transcription factors. At the cytoplasm activated ERK1/2 mediate several mechanisms including the phosphorylation of other proteins and receptors internalization. Modified from Ji et al. 2009. B) Detailed photomicrograph of a brain section (30µm thick) from a naïve rat showing pyramidal neurons in the prefrontal cortex labeled against the pERK1/2 activated isoforms. The immunolabeling was revealed by using a ABC kit and 3,3'-diaminobenzidine. Original photomicrograph from the author. Scale bar =100µm.

2.7.1. ERK1/2 in pain

The expression of ERK1/2 was studied in different pain types and experimental models and this has been the focus of several reviews (Ji et al. 2009; White et al. 2011; Borges et al. 2015). Indeed, several data suggest that these kinases may be an exceptional target for studying the signaling mechanisms underlying the processing of noxious stimuli in painful conditions, and that they may even represent a valid therapeutic target (Ciruela et al. 2003; Zhang et al. 2005; Ji et al. 2007). Accordingly, the use of specific upstream ERK inhibitors resulted in analgesia in several pain models (Cruz et al. 2005) and, in the same way, analgesic pharmacological approaches were also able to reduce pain-induced increases in ERK1/2 levels in the spinal cord (Cruz et al. 2005; Borges et al. 2014).

In the LC, some studies addressed the expression of pERK1/2 in painful conditions. Thus, the formalin injection into the hind paw resulted in increased ERK1/2 activation in the LC for 1 hour after the injection, while acute ERK1/2 activation after hind paw injection of CFA was not that prolonged (Imbe et al. 2009). It was shown that a great part (90%) of the cells expressing pERK1/2 were TH-positive neurons (Imbe et al. 2009). On the contrary, in the CCI model (7 days), it was shown that pERK1/2 was significantly decreased in the LC neurons (Borges et al. 2013). Despite the indication of a higher or lesser activation of LC neurons in certain pain conditions, this data is quite unsatisfactory when we are looking for the specificity of ERK1/2 role in those conditions.

2.7.2. ERK1/2 in pain-associated disorders

Apart from the well-known role in pain signaling, at least at the spinal cord level, new roles have been attributed to ERK1/2 pain signaling at supraspinal levels in the last few years. Indeed, more than to the sensory component, ERK1/2 has also been associated to the affective/emotional component of pain (Borges et al. 2015). Important contributions for this knowledge were provided by Bravo and colleagues (2012) who showed for the first time that animals submitted simultaneously to the CCI model of pain and to a chronic mild stress protocol, as a model of depression, have increased aversion to painful experiences (Bravo et al. 2012). Interestingly, this behavioral response was accompanied by higher pERK1/2 expression and decreased neuronal density in the ACC (Bravo et al. 2012), suggesting that, although this cascade is highly activated, there is an evident neuronal loss under these experimental conditions. Thus, these studies represent undeniable evidences that depression might lead to impairments in pain interpretation in patients suffering from chronic pain, and that the ERK1/2 cascade in the ACC has a role on it (Bravo et al. 2012). Another important contribution was provided by Cao and colleagues (Cao et al. 2009). They reached similar conclusions by demonstrating the engagement of the ACC, which is part of the PFC, in the processing of the affective component of pain (Johansen et al. 2001) and a role for ERK1/2-mediated signaling in these events. They used the formalin model of acute pain and observed that ERK1/2 activation in the ACC is needed for the induction and expression of the affective pain component (Cao et al. 2009), and that the ERK cascade in the ACC plays a central role in managing the pain-related negative

emotion and pain-related aversive learning underlying pain-predictive recognition and avoidance (Cao et al. 2009).

The mechanisms associated to ERK signaling during anxio-depressive conditions are still barely known. It has been observed that ERK1/2 are involved in CRF receptor signaling in neuronal cells (Hauger et al. 2006). Interestingly, increased CRF neurotransmission has been associated with depression and anxiety and, more recently, its implication in pain was also shown (Hummel et al. 2010). Thus, it is possible that ERK1/2 modulate pain perception partly through mechanisms associated to CRF-mediated neurotransmission.

Altogether, these important studies reinforce the fact that the study of ERK1/2 expression might be an appropriate target in the search of the mechanisms underlying chronic pain conditions and/or anxio-depressive phenotypes associated to chronic pain.

Aims

3. Aims

There is still sparse data regarding the role of LC neuronal activity in chronic pain conditions. In addition, it is barely understood the role of this nucleus and how its activity is modulated in chronic pain conditions that lead to the development of anxiety and depressive behaviors. Moreover, although several evidences implicate the activation of the ERK1/2 cascade in pain signaling mechanisms in the spinal cord, at supraspinal levels less is known. Particularly in the LC, the associated molecular mechanisms are hardly explored, and the possible contribution of ERK1/2 might be much more complex, especially when anxiety and depressive behaviors are present as a result of the chronic painful condition.

Considering the above mentioned, the main aim of the present work was **to understand how chronic pain conditions affect the activity of the Locus coeruleus neurons and if the signaling-mediated by the ERK1/2 cascade would be correlated with that activity.** To achieve this goal several tasks, each devoted to a specific objective, were planned. First, to understand how ERK1/2-mediated signaling could be implicated in the development of neuropathic pain induced by the CCI model, we performed an extensive evaluation of the expression of phosphorylated ERK1/2 in the spinal cord, LC and other brainstem nuclei of CCI animals, either submitted or not to an acute noxious stimulation protocol (**Publication I**)**.** To correlate with existing data (Alba-Delgado et al. 2011), we used CCI animals with 7 days of disease development

(**Publication I**). Sham (control) rats were submitted to an identical experimental protocol. We are planning to repeat the procedures in stimulated and non-stimulated CCI animals at a longer disease duration equal to that used in MA animals (**Publication II**), but no additional behavioral or electrophysiological approaches were performed because there was already data regarding this in the CCI neuropathic pain model. Indeed, the development of anxiety- and depression-like behaviors has also been shown in the CCI model at prolonged time-points (4 weeks; Alba-Delgado et al. 2013). Additionally, electrophysiological studies had already shown that the tonic and phasic activity of the LC neurons was not altered at earlier time-points of the CCI condition, namely at 7 (Alba-Delgado et al. 2011) and 14 days (Bravo et al. 2013), but that they were significantly altered, particularly the phasic activity, at 28 days after the CCI surgery (Alba-Delgado et al. 2013).

At the time these experiments were performed it was unclear if a prolonged chronic inflammatory joint pain condition would also trigger the affective component of pain and what was the necessary disease duration for that to occur. Thus, we used the monoarthritis model induced by CFA injection into the tibiotarsal joint, and assessed the development of anxio-depressive behaviors in that chronic inflammatory painful condition (**Publication II**). Additionally, at supraspinal level, some sparse studies suggested the involvement of ERK1/2 signaling in the regulation of inflammatory pain (Imbe et al. 2009). To understand if these behavioral changes would be somehow associated with changes in ERK1/2 activation in the LC neurons, and in the main regions projecting to (PGi) or receiving afferents from (the spinal cord and

the PFC) the LC, we then evaluated the level of activation/phosphorylation of ERK1/2 following different time-points of monoarthritis duration (**Publication II**). This allowed us to correlate with our data in the CCI model and to contribute to the existing information regarding ERK1/2 implication in chronic pain processing at supraspinal level. Secondly, in order to confirm that the inflammatory nociceptive input was the main cause of behavioral and molecular changes, namely in the activation of the ERK1/2 cascade, we studied the effect of the application to the inflamed paw of a topic anti-inflammatory drug. With this we were able to study the effect of the successful blockade of the nociceptive inputs arising from the inflamed paw on the affective dimension of pain, as well as on the level of activation of ERK1/2 in the PGi-LC-PFC pathway (**Publication II**).

The electrophysiological activity of the LC neurons in response to exacerbated noxious input is also a hardly studied topic in chronic inflammatory pain models. Electrophysiological recordings of the LC neurons provide good quality data and allow the study of the two most important and recognized modes of LC function, the tonic and the phasic activities, as described (section 2.6.2.), which is a difficult, or even impossible, task to attain in studies devoted to the immunodetection of activation markers as are the pERK1/2 or the pCREB. Thus, to understand how the LC neurons activity is affected by the monoarthritic condition, we evaluated the electrophysiological properties of the tonic and evoked phasic activity of the noradrenergic neurons in the LC, in control and monoarthritic rats at different time-points of evolution of the inflammatory pain condition (**Publication III**). A significant

increase in the phasic activity of the LC neurons was observed following long-term monoarthritis, which correlated with the pattern of pERK1/2 expression in the LC. Consequently, to functionally associate LC neurons electrical activity to ERK1/2-mediated signaling, additional electrophysiological recordings of the phasic activity of the LC neurons were performed after local inhibition of ERK1/2 activation by the LC-intra administration of the SL327 compound (**Publication III**).

To evaluate the behavioral effects of blocking ERK1/2 activation directly in the LC, the nociceptive and anxiety-like behaviors were also evaluated in rats submitted to prolonged monoarthritis following an intra-LC administration of the same inhibitor of ERK1/2 activation (**Publication III**). Moreover, the expression of pERK1/2 in the ACC of the PFC was also addressed in those conditions (**Publication III**). To understand how the signaling mechanisms in the nociceptive system react to the monoarthritic chronic condition, the expression of activated/phosphorylated ERK1/2 and the TH expression were studied by immunohistochemistry in the LC of one week and four weeks monoarthritic rats, as compared to control non-inflamed animals (**Publication III**).

Finally, it has been described that CRF activates the ERK1/2 signaling cascade to produce internalization of CRF receptors (Bonfiglio et al. 2013). Additionally, there are strong evidences supporting a role played by CRF in the LC, particularly in stress-induced depression and anxiety conditions (Van Bockstaele et al. 2010). Taking into account that chronic inflammatory pain has already been considered as a “stressor” agent activating the CRF pathway (Hummel et al. 2010), we then explored the role of CRF-mediated neurotransmission in the LC in the nociceptive and anxiety-like

behaviors associated to prolonged inflammatory conditions by using a CRF receptor antagonist. We then evaluated the expression of CRF receptors in the LC as well as of pERK1/2 in the PVN, the main CRF supplier in the CNS, and in the LC, in the monoarthritic animals at prolonged time-points of disease and after the antagonist administration (**Publication IV**).

In summary, in order to accomplish the objectives proposed for this thesis, and attain the main aim of the present thesis, this study was divided into six aims, each with specific tasks, as follows:

- **Aim I – To evaluate if the pattern of ERK1/2 activation is altered in the CCI model of neuropathic pain, by:**
  - a) Evaluating ERK1/2 activation in the spinal cord and brainstem nuclei following seven days of chronic constriction injury (Publication I).
  - b) Analyzing if this pattern of ERK1/2 activation is altered following an additional mechanical stimulation of the area innervated by the constricted sciatic nerve (Publication I).

- **Aim II – To evaluate if a monoarthritic painful condition triggers the affective component of pain and if is this correlated with changes in ERK1/2 activation in the PGi-LC-PFC pathway, by:**
  - a) Evaluating the development of anxiety- and depressive-like behaviors at different time-points of the monoarthritic disease (Publication II).

- b) Assessing if ERK1/2 activation, evaluated by pERK1/2 expression, is altered in the PGi-LC-PFC pathway in those pathological conditions (Publication II).

- **Aim III – To evaluate if the behavioral and molecular changes observed in the monoarthritic animals (Aim II) are reversed by topical analgesia, by:**
  - a) Studying the effect of a local maintained analgesia in the anxiety- and depressive-like behaviors that had been triggered by the prolonged monoarthritic states (Publication II).
  - b) Investigating the effect of a local maintained analgesia in the monoarthritis-induced increased ERK1/2 activation in the PGi-LC-PFC pathway (Publication II).

- **Aim IV – To evaluate if the electrophysiological activity of LC neurons is altered at an earlier (when only nociceptive behavior is detected) and/or at a late-phase (when there is also an anxio-depressive phenotype) of the monoarthritic condition, by:**
  - a) Studying the tonic-spontaneous activity and the phasic-evoked activity of the LC neurons in anaesthetized rats bearing a monoarthritic condition (Publication III).

- **Aim V – To evaluate if the increased ERK1/2 activation (observed in Aim II) is correlated with an increased phasic activity of LC neurons (detected in Aim IV), and how is the noradrenergic demand and pERK1/2 in cortical areas (Aim II) in those conditions, by:**
  - a) Investigating the effect of inhibiting ERK1/2 activation directly in LC neurons (intra-LC injections) on the phasic activity of the LC neurons (Publication III).
  - b) Evaluating if ERK1/2 activation (pERK1/2 expression) in the LC and ACC is altered in monoarthritis-induced chronic inflammatory painful condition (Publication III).
  - c) Analyzing tyrosine hydroxylase expression in the LC of the same monoarthritic animals (Publication III).

- **Aim VI - To evaluate if the inhibition of the ERK1/2 activation in the LC in prolonged monoarthritis has effects on the nociceptive and anxiety-like behaviors, by:**
  - a) Investigating the effect of inhibiting ERK1/2 activation directly in LC neurons (intra-LC injections) on the nociceptive behavior (Publication III).
  - b) Investigating the effect of inhibiting ERK1/2 activation directly in LC neurons (intra-LC injections) on the anxiety-like behavior (Publication III).

- **Aim VII – To evaluate if the CRF neurotransmission in the LC upon prolonged inflammatory conditions plays a role in the nociception and anxiety-like behaviors and ERK1/2-mediated signaling, by:**
  - a) Evaluating changes in the expression of CRF receptors in the LC during monoarthritis (Publication IV).
  - b) Analyzing the activation of ERK1/2 in the PVN of the hypothalamus, the main supplier of CRF, during monoarthritis (Publication IV).
  - c) Studying the effect of blocking CRF neurotransmission within the LC (intra-LC injection of a CRF receptor antagonist) on the nociceptive and anxiety-like behaviors (Publication IV).
  - d) Studying the effect of blocking CRF neurotransmission within the LC (intra-LC injection of a CRF receptor antagonist) on the activation of ERK1/2 in LC neurons (Publication IV).

Results

4. Results

4.1. Publication I

Extracellular signal-regulated kinase activation in the chronic constriction injury model of neuropathic pain in anaesthetized rats.

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ORIGINAL ARTICLE

Extracellular signal-regulated kinase activation in the chronic constriction injury model of neuropathic pain in anaesthetized rats

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Conflicts of interest
The authors declare no conflict of interest.

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Abstract

Background: The role of extracellular signal-regulated kinases (ERKs) in nociception has been explored in the last years. While in spinal cord their activation is frequently correlated with pain or acute noxious stimuli, supraspinally, this association is not so evident and remains unclear. This study aims to evaluate ERK1/2 activation in the spinal cord and brainstem nuclei upon neuropathy and/or an additional mechanical stimulus.
Methods: Acute noxious mechanical stimulation was applied in the left hindpaw of anaesthetized SHAM-operated and chronic constriction injured (CCI, neuropathic pain model) rats. Other SHAM or CCI rats did not receive any stimulus. Immunohistochemistry against the phosphorylated isoforms of ERK1/2 (pERK1/2) was performed in lumbar spinal cord and brainstem sections to assess ERK1/2 activation.
Results: In the spinal cord, stimulation promoted an increase in pERK1/2 expression in the superficial dorsal horn of SHAM rats. No significant effects were caused by CCI alone. At supraspinal level, changes in ERK1/2 activation induced by CCI were observed in A5, locus coeruleus (LC), raphe obscurus (ROb), raphe magnus, dorsal raphe (DRN), lateral reticular and paragigantocellularis nucleus. CCI increased pERK1/2 expression in all these nuclei, with exception of LC, where a significant decrease was verified. Mechanical noxious stimulation of CCI rats decreased pERK1/2 expression in ROb and DRN, but no further changes were detected in either SHAM- or CCI-stimulated animals.
Conclusion: ERK1/2 are differentially activated in the spinal cord and in selected brainstem nuclei implicated in nociception, in response to an acute noxious stimulus and/or to a neuropathic pain condition.

1. Introduction

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family, translating extracellular stimuli into intracellular responses (Ji et al., 2009). Immunodetection of ERK1/2 phosphorylation/activation (pERK1/2) is observed in multiple compartments as neuronal perikarya and dendrites, allowing cell morphology delimitation (Flood et al., 1998; Koh et al., 2002). pERK1/2 expression in spinal cord dorsal horn neurons depends on nociceptive activity, therefore being used as a marker in pain studies (Ji et al., 2009). Indeed, spinal ERK1/2 activation was observed

What's already known about this topic?
- Expression of extracellular signal-regulated kinases (pERK1/2) in spinal cord dorsal horn neurons depends on nociceptive activity.
- The use of specific ERK inhibitors resulted in analgesia.
- ERK1/2 seem to be implicated in chronic pain and may even represent a therapeutic target.

What does this study add?
- Acute mechanical noxious stimulation modulates ERK1/2 activation in the spinal cord; supraspinally, the CCI is prevailing, in relation to acute stimulation promoting pERK1/2 increases or decreases; ERK1/2 activity in descending modulation pathways is differentially altered.

in the presence of allodynia and/or hyperalgesia (Gao and Ji, 2010; Han et al., 2011), two types of exacerbated nociception characteristic of persistent painful conditions. ERK1/2 involvement in the signalling of noxious stimuli has been studied in different pain models, and its spinal activation has been correlated with chronic inflammatory pain (Cruz et al., 2005, 2007; Imbe et al., 2009; Ji et al., 2009). Also, ERK1/2 seem to be implicated in neuropathic pain and may even represent a therapeutic target (Ciruela et al., 2003; Zhang et al., 2005). Accordingly, the use of specific upstream ERK inhibitors resulted in analgesia in several pain models (Ji et al., 2002; Galan et al., 2003; Cruz et al., 2005).

Despite the numerous studies evaluating pERK1/2 in spinal cord in distinct pain conditions (Song et al., 2005; Zhang et al., 2005), equivalent knowledge in supraspinal structures is scarce. Indeed, ERK1/2 activation in pain-related encephalic areas has been studied in only a few regions. Exemplifying, in locus coeruleus (LC or A_6), the main source of noradrenaline (NA) in central nervous system (CNS), high ERK1/2 activation induced by acute noxious stimulation was shown (Imbe et al., 2009). Spinal cord receives noradrenergic inputs from brainstem pontine A_5, LC and A_7 (Millan, 2002), and all these nuclei are connected with periaqueductal grey (PAG, Bajic and Proudfit, 1999), a region involved in descending pain modulation. Serotonergic nuclei, as the raphe magnus (RMg), dorsal raphe (DRN) and median raphe, are also important in pain modulation (Suzuki et al., 2004). RMg has been proposed as one region responsible for the increased serotonin (5-HT) release into the spinal cord verified upon PAG stimulation (Cui et al., 1999). Interestingly, attenuation of formalin-evoked spinal ERK1/2 activation was observed upon depletion of spinal 5-HT (Svensson et al., 2006).

In chronic inflammatory pain, we showed that spinal ERK1/2 activation requires movement of the arthritic joint (Cruz et al., 2005). We also reported increased pERK1/2 expression in spinal cord, paragigantocellularis nucleus (PGi) and DRN in the chronic constriction injury (CCI, Bennett and Xie, 1988) model of neuropathic pain (Alba-Delgado et al., 2012). However, it is unknown if this happens in other supraspinal structures implicated in cognitive, affective and sensory functions following neuropathic pain or if this is altered by additional noxious stimulation. For effective therapeutic purposes this is important to check. We evaluated, by immunohistochemistry, CCI-induced pERK1/2 expression in lumbar spinal cord and selected brainstem areas, and the effect of a mechanical noxious stimulation applied in the hindpaw of anaesthetized rats.

2. Methods

2.1 Animals

Experiments were carried out in 39 adult male Harlan Sprague-Dawley rats (250–300 g), housed 4 per cage under controlled conditions of lighting (12 h light/12 h dark cycle), temperature (22 °C) and humidity (45–60%), with food and water *ad libitum*. All efforts were made to minimize distress and reduce the number of animals. Procedures followed the European Communities Council Directive (86/609/EEC), the Spanish Law (RD 1201/2005) and the ethical guidelines for pain investigation in animals (Zimmermann, 1983).

2.2 Neuropathic pain model

Neuropathic pain was induced by sciatic nerve constriction (CCI model) following established surgical procedures (Bennett and Xie, 1988; Berrocoso et al., 2007) in anaesthetized rats (intraperitoneal sodium pentobarbital, 50 mg/kg; Sigma, Madrid, Spain). Briefly, four chromic gut (4/0) ligatures were loosely tied around the left sciatic nerve, about 1.0 mm apart (CCI operated) and the surgical wound was sutured. Control animals (SHAM) experienced similar procedures except for the sciatic nerve ligation. All animals were used 7 days after SHAM or CCI surgeries. Behavioural tests were performed in an additional set of SHAM and CCI animals to avoid further paw stimulation.

2.3 Validation of the neuropathic pain model

A parallel set of animals (SHAM $n = 8$ and CCI $n = 8$) was used to evaluate mechanical and thermal hyperalgesia, as described (Alba-Delgado et al., 2012). Rats were habituated for 3 days before experiments. Randall–Selitto's paw pressure apparatus (Ugo Basile, Comerio, Italy) was used to evaluate the withdrawal threshold to paw mechanical stimulation (Randall and Selitto, 1957). The hindpaw was submitted to an increasing force (250 g cut-off) until rats vocalized or withdrew the paw. Reduced withdrawal latency indicates mechanical hyperalgesia.

Withdrawal threshold to paw thermal stimulation was evaluated by the Hargreaves' plantar test apparatus (Ugo Basile, Hargreaves et al., 1988). Animals were placed on a 2-mm-thick glass floor with an infrared heat generator under the floor targeted at the rat's hindpaw. Rats could freely withdraw the paw and the paw withdrawal latency was recorded automatically. Reduced withdrawal latency indicates thermal hyperalgesia. In both tests, three measures taken on each paw at 5-min intervals were averaged.

2.4 Noxious mechanical stimulation

To evaluate the effect of noxious mechanical stimulation on ERK1/2 activation, SHAM or CCI animals were anaesthetized by intraperitoneal injection of chloral hydrate (35%, 350 mg/kg; Panreac, Barcelona, Spain) to avoid confounding factors resultant from painful stimulation (e.g., arousal, anxiety or stress) which, by themselves, could trigger ERK1/2 activation, and were submitted to mechanical noxious stimulation. This consisted of 4 pinches/min (1–2 s/pinch) for 20 min, manually applied with a similar and constant force with a rat-tooth forceps (Pinto et al., 2003) in the left hindpaw area innervated by the sciatic nerve [SHAM-stimulated (SS) and CCI stimulated (CCIS), $n = 6$ per group]. Other two groups of anaesthetized rats were not submitted to noxious stimulation [SHAM not stimulated (SNS), $n = 6$; CCI not stimulated (CCINS), $n = 5$].

2.5 Tissue processing and immunohistochemistry

After noxious mechanical stimulation (or not), the anaesthetized animals were transcardially perfused through the ascending aorta with 250 mL of oxygenated Tyrode's solution followed by 750 mL of paraformaldehyde 4% in phosphate buffer, 0.1 mol/L pH 7.2. Brains and spinal cords were processed for free-floating immunohistochemistry (Cruz et al., 2005; Nascimento et al., 2011) against pERK1/2. One in each five 40-μm brainstem or L4, L5 and L6 spinal cord sequential transverse sections from each rat went through washes and incubation in blocking solution (Cruz et al., 2005), and were incubated in antibody raised in rabbit against the phosphorylated isoforms ERK1 and ERK2 (pERK1/2; 1:1000; 48 h at 4–8 °C; Neuromics, Edina, Minnesota, USA). Immunodetection was achieved upon incubation in biotinylated swine anti-rabbit antiserum (1:200; 1 h; DAKO, Glostrup, Denmark), followed by ABC solution (1:200, 1 h; ABC Elite kit; Vector Laboratories, Peterborough, UK) and reaction with 3,3-diaminobenzidine tetrahydrochloride (DAB; 10 min) (Cruz et al., 2005).

To evaluate the nature of the pERK1/2 IR cells in spinal cord sections of CCI rats, double immunolabelling against pERK1/2 and the anti-neuronal nuclear antigen (NeuN), specific for mature neurons in vertebrates (Mullen et al., 1992), was performed by incubation (two overnights) in rabbit anti-pERK1/2 (1:1000) and mouse anti-NeuN (1:100; Millipore, Chemicon, CA, USA) primary antibodies. The immunoreaction was revealed using as secondary antibodies biotinylated donkey anti-rabbit (1:200) followed by incubation in Streptavidin conjugated with alexa®488 (1:200; Invitrogen, Molecular Probes, USA) and donkey anti-mouse conjugated with alexa®568 (1:1000; Invitrogen, Molecular Probes). A similar procedure was used to evaluate the neurochemical nature of noradrenergic [sheep anti-tyrosine hydroxylase (TH), 1:2000; Abcam, Cambridge, UK] or serotonergic [sheep anti-tryptophan hydroxylase (TrpOH), 1:500; Millipore, Chemicon] areas in supraspinal adjacent sections. Biotinylated donkey anti-rabbit (1:200), streptavidin conjugated with alexa®488, and donkey anti-sheep conjugated with alexa®568 (1:1000) were used. Specificity was controlled by omitting each primary antibody in various sets of immunoreactions.

2.6 Data analysis and statistics

pERK1/2 labelling was studied in spinal cord segments L4, L5 or L6, and in the following brainstem nuclei: A_5, LC (A_6), A_7, raphe obscurus nucleus (ROb), RMg, DRN, lateral reticular nucleus (LRt), PGi, gigantocellular reticular pars alpha nucleus (GiA), PAG, lateral parabrachial nucleus (LPB) and Edinger–Westphal nucleus (EW). Histological delimitation of brain areas and respective rostrocaudal extensions was made

according to The Rat Brain Atlas (Paxinos and Watson, 2009) and co-localization with TH or TrpOH, for noradrenergic or serotonergic nuclei, respectively. Quantification of pERK1/2 immunoreactive (IR) cells in spinal cord was performed, as described (Cruz et al., 2005), by using an optical light microscope (Axioskop 40; Zeiss, Hertfordshire, UK) and counting the number of cell bodies with a brownish labelling for DAB in laminae I–III, IV–VI, VII–IX and X (Molander et al., 1984; Cruz et al., 2005), in a total of 15 sections per animal (5 sections of the caudal portion of segment L4 and 10 sections of segments L5 and L6).

At supraspinal level, a similar approach was used to count pERK1/2 IR cells in all sections containing the analysed regions, per animal, except for LC and LPB. The average number of pERK1/2 IR cells/section/animal was statistically analysed. In LC and LPB, a distinct pERK1/2 fibre labelling intensity was visually detected in rostrocaudal extensions, and so separate quantification was performed. Photomicrographs of all sections containing the LC or LPB, of regions with only non-specific labelling, and of the glass in each slide, per animal, were taken, as described (Alba-Delgado et al., 2011). Acquisition conditions (objective amplification, light intensity, contrast and hue) were maintained constant. Densitometric analysis of each photomicrograph was performed using free access software (Image J 1.42). Mean grey densitometric values/section/animal were calculated by using a specific equation as described (Alba-Delgado et al., 2012), and were averaged. Values of pERK1/2 expression in LC or LPB were expressed as densitometry optic units/section/animal and were statistically analysed. Co-localization of pERK1/2 with NeuN, TH or TrpOH was evaluated by using an AxioImager Z1 (Zeiss) fluorescence microscope.

Behavioural data were statistically compared by two-way analysis of variance (ANOVA) for factors 'surgery' and 'time' followed by the Bonferroni *post hoc*. For the immunohistochemistry data, at spinal level, only data from the ipsilateral side were considered since contralateral pERK1/2 expression was insignificant. At supraspinal level, as no statistically significant differences between ipsi- and contralateral sides were detected (independent Student's *t*-test), values from both sides were pooled to calculate the mean number of IR cells or densitometry per section and per animal. Two-way ANOVA for the factors 'stimulation' and 'surgery' was performed followed by the Fisher least significant difference (LSD) *post hoc* test. The level of significance accepted was 0.05. GraphPad Prism 5 and STATISTICA software were used.

3. Results

3.1 Chronic neuropathic pain

In 7 days, CCI rats developed a stable mononeuropathy in the operated (ipsilateral) paw, characterized by mechanical and thermal hyperalgesia. Both the mechanical pressure supported by animals and the withdrawal latency to thermal stimuli, on the ipsilateral paw, were significantly reduced in CCI when compared with SHAM animals ($p < 0.001$ and $p < 0.01$, respectively; Supporting Information Fig. S1). No differences were observed contralaterally. SHAM rats did not develop any neuropathic reaction. All animals were healthy (no abnormal behaviour).

3.2 Spinal ERK1/2 activation

pERK1/2 expression in L4, L5 and L6 spinal cord segments of SHAM or CCI rats either receiving, or not, a mechanical stimulation is depicted in Supporting Information Table S1 and Fig. 1. Positive immunolabelling to pERK1/2 was observed in perikarya and fibres, and co-localizes with NeuN indicating that ERK1/2 are activated in neurons (Supporting Information Fig. S2). Few (less than 10) pERK1/2 IR cells/section were detected in SNS rats in every lamina of the lumbar spinal cord, both ipsi- and contralaterally. Noxious mechanical stimulation of SHAM rats (SS group) induced a significant increase of pERK1/2 IR cells/section in the ipsilateral laminae I–III ($p < 0.001$), when compared with the same laminae of SNS rats (Supporting Information Table S1 and Fig. 1A). Moreover, in SS rats, pERK1/2 IR cells were concentrated in the medial region of the ipsilateral superficial dorsal horn (Fig. 1A, SS). Additionally, the number of pERK1/2 IR cells/section in SS was significantly higher than in CCINS and CCIS animals, in laminae I–III ($p < 0.01$ and $p < 0.05$, respectively, Supporting Information Table S1 and Fig. 1A). Thus, the noxious mechanical stimulation was sufficient to induce a higher ERK1/2 activation than the CCI condition alone or combined with the stimulus. In CCINS, the number of pERK1/2 IR cells/section in laminae I–III was higher than in SNS (but without statistical significance) and positive neurons were distributed in all superficial dorsal horn (Fig. 1B, CCINS), contrary to that observed in SS and CCIS. Noxious mechanical stimulation of CCI rats produced a significantly higher number of pERK1/2 IR cells ($p < 0.05$) in superficial dorsal horn than that observed in SNS, but also significantly lesser than that observed in SS animals ($p < 0.05$, Supporting Information Table S1 and Fig. 1A), suggesting a different effect of

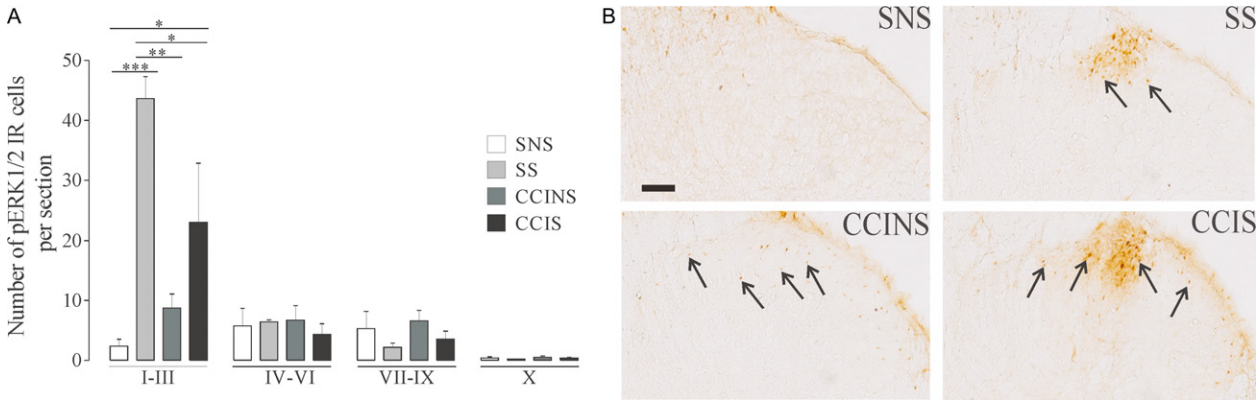

Figure 1 Effect of chronic constriction injury (CCI) in the left sciatic nerve and/or application of a noxious mechanical stimulation to the ipsilateral hindpaw on the average number of pERK1/2 IR cells/section counted in four different regions of the lumbar spinal cord grey matter. (A) In the superficial dorsal horn laminae I–III, the CCI condition (CCI not stimulated group, CCINS) induced an increase (not statistically significant) in the number of pERK1/2-labelled cells when compared with SHAM (SHAM not stimulated group, SNS). Acute mechanical noxious stimulation significantly increased pERK1/2 expression in the same region of SHAM (SHAM stimulated group, SS) but had no significant effect on CCI rats (CCI stimulated group, CCIS), when compared with the respective not stimulated groups. No changes were detected in deep dorsal horn (laminae IV– VI), ventral horn (laminae VII–IX) and central canal (lamina X). (B) Photomicrographs showing that pERK1/2 labelling in the spinal cord from SS and CCIS rats has a medial distribution in ipsilateral superficial dorsal horn while in the CCINS, this distribution pattern was not observed; scale bar = 100 μm. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ by two-way analysis of variance followed by Fisher least significant difference *post hoc* test.

the noxious stimulation in SHAM and in neuropathic animals, confirmed by the significant value ($p < 0.05$) obtained for the 'interaction' between the factors 'surgery' and 'stimulation', in laminae I–III (Supporting Information Table S1). However, similar to SS rats, a medial distribution of pERK1/2 IR cells was observed (Fig. 1B, CCIS).

In laminae IV–VI, VII–IX and X, no changes were observed between any group (Supporting Information Table S1 and Fig. 1A).

3.3 Brainstem ERK1/2 activation

pERK1/2 immunolabelling in almost all brainstem nuclei consisted of a brownish precipitate in perikarya and dendrites (Supporting Information Fig. S2). In general, a baseline labelling was observed in all nuclei analysed in the SNS group (Supporting Information Table S2). Double immunoreactions confirmed the localization of the A_5, LC and A_7 (TH-positive regions) or ROb, RMg and DRN (TrpOH-positive regions). Overall, statistical analysis of the factor 'stimulation' did not detect significant changes between the experimental groups in any brainstem nuclei studied. Thus, the mechanical noxious stimulation, *per se*, did not seem to induce significant changes in ERK1/2 activation in brainstem nuclei, either in SHAM or CCI rats. However, two-way ANOVA for the factor 'surgery' indicated that the neuropathic condition induced significant changes in ERK1/2 activation in some nuclei, as subsequently described.

3.3.1 A_5, LC and A_7 regions

Statistically significant changes between groups were identified for the factor 'surgery' in A_5 ($p < 0.05$) and LC ($p < 0.05$, $p < 0.01$ and $p < 0.01$ in total, caudal or rostral portions, respectively, Supporting Information Table S2). In A_5, a significantly increased number of pERK1/2 IR cells/section in CCINS was observed, when compared with SNS ($p < 0.01$) or SS rats ($p < 0.05$, Supporting Information Table S2 and Fig. 2A). In contrast, pERK1/2 IR-fibre intensity was significantly decreased in LC in CCINS ($p < 0.05$, for total LC, LC caudal or LC rostral) or CCIS ($p < 0.05$ in total LC or in the caudal extension; $p < 0.01$ in LC rostral), when each was compared with SNS animals (Supporting Information Table S2 and Fig. 2B). No significant changes were observed in A_7.

3.3.2 ROb, RMg and DRN regions

In rostral ROb, a significant interaction ($p < 0.01$, Supporting Information Table S2) between the 'surgery' and 'stimulation' factors was observed, meaning that noxious mechanical stimulation differently affects SHAM and CCI animals. The Fisher LSD *post hoc* test detected a significant increase in the number of pERK1/2 IR cells/section in SS ($p < 0.05$) and CCINS ($p < 0.05$) when compared with SNS rats (Supporting Information Table S2 and Fig. 3A). In contrast, a significantly decreased number of pERK1/2 IR cells/section was observed in CCIS when compared with

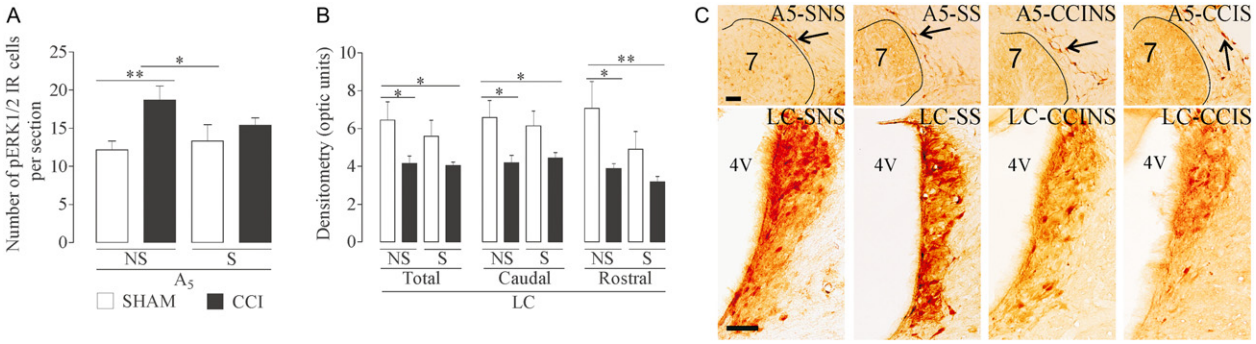

Figure 2 Effect of chronic constriction injury (CCI) in the left sciatic nerve and/or application of a noxious mechanical stimulation to the ipsilateral hindpaw on the average number of pERK1/2 IR cells/section or in the densitometry/section in the noradrenergic nuclei. (A) The CCI condition significantly increased pERK1/2 expression in the A5. No changes were observed following noxious mechanical stimulation of either SHAM or CCI animals. (B) CCI groups (either stimulated or not) showed significant decreases in pERK1/2 expression at all levels of LC (total, caudal and rostral). (C) Photomicrographs showing the localization or pERK1/2 labelling in A5 and in the caudal part of LC in SHAM not stimulated (SNS), SHAM stimulated (SS), CCI not stimulated (CCINS) and CCI stimulated (CCIS) rats; scale bar = 100 μm. 7 – facial nucleus, 4V – fourth ventricle. *$p < 0.05$ and **$p < 0.01$ by two-way analysis of variance followed by Fisher least significant difference *post hoc* test.

CCINS ($p < 0.05$) or SS animals ($p < 0.05$, Supporting Information Table S2 and Fig. 3A). In RMg, a significant value for the factor 'surgery' was verified ($p < 0.05$, Supporting Information Table S2), and the Fisher LSD *post hoc* test revealed a significantly increased number of pERK1/2 IR cells in CCINS when compared with SNS animals ($p < 0.01$, Supporting Information Table S2 and Fig. 3A). In DRN, the factor 'surgery' was significant ($p < 0.01$, Supporting Information Table S2), and a significantly increased number

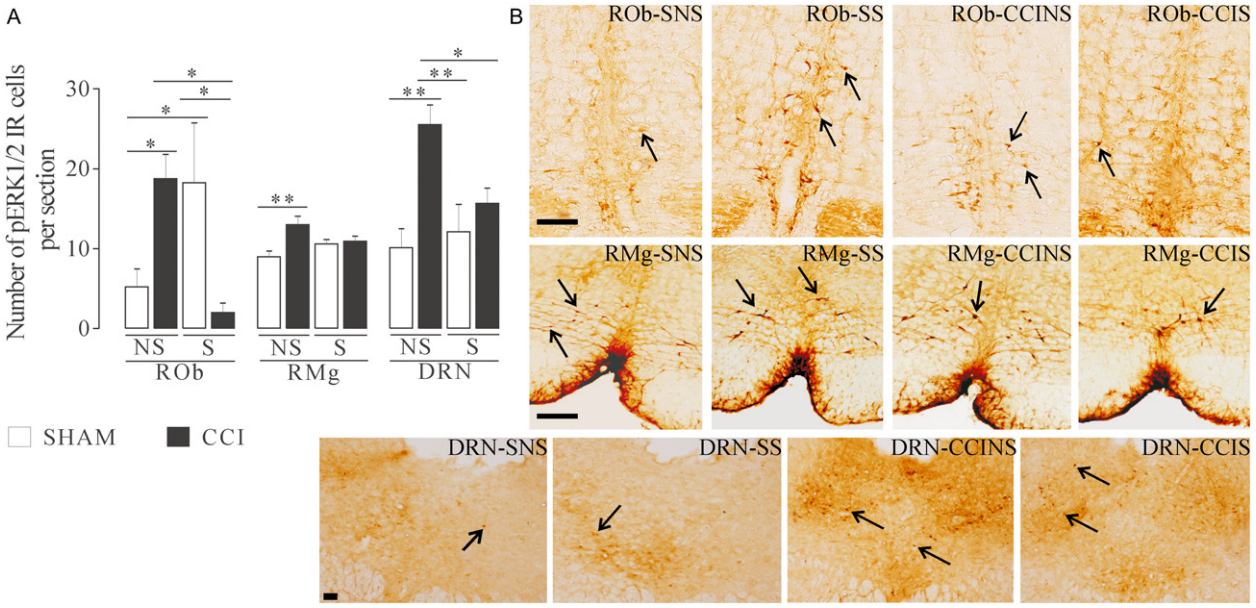

Figure 3 Effect of chronic constriction injury (CCI) in the left sciatic nerve and/or application of a noxious mechanical stimulation to the ipsilateral hindpaw on the average number of pERK1/2 IR cells/section in the serotonergic nuclei. (A) The CCI condition significantly increased pERK1/2 expression in ROb, RMg and DRN. Acute noxious stimulation applied to SHAM animals also resulted in increased pERK1/2 expression in ROb. In RMg, no changes were observed in stimulated (either SHAM or CCI) groups. In ROb and DRN, acute noxious stimulation of CCI animals induced a decrease in the number of pERK1/2 IR cells. (B) Photomicrographs showing the localization or pERK1/2 labelling in ROb, RMg and DRN in SHAM not stimulated (SNS), SHAM stimulated (SS), CCI not stimulated (CCINS) and CCI stimulated (CCIS); scale bar = 100 μm. *$p < 0.05$ and **$p < 0.01$ by two-way analysis of variance followed by Fisher least significant difference *post hoc* test.

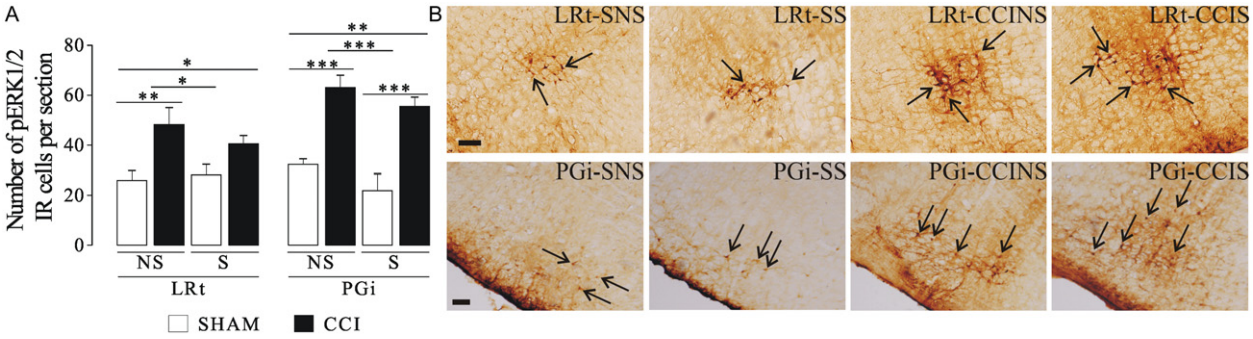

Figure 4 Effect of chronic constriction injury (CCI) in the left sciatic nerve and/or application of a noxious mechanical stimulation to the ipsilateral hindpaw on the average number of pERK1/2 IR cells/section in the LRt and PGi. (A) The CCI condition significantly increased pERK1/2 expression in LRt. Acute noxious stimulation had no significant effects either in SHAM or CCI when compared with the respective not stimulated groups. In PGi, a significant increase in pERK1/2 expression was detected in CCI groups (either stimulated or not) in comparison to the respective SHAM groups. (B) Photomicrographs showing the localization or pERK1/2 labelling in LRt and PGi in SHAM not stimulated (SNS), SHAM stimulated (SS), CCI not stimulated (CCINS) and CCI stimulated (CCIS); scale bar = 100 μm. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ by two-way analysis of variance followed by Fisher least significant difference *post hoc* test.

of pERK1/2 IR cells was detected in CCINS when compared with SNS ($p < 0.01$), SS ($p < 0.01$) and CCIS animals ($p < 0.05$, Fig. 3A).

3.3.3 Rostroventromedial and ventrolateral medulla

In LRt [from ventrolateral medulla (VLM)] and PGi [from rostroventromedial medulla (RVM)], a significantly increased number of pERK1/2 IR cells/section was observed in CCINS when compared with SNS ($p < 0.01$ for LRt and $p < 0.001$ for PGi) or with SS ($p < 0.05$ for LRt and $p < 0.001$ for PGi), or in CCIS when compared with SNS ($p < 0.05$ for LRt and $p < 0.01$ for PGi) or with SS ($p < 0.001$ for PGi only, Supporting Information Table S2 and Fig. 4A). No significant changes were observed in GiA.

3.3.4 Other brainstem nuclei

In the ventrolateral PAG (VLPAG), LPB and EW no statistical significant differences were detected.

4. Discussion

This study shows that in 7-day CCI rats, ERK1/2 are activated in the spinal cord and in several brainstem monoaminergic regions implicated in pain modulation (Pinto et al., 2003; Almeida et al., 2004; Rouwette et al., 2010). Additionally, mechanical noxious stimulation increased pERK1/2 expression in the superficial dorsal horn of SHAM but not of CCI rats, while, supraspinally, the acute stimuli *per se* did not induce any change.

The spinal cord laminae I–VI are majorly implicated in the reception and transmission of nociceptive information (Rexed, 1952; Willis and Coggeshall, 1991). Few IR cells were observed in SHAM rats, but pinching the ipsilateral hindpaw promoted an increase in pERK1/2 IR cells in laminae I–III, indicating that 20 min of noxious mechanical stimulation is sufficient to induce ERK1/2 activation in spinal cord. While mechanical stimulation induced an increase of pERK1/2 IR cells in laminae I–III of SHAM rats, when applied to CCI rats no changes were detected, suggesting that the processing of this stimulus is altered at the spinal cord level, when other nociceptive inputs, which are probably more deleterious to the organism, are already being chronically perceived. Thus, CCI seems to be sufficient to induce some degree of tonic ERK1/2 activation while additional noxious stimulation of these animals only provokes a redistribution of IR cells. Although ERK1/2 activation in superficial dorsal horn during neuropathic pain has already been demonstrated (Song et al., 2005; Alba-Delgado et al., 2011), consistent with an increased number (although not statistically significant) of pERK1/2 cells in laminae I–III of CCINS rats, the effect of noxious stimulation during neuropathic pain conditions had never been addressed. Actually, the prevalence of ERK1/2 activation in laminae I–III in SS and CCIS animals is expected since superficial dorsal horn neurons have been implicated in acute pain processing (Coghill et al., 1991; Porro and Cavazzuti, 1996) and in noxious stimuli response through pERK1/2 (Cruz et al., 2005; Yanagidate and Strichartz, 2007). Overall, these data point to a relation between high levels of pERK1/2 in laminae I–III

and the processing of nociceptive input from the injured paw.

Supraspinally, changes in pERK1/2 immunolabelling occurred in a few nuclei, mostly in CCI animals when compared with SHAM, but they were not significantly influenced by noxious stimulation in any region analysed. However, significant interactions between factors were obtained for some nuclei.

4.1 A_5, LC and A_7 regions

The A_5, LC and A_7 provide the main noradrenergic inputs into the spinal cord (Proudfit, 1988; Kwiat and Basbaum, 1992). The A_5 is involved in pain modulation, playing both antinociceptive (Miller and Proudfit, 1990; Burnett and Gebhart, 1991) and pronociceptive roles (Sagen and Proudfit, 1986; Tavares and Lima, 2002; Marques-Lopes et al., 2010). Increased pERK1/2 immunolabelling in CCINS suggests an enhanced activity of A_5 neurons during neuropathic pain, which may be related with increased NA synthesis and/or release into the spinal cord. In fact, an increased activation of cAMP response element-binding protein (another neuronal activation marker) and TH (an NA synthesis rate-limiting enzyme) labelling in A_5 upon painful diabetic neuropathy was observed along with increased NA at spinal level (Morgado et al., 2011). In mononeuropathy, as is CCI, this remains to be clarified. The role of the LC in pain modulation is well established (Tsuruoka et al., 2003; Maeda et al., 2009; Khakpay et al., 2010) at both ascending and descending pathways. In LC, both pronociceptive and antinociceptive roles were described. Hence, lesions of LC's noradrenergic neurons resulted in increased thermal hyperalgesia induced by inflammation and also in increased responsiveness of dorsal horn neurons (Tsuruoka and Willis, 1996; Wei et al., 1999; Tsuruoka et al., 2003), suggesting a role in pain inhibition. Contrarily, similar lesions in LC neurons also resulted in decreased tonic behavioural responses to intraplantar formalin injection (Martin et al., 1999; Taylor et al., 2000), and reduced mechanical hyperalgesia, tactile and cold allodynia after spared sural nerve injury (Brightwell and Taylor, 2009), suggesting a role in pain facilitation. Here, a decreased pERK1/2 labelling was observed in CCI animals when compared with SHAM, indicating a general decrease in LC neurons activity following CCI. In contrast, ERK1/2 were activated in LC for 1 h following formalin injection into the hindpaw (Imbe et al., 2011), while complete Freund's adjuvant (CFA)-evoked chronic inflammation did not induce prolonged ERK1/2 activation (Imbe et al., 2009). Thus, different pain modalities might exert unique effects on ERK1/2 activation in LC neurons. Interestingly, chronic restraint stress produced hyperalgesia and significantly decreased pERK1/2 IR cells in LC and the analgesia produced by acute stress is accompanied by mild pERK1/2 IR cells increase (Imbe et al., 2004). This suggests that LC responds to acute stimuli by increasing ERK1/2 activity, probably activating the descending inhibitory pathway, but when nociceptive stimulation becomes chronic such activation is compromised. Overall data suggest that nerve injury modified the evoked LC activity pattern leading to an altered descending modulation of painful stimuli and contributing to the pain state. In A_7, no changes were detected, indicating that in this region the nociceptive processing does not involve ERK1/2 activation. Interestingly, no changes in TH expression in A_7 during diabetic neuropathy were observed (Morgado et al., 2011).

4.2 ROb, RMg and DRN regions

ERK1/2 phosphorylation was also evaluated in ROb, RMg and DRN. ROb involvement in pain modulation has been proposed (Aicher and Randich, 1990) but the underlying mechanisms remain largely unknown. Increased pERK1/2 expression was observed following either noxious stimulation of SHAM or CCI animals, suggesting that ERK1/2 activation is implicated in nociceptive-related signalling events occurring at ROb. However, when the stimuli were applied to neuropathic animals, no further increase was produced, suggesting that this extra input is minor when compared with the input resultant from nerve injury, and that, its processing does not require ERK1/2 activation. Interestingly, C-Fos expression, another neuronal activation marker, is increased in ROb after noxious mechanical stimulation (Pinto et al., 2003), and this occurred mainly in neurons expressing gamma aminobutyric acid type B (GABAB) receptors (Pinto et al., 2003), pointing to the involvement of the GABAergic system. In RMg, a small increase was detected in pERK1/2 immunolabelling upon CCI. Others obtained a similar result upon chronic stress (Imbe et al., 2004), suggesting some analogy between these two chronic situations. In DRN, CCI induced an increased ERK1/2 expression, consistent with our previous data (Alba-Delgado et al., 2011). The DRN constitutes the major 5-HT source in CNS (Dahlstrom and Fuxe, 1964), and is involved in other (patho)physiological functions beyond pain processing (Greenwood et al., 2003). Moreover, the DRN connects directly to the LC (Kim et al., 2004) providing a potent negative input (Segal,

1979), which might explain why, in LC, a decreased ERK1/2 activation was verified during CCI.

4.3 RVM and VLM

In PGi, a nucleus of the RVM, there was increased pERK1/2 immunolabelling in CCI animals, compared with SHAM, suggesting a highest neuronal activation during neuropathic pain consistent with previous results (Alba-Delgado et al., 2012). The RVM plays an important role in the nociceptive activity of spinal cord neurons and in pain transmission (Porreca et al., 2002). Activation of distinct groups of RVM neurons results in descending inhibition or facilitation and, in particular, the PGi constitutes the main excitatory LC afferent (Carlson et al., 2007; Heinricher et al., 2009). Additionally, the LC is under the inhibitory control of the serotonergic DRN (Kim et al., 2004), being puzzling how these excitatory and inhibitory drives might be affecting ERK1/2 activation in LC. Mechanisms still unknown are probably implicated. Moreover, in LRt, which belongs to the VLM, another region highly engaged in pain modulation (Heinricher et al., 2009), similar data were obtained. The increase in pERK1/2 IR cells during CCI suggests the involvement of ERK1/2 cascade in neuropathic pain modulation in those nuclei of the RVM and VLM.

4.4 Other brainstem nuclei

PAG acts in ascending and descending nociceptive systems and electrical stimulation of its ventrolateral column produces antinociception (Porreca et al., 2002). Despite the reasonable pERK1/2 baseline labelling found in VLPAG, no significant changes were observed, indicating a low recruitment of the ERK1/2 signalling cascade in VLPAG for either acute or neuropathic pain modulation. Consistently, few studies have detected ERK1/2 activation in PAG. Exemplifying, discrete increases in pERK1/2 neuronal density were found in ether-anaesthetized noxious-stimulated rats (Gioia et al., 2005). Also in LPB and EW, no changes in the ERK1/2 activation pattern were observed in CCI or after acute noxious stimulation, despite their roles in pain modulation (Basbaum and Fields, 1979; Gauriau and Bernard, 2002). Therefore, chronic pain modulation in these nuclei may not involve the ERK1/2 cascade.

Concluding, we demonstrate that ERK1/2 activation is involved in neuropathic pain and/or acute mechanical noxious stimulation processing, in spinal cord and selected brainstem regions. While in spinal cord acute noxious stimulation modulates ERK1/2 activation, the same does not happen in most of the brainstem nuclei analysed. Supraspinally, the CCI condition is prevailing, in relation to acute stimulation, inducing pERK1/2 increases in some nuclei and decreases in LC, suggesting that ERK1/2 activity in descending modulation pathways is differentially altered, contributing to the pain state. Therefore, the use of MAPK/ERK kinase (MEK) inhibitors must be carefully considered in neuropathic pain treatment.

Author contributions

The first author, Gisela Borges, performed all the behavioural tests, immunohistochemistry and further analysis (quantification and statistics). Esther Berrocoso and Antonio Ortega-Alvaro performed the SHAM and CCI surgeries. Esther Berrocoso, Juan A. Micó and Fani Neto conceived and designed the experiments. Esther Berrocoso supervised the behavioural studies and Fani Neto supervised the immunohistochemistry studies. All authors discussed the results. Gisela Borges was responsible for writing the first drafts of the manuscript and revising it. All other authors were involved in revising it critically for important intellectual content, and all authors approved the final version.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Graphs depicting the effect of chronic constriction injury (CCI) of the left sciatic nerve on the mechanical and thermal threshold of the ipsilateral paw (A and C, respectively) and contralateral paw (B and D, respectively). In the ipsilateral paw, both mechanical (A) and thermal (B) hyperalgesia was observed at 7 days after the CCI surgery. No significant changes were observed in the contralateral paw. $^{**}p < 0.01$ and $^{***}p < 0.001$ by two-way ANOVA followed by Bonferroni *post hoc* test.

Figure S2. Photomicrographs showing the co-localization of pERK1/2 (green) with NeuN, TH or TrpOH (red). Double labelling was used to evaluate the nature of the cells expressing pERK1/2 in spinal cord (neurons, co-localization with NeuN) and also to facilitate the delimitation of noradrenergic (A_5, LC and A_7, co-localization with TH) and serotonergic (ROb, RMg and DRN, co-localization with TrpOH) populations. Double-labelled cells are indicated by yellow colour in the merged figure (arrows). Scale bar = 100 μm in all fluorescent photomicrographs. Note the localization of DAB brownish precipitate in the perikarya, dendrites and nucleus of the neuron (right corner of figure). Scale bar = 10 μm.

Table S1. Table depicting the number of pERK1/2 IR cells per section for each spinal cord region analysed in the ipsilateral side in each experimental group. Values expressed as mean ± SEM *p*-values were obtained by two-way ANOVA followed by Fisher LSD *post hoc* test.

Table S2. Table depicting the number of pERK1/2 IR cells or the densitometric values (LC and LPB) per section for each brainstem nucleus studied in each experimental group. Values expressed as mean ± SEM *p*-values were obtained by two-way ANOVA followed by Fisher LSD *post hoc* test.

Appendix S1. Additional discussion.

Figure S1

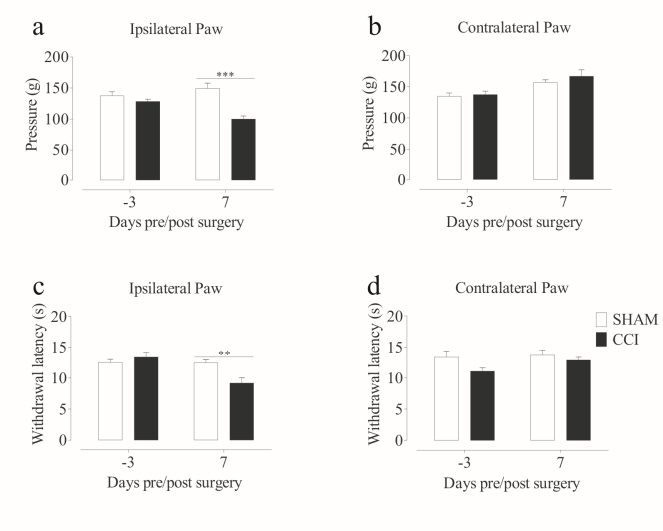

Figure S2

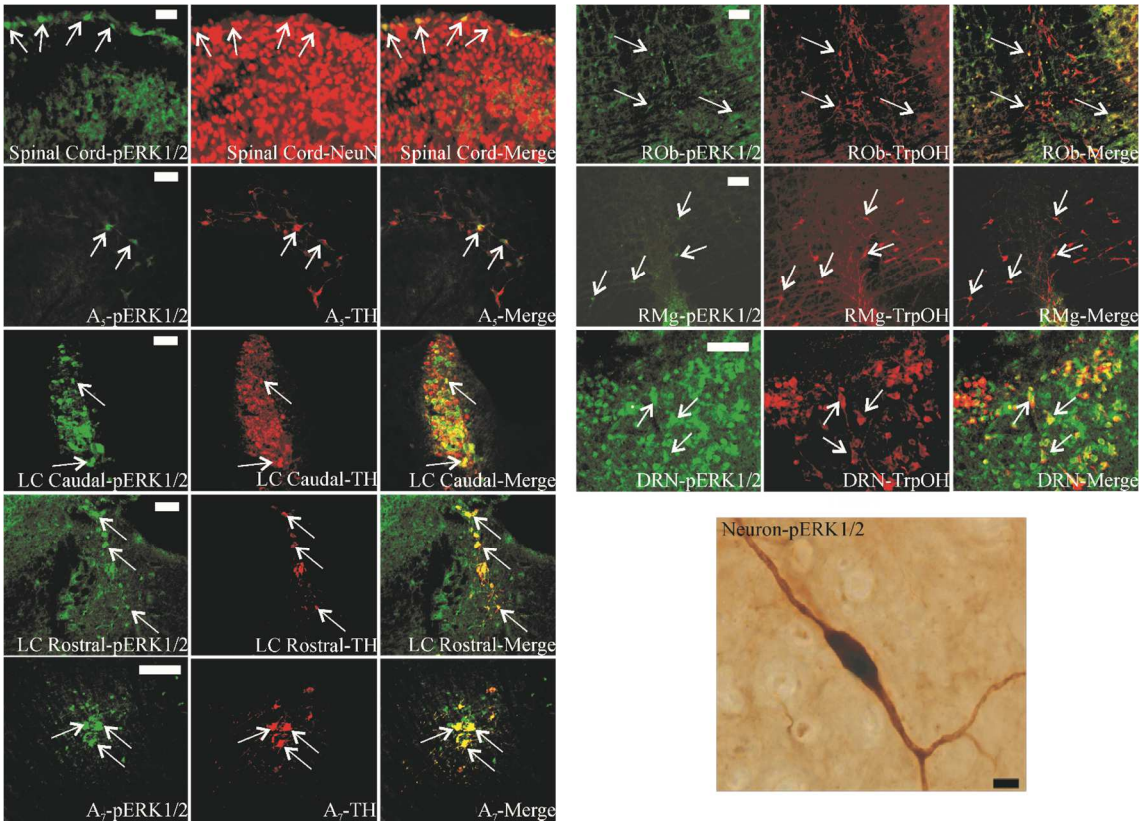

Table S1 - Number of pERK1/2 IR-cells per section (expressed as mean ± S.E.M.) for each spinal cord region analyzed in the ipsilateral side in each experimental group.

Lumbar Spinal Cord	SHAM		CCI		P Value (Two-Way ANOVA)			Fisher LSD (*post-hoc*)
	Non Stimulated (SNS - a)	Stimulated (SS - b)	Non Stimulated (CCINS - c)	Stimulated (CCIS - d)	Stimulation	Surgery	Interaction	
Laminae I-III	2.3±1.1	43.6±3.6	9.4±2.0	23.0±9.7	**<0.001*** **	0.256	**0.032***	**a/b*** ; a/d* ; b/c** ; b/d***
Laminae IV-VI	5.7±2.9	6.4±0.3	6.7±2.4	4.3±1.7	0.718	0.812	0.514	-
Laminae VII-IX	5.3±2.8	2.1±0.6	6.6±1.7	3.5±1.3	0.161	0.527	0.986	-
Lamina X	0.4±0.2	0.2±0.0	0.5±0.2	0.4±0.1	0.305	0.305	0.833	-

*** p<0.05; ** p<0.01 and *** p<0.001**

Note: In the *post-hoc* column, a/b* means that SNS was statistically different from SS with p<0.001.**

Table S2 - Number of pERK1/2 IR-cells or densitometric values (LC and LPB) per section (expressed as mean ± S.E.M.) for each brainstem nuclei studied in each experimental group.

Encephalic Nuclei	Extension	SHAM		CCI		P Value (Two-Way ANOVA)			Fisher LSD (*post-hoc*)
		Non Stimulated (SNS - a)	Stimulated (SS - b)	Non Stimulated (CCINS - c)	Stimulated (CCIS - d)	Stimulation	Surgery	Interaction	
TH-positive Regions									
A_5 (Noradrenergic Cells)	**Total**	12.1±1.2	13.3±2.1	18.7±1.9	15.4±1.0	0.498	**0.012***	0.161	**a/c**; b/c***
LC (Locus Coeruleus)	**Total**	6.4±1.0	5.6±0.8	4.2±0.4	4.0±0.2	0.489	**0.011***	0.601	**a/c*; a/d***
	Caudal[A]	6.6±0.9	6.1±0.8	4.2±0.4	4.4±0.3	0.890	**0.005****	0.602	**a/c*; a/d***
	Rostral[B]	7.1±1.4	4.9±0.9	3.9±0.2	3.2±0.3	0.075	**0.005****	0.352	**a/c*; a/d****
A_7 (Noradrenergic Cells)	**Total**	31.2±8.6	50.6±1.6	54.7±13.2	49.7±4.9	0.419	0.216	0.186	-
TrpOH-positive Regions									
ROb (Raphe Obscurus)	**Rostral[C]**	5.2±2.2	18.3±7.5	18.8±3.1	2.0±1.2	0.681	0.764	**0.005****	**a/b*; a/c*; b/d*; c/d***
RMg (Raphe Magnus)	**Total**	9.0±0.5	10.6±0.5	13.0±1.0	10.9±0,6	0.815	**0.039***	0.076	**a/c****
DRN (Dorsal Raphe)	**Total**	9.9±3.2	12.1±3.4	26.4±2.5	15.6±1.9	0.177	**0.006****	0.057	**a/c**; b/c**; c/d***
RVM and VLM									
LRt (Lateral Reticular)	**Rostral[D]**	25.9±3.9	28.1±4.3	48.2±6.9	40.5±3.3	0.567	**0.003****	0.302	**a/c**; a/d*; b/c***
PGi (Paragigantocellularis)	**Total**	30.±2.2	21.8±6.7	63.0±5.0	55.5±3.8	0.067	**<0.001*****	0.751	**a/c***; a/d**; b/c***; b/d*****
GiA (Gigantocellular Reticular Pars Alpha)	**Total**	18.2±1.8	17.4±3.7	18.0±1.8	16.8±0.5	0.662	0.877	0.940	-
Other Nuclei									
VLPAG (Ventrolateral Periaqueductal Grey)	**Total**	32.8±4.2	27.7±6.6	42.5±9.2	29.3±3.6	0.183	0.397	0.541	-
LPB (Lateral Parabrachial)	**Total**	5.8±0.9	6.9±1.1	7.7±2.5	6.5±0.6	0.958	0.618	0.409	-
	Caudal[E]	5.9±0.7	7.1±1.0	8.4±2.9	7.1±0.8	0.991	0.401	0.404	-
	Rostral[F]	5.2±1.8	6.3±1.3	6.5±1.9	5.4±0.6	0.988	0.903	0.436	-
EW (Edinger-Westphal)	**Total**	39.7±9.5	45.6±1.9	43.2±3.7	43.7±2.3	0.514	0.869	0.575	-

*** p<0.05; ** p<0.01 and *** p<0.001**

Coordinates from bregma: [A] -9.8 to -10.12mm; [B] -9.16 to -9.8mm; [C] -13.68 to -11.60mm; [D] -13.68 to -13.24mm; [E] -9.16 to -10.04mm; [F] -8.80 and -9.16mm.

Note: In the *post-hoc* column, a/c means that SNS was statistically different from CCINS with p<0.01.**

Discussion - Supplementary Material

Spinal ERK1/2 activation

In the spinal cord superficial dorsal horn, the application of mechanical noxious stimulation induced a specific pattern in the distribution of pERK1/2 of IR-cells. In fact, in these conditions positive pERK1/2 profiles were confined to a medial zone within this region. Interestingly, C-fiber activation by capsaicin also induced pERK1/2 IR-cells in the medial part of laminae I-III, where nociceptive inputs from the hindpaw are received (Ji et al., 1999; Ji et al., 2009). Additionally, a medial distribution of the pERKs1/2 IR-cells was also observed in CCIS rats. Conversely, in CCINS rats, pERK1/2 IR-cells were numerous but detected along all the superficial dorsal horn, suggesting a distinctive spatial distribution depending on the presence of chronic nociceptive stimulation (neuropathic pain) or of acute nociceptive stimuli (mechanical noxious stimulation). Another relevant observation was that in the remaining spinal cord laminae (IV-VI, VII-IX, X) studied, pERK1/2 immunolabeling was not changed by neuropathic pain and/or mechanical noxious stimulation, suggesting that ERK1/2 activation in these regions is not a strategic event in the processing of these noxious stimuli, at least at 7 days of CCI. Others showed different regulation of ERK1/2 phosphorylation at both superficial and deep dorsal horn after noxious stimulation of 14 days monoarthritic rats (Cruz et al., 2005). Thus it is possible that a prolonged time of disease is needed to trigger ERK1/2 activation in the remaining laminae of the spinal cord.

Supraspinal ERK1/2 activation

In opposition to the spinal cord, at supraspinal level, ERK1/2 phosphorylation occurred even in SHAM animals, pointing to a baseline activation of these kinases, as already described in some areas (Imbe et al., 2011). This supraspinal baseline ERK1/2 phosphorylation might be enough to trigger any mechanistic event in response to acute noxious mechanical stimulation. This would rather explain the lack of changes upon noxious mechanical stimulation of either SHAM or CCI rats. Additionally, unilateral CCI induced bilateral ERK1/2 activation in almost all nuclei analyzed (with exception of DRN, ROb and EW, the medial nuclei), suggesting that ERK activation in brainstem, due

to noxious input, is not confined to the side somatotopically related to the neuropathic paw. In fact, our study is consistent with the finding of bilateral increased metabolic activity in many brain regions during CCI (Mao et al., 1993).

One interesting observation of our study was the fact that in the LC, pERK1/2 expression was decreased in the CCI condition. However, it is possible that LC neuronal activity is enhanced but without requiring ERK1/2 signaling, since although pERK1/2 expression is considered a neuronal activation marker, this does not mean that when ERK1/2 phosphorylation is not observed, neurons are not activated. Indeed, further studies are needed to fully understand the pattern of pERKs expression in LC during CCI conditions.

Methodological considerations

In CCI rats the source of pain is the constriction of the sciatic nerve. Therefore, as mechanical noxious stimulation, we chose to apply a standardized protocol of pinches to the limb area innervated by the sciatic nerve. Since ERK1/2 are activated upon different types of stimulus (Ailing et al., 2008; Dai et al., 2011; Wang et al., 2010), the animals were previously anesthetized to avoid all external confounders (e.g. stress, anxiety) caused by noxious mechanical stimulation. However, different anesthetic procedures and the duration of anesthesia seem to affect ERK phosphorylation in different brain regions (Planel et al., 2007; Springell et al., 2005; Takamura et al., 2008). Additionally, the use of anesthetics may also interfere with some of the pain-related responses (such as cognition and affection), which could alter/mask the pattern of pERK1/2 expression in encephalic nuclei involved in these functions, as is the case of some brainstem regions analyzed in this study. Thus, anesthesia might have interfered with the level of ERKs1/2 activation in some of those regions. However, if that happened, anesthesia affected ERK1/2 phosphorylation in a similar way/magnitude in all the experimental groups, as all animals were submitted to similar anesthesia protocols and experimental conditions. Thus, interindividual variability in ERK1/2 detection has been minimized, and in what respects to the relative changes (increases/decreases) in ERK1/2 expression we found in each analyzed region in response to CCI and/or acute noxious stimulation, our findings remain valid and are important. Since we did not study matched un-anesthetized groups of animals, the data obtained does not allow inferring how the anesthetic agents interact with ERK1/2 activation in the analyzed regions and in the conditions of this specific

experimental protocol (CCI and/or noxious mechanical stimulation). Moreover, to our best knowledge, there are no such studies yet and so this is still lacking verification and/or comphreension. It would be of great value the evaluation of the effect of anesthesia (in what regards to the substance, duration, doses, and other parameters) in respect to ERK1/2 activation during modulation of the pain experience, in comparison with an anesthetized situation, since our data was restricted to studying pERK1/2 expression in supraspinal nuclei of SHAM and CCI rats, submitted to a particular anesthesia protocol. Also, both anesthetics and pain are able to change cardiovascular and respiratory responses (Guyenet et al., 2001; Jin et al., 2008; Pilowsky and Goodchild, 2002; Randich and Maixner, 1984), and some of the encephalic areas studied are implicated in the control of cardiovascular and respiratory functions. Thus, this aspect must be also considered when interpreting the data. Although the respiratory, arterial pressure or heart beat rate were not controlled, all the animals were submitted to identical anesthesia and stimulation protocols and so, it is expectable that interindividual differences in cardiovascular or respiratory changes that may have occurred due to anesthesia or to acute noxious stimulation were minimized/standardized. The same rationale is not possible to infer in what concerns the autonomic responses that may occur due to the CCI condition, and that may have affected, somehow, ERK1/2 activation in regions involved in autonomic control. Studies aiming at correlating the cardiovascular and respiratory changes, upon acute or chronic noxious stimulation, with supraspinal ERK1/2 activation in nuclei specifically implicated in autonomic control would be of great significance.

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4.2. Publication II

Reversal of monoarthritis-induced affective disorders by diclofenac in rats.

Anesthesiology (2014)

Reversal of Monoarthritis-induced Affective Disorders by Diclofenac in Rats

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ABSTRACT

Background: Nonsteroidal anti-inflammatory drugs are effective for arthritic pain, but it is unknown whether they also benefit anxiety and depression that frequently coexist with pain. Using the monoarthritis model, the authors evaluated the activation of extracellular signal–regulated kinases 1 and 2 ($ERK_{1/2}$) in structures implicated in both sensorial and emotional pain spheres, and it was verified whether analgesia can reverse monoarthritis-mediated affective responses.

Methods: Monoarthritis was induced in male rats by complete Freund's adjuvant injection. Allodynia (ankle-bend test), mechanical hyperalgesia (paw-pinch test), anxiety- and depression-like behaviors (elevated zero maze and forced swimming tests, respectively), and $ERK_{1/2}$ phosphorylation (Western blot) in the spinal cord, paragigantocellularis nucleus, locus coeruleus, and prefrontal cortex were evaluated at 4, 14, and 28 days postinoculation (n = 6 per group). Changes in these parameters were evaluated after induction of analgesia by topical diclofenac (n = 5 to 6 per group).

Results: Despite the pain hypersensitivity and inflammation throughout the testing period, chronic monoarthritis (28 days) also resulted in depressive- (control [mean ± SEM]: 38.3 ± 3.7 *vs.* monoarthritis: 51.3 ± 2.0; $P < 0.05$) and anxiogenic-like behaviors (control: 36.8 ± 3.7 *vs.* monoarthritis: 13.2 ± 2.9; $P < 0.001$). These changes coincided with increased $ERK_{1/2}$ activation in the spinal cord, paragigantocellularis, locus coeruleus, and prefrontal cortex (control *vs.* monoarthritis: 1.0 ± 0.0 *vs.* 5.1 ± 20.8, $P < 0.001$; 0.9 ± 0.0 *vs.* 1.9 ± 0.4, $P < 0.05$; 1.0 ± 0.3 *vs.* 2.9 ± 0.6, $P < 0.01$; and 1.0 ± 0.0 *vs.* 1.8 ± 0.1, $P < 0.05$, respectively). Diclofenac decreased the pain threshold of the inflamed paw and reversed the anxio-depressive state, restoring $ERK_{1/2}$ activation levels in the regions analyzed.

Conclusion: Chronic monoarthritis induces affective disorders associated with $ERK_{1/2}$ phosphorylation in paragigantocellularis, locus coeruleus, and prefrontal cortex which are reversed by diclofenac analgesia. **(ANESTHESIOLOGY 2014; 120:1476-90)**

PATIENTS with arthritis primarily seek medical assistance due to persistent pain caused by the inflammation and destruction of joints.[1] This pain results from a complex interaction between the peripheral and central nervous systems. In general consensus, chronic pain provokes neuroplastic alterations that contribute to the maintenance and amplification of painful sensation and to the onset of related disorders.[2] One of the most common consequences of chronic pain is the development of anxio-depressive disorders, manifested as helplessness, pessimism, rumination, and catastrophism.[3] These affective alterations originate due to the disruption of central mechanisms and should be carefully monitored, because their presence is directly related with pain severity.[4] Indeed, such modifications establish a vicious circle whereby chronic pain triggers profound emotional changes, which in turn enhance pain perception.[4] It is therefore critical to determine whether these plastic changes are reversible, because this could strongly condition the treatment strategies used. One potentially effective approach is to explore the effect of topically administering nonsteroidal anti-inflammatory drug in animal models which are among the first-line treatments to alleviate joint pain[5] and are frequently administered topically to avoid the side effects associated with their oral administration.[6]

What We Already Know about This Topic

- Many types of chronic pain including pain related to arthritis are associated with depressed mood and anxiety

What This Article Tells Us That Is New

- Using a rat model of arthritis, diclofenac reduced nociceptive sensitization and reduced additional behaviors suggesting anxiety- and depression-like changes in the animals
- The activation of extracellular signaling-related kinase in several brain regions was implicated in these changes

We focused on the pathway formed by the spinal cord (SC), paragigantocellularis nucleus (PGi), locus coeruleus (LC), and prefrontal cortex (PFC). The LC is involved in

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ascending and descending pain pathways and constitutes the main source of noradrenaline in the central nervous system, a neurotransmitter implicated in pain, emotion, stress, depression, anxiety, and other disorders.[7] The LC receives ascending nociceptive inputs from the SC through the PGi[8] and it projects to forebrain structures similar to the anterior cingulate cortex in the PFC, which is implicated in cognition and pain-related emotion.[9–13] Although it is clear that the anatomical and modulatory link between these structures may mediate the integration of pain processing at the emotional level, the underlying molecular and cellular mechanisms remain poorly understood. The extracellular signal–regulated kinases 1 and 2 ($ERK_{1/2}$), members of the mitogen-activated protein kinase superfamily, have been used as markers of activity in the SC after noxious stimulation and tissue injury.[14] Beneficial results were demonstrated in a clinical trial using a mitogen-activated protein kinase inhibitor to treat neuropathic pain,[15] suggesting this kinase as a target in the treatment of pathological pain.[16] Interestingly, $ERK_{1/2}$ activation is also strongly implicated in the regulation of pain-associated disorders. Indeed, recent studies indicate that $ERK_{1/2}$ is activated in the anterior cingulate cortex of the PFC after tissue or nerve injury,[9,10,13] suggesting that $ERK_{1/2}$ is involved in both the sensorial and emotional aspects of pain.

We hypothesized that an effective treatment of the inflammatory condition is able to reverse the emotional and molecular responses produced by this condition. Thus, we used an experimental rat model of rheumatoid arthritis (monoarthritis) and we assessed the subsequent effects of diclofenac treatment on pain-induced anxiety, depression, and $ERK_{1/2}$ activation in the SC–PGi–LC–PFC pathway.

Materials and Methods

Animals

Adult male Harlan Sprague–Dawley rats (n = 116) weighing 250 to 300 g were provided by the Experimental Unit of the University of Cádiz, Cádiz, Spain (registration number: ES110120000210). The animals were housed 2 to 4 per cage, with an *ad libitum* access to food and water, and they were maintained on a 12-h light–dark cycle at 22°C and with 45 to 60% humidity. All experimental procedures were carried out in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EC), Spanish Law (RD 1201/2005), and ethical guidelines for the study of experimental pain in animals.[17] The experimental protocols were reviewed and approved by the Institutional Ethical Committee for animal care and use (Cádiz, Andalucía, Spain).

Inflammatory Pain Model: Monoarthritis

Monoarthritis was induced as described previously.[18] In brief, anesthesia was induced and maintained with isoflurane (4 and 2%, respectively; Abbott, Madrid, Spain), and the rats were injected in the left tibiotarsal joint with 50 µl of complete Freund's adjuvant (CFA) solution containing 30 mg of desiccated *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI) diluted in the vehicle solution (3 ml paraffin oil, 2 ml saline, and 500 µl Tween-80). Control rats were injected with the vehicle solution alone and any animal exhibiting sign of polyarthritis was excluded from the study.

Inflammation Assessment

After CFA or vehicle injection, all the animals were monitored weekly for 4 weeks, measuring their body weight, rectal temperature (Chy 580BR Thermometer; Bioseb, Vitrolles, France), paw volume (using a plethysmometer apparatus; Ugo Basile, Comerio, Italy), and inflammation score. The inflammation score was a subjective scoring based on the signs of inflammation at the injection site and locomotion, whereby: 0 corresponds to the absence of inflammation and 4 to severe inflammation with persistent flexion of the affected limb and motor activity effects.[19]

Nociceptive Behavioral Assessment

Each week, nociceptive behavior was assessed in both the ipsi- and contralateral paws. Physiological evaluation of movement-induced nociception (allodynia) was performed using the ankle-bend test,[20] which involves the assessment of squeak and/or struggle reactions in response to five alternate flexions and extensions of the ankle joint. Higher scores (score 2) indicate squeak responses to moderate manipulations of the inflamed joint, whereas lower scores (score 0) indicate the absence of a response to manipulation. The reactions recorded in response to each extension or flexion are summed to obtain the ankle-bend score, giving a maximum value of 20, and a high ankle-bend score is indicative of allodynia. In addition, the presence of mechanical secondary hyperalgesia was determined using the paw-pinch test.[21] In brief, increasing pressure was gradually applied to the dorsal side of the paw using a graded motor-driven device (Ugo Basile) and beginning at 30 g of pressure. Three measurements were taken for each paw at 5-min intervals and the average value determined, with a 250 g cutoff applied to prevent damage to the paw. Secondary hyperalgesia is indicated by a reduction in the pressure-provoking withdrawal.

Anxiety- and Depression-like Behavior

Pain-induced emotional and affective changes were assessed in a separate set of animals at 4 (monoarthritis [MA]4D), 14 (MA14D), and 28 days (MA28D) after inducing monoarthritis. Anxiety-like behavior was evaluated by using the marble-burying and elevated zero maze (EZM) tests.

In the marble-burying test,[22] after a 30-min acclimatization, the rats were placed individually in a transparent plastic cage (51 × 22 × 15 cm) illuminated by a 100-W light and containing bedding (5 cm deep) in which 20 marbles were arranged in four columns and five rows. After 30 min,

the rats were removed and the number of buried marbles was counted, considering the marbles to be buried if they were at least two thirds covered with bedding. A larger number of buried marbles are taken as an indicator of increased anxiety-like behavior.

The EZM consisted of a black circular platform 105 cm in diameter that was elevated 65 cm above the floor. This maze was divided into four equal length quadrants: the two opposing open quadrants had 1-cm-high clear lips to prevent falls, whereas the two opposing closed quadrants were enclosed by 27-cm-high black walls. Each 5-min trial was carried out under the same lighting conditions and commenced with the animal being placed in the center of a closed quadrant. Spontaneous Motor Activity Recording and Tracking software (Panlab S.L.U., Barcelona, Spain) was used to analyze the percentage time spent in the open arms and the total distance travelled by each rat. An increase in the amount of time spent in the closed areas is correlated with anxiety-like behavior.

Depression-like behavior was evaluated by using a modified version of the forced swimming test (FST), as described previously.[23] When confined to a limited space, rodents engage in vigorous escape behavior. When it becomes clear that escape is impossible, these animals adopt an immobile posture, performing only the necessary movements required to keep their head above the water. Accordingly, in the FST, rats were placed for 15 min (pretest) in a clear cylindrical plastic container (46 cm high and 20 cm in diameter) filled with 30 cm of water (25° ± 1°C). After 24 h, the rats were once again exposed to the same conditions for 5 min (test) and they were videotaped to score their immobility (floating without struggling, using small movements to maintain the head above water), swimming (actively moving limbs more than is required to maintain the head above water), and climbing (active forepaw movements in and out of the water, often directed at the wall of the tank).[23] Customized software (Red Mice, Cádiz, Spain) was used to analyze the videos and to determine the predominant behavior at 5-s intervals. The total counts for each behavior during a 5-min test were averaged and although longer periods of immobility are taken as an indication of depression-like behavior, changes in the time spent climbing or swimming have been correlated with alterations in the availability of noradrenaline and serotonin, respectively.[23] As a positive control of antidepressant activity in the FST, the antidepressant desipramine (20 mg/kg; Sigma-Aldrich, St Louis, MO) was administered intraperitoneally to naive rats at 23.5, 5, and 1 h before testing. The behavioral tests were separated by a 3-day interval.

Western Blotting

Fresh tissue samples from the ipsilateral SC segments L3–L6, PGi, LC, and PFC regions were collected at 4, 14, and 28 days after injection of the CFA or vehicle. All the samples were processed for Western blotting and after the tissue was lysed, an aliquot (50 μg) was separated on a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA). After washing in Tris-buffered saline containing 0.1% Tween-20 (TBST), the blots were blocked with 5% bovine serum albumin (Sigma) in TBST and probed overnight at 4°C with rabbit anti-phospho-$ERK_{1/2}$ (1:5,000; Acris Antibodies, Herford, Germany), mouse-anti $ERK_{1/2}$ (1:2,000; Cell Signaling Technology, Danvers, MA), and mouse anti-tubulin (1:250,000; Sigma) antibodies diluted in 5% bovine

Table 1. Descriptive Statistics and Exact *P* Values for the Repeated-measures ANOVA

	Control				
	Basal (a)	First Week (b)	Second Week (c)	Third Week (d)	Fourth Week (e)
Weight (g)	303.7 ± 13.3 (6)	329.8 ± 10.7 (6)	352.3 ± 9.7 (6)	374.8 ± 9.3 (6)	388.9 ± 9.1 (6)
Temperature (°C)	37.5 ± 0.1 (6)	37.5 ± 0.1 (6)	37.1 ± 0.3 (6)	37.3 ± 0.1 (6)	36.9 ± 0.2 (6)
Paw volume Ip (ml)	1.7 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)
Paw volume Ctr (ml)	1.7 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)
Inflammation score	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)
Ankle-bend score	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)
Paw withdrawal Ip (g)	223.8 ± 17.0 (6)	196.3 ± 4.5 (6)	222.5 ± 6.0 (6)	223.8 ± 7.1 (6)	218.8 ± 7.9 (6)
Paw withdrawal Ctr (g)	248.8 ± 17.9 (6)	218.8 ± 9.6 (6)	225.0 ± 13.3 (6)	252.5 ± 17.8 (6)	238.8 ± 9.0 (6)

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). In the *post hoc* column, c/h ($P = 0.0008$) means that at the 2nd week, controls were statistically different from monoarthritic rats with a *P* value of 0.0008.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Ctr = contralateral; Ip = ipsilateral; MA = monoarthritis; ns = no significancies.

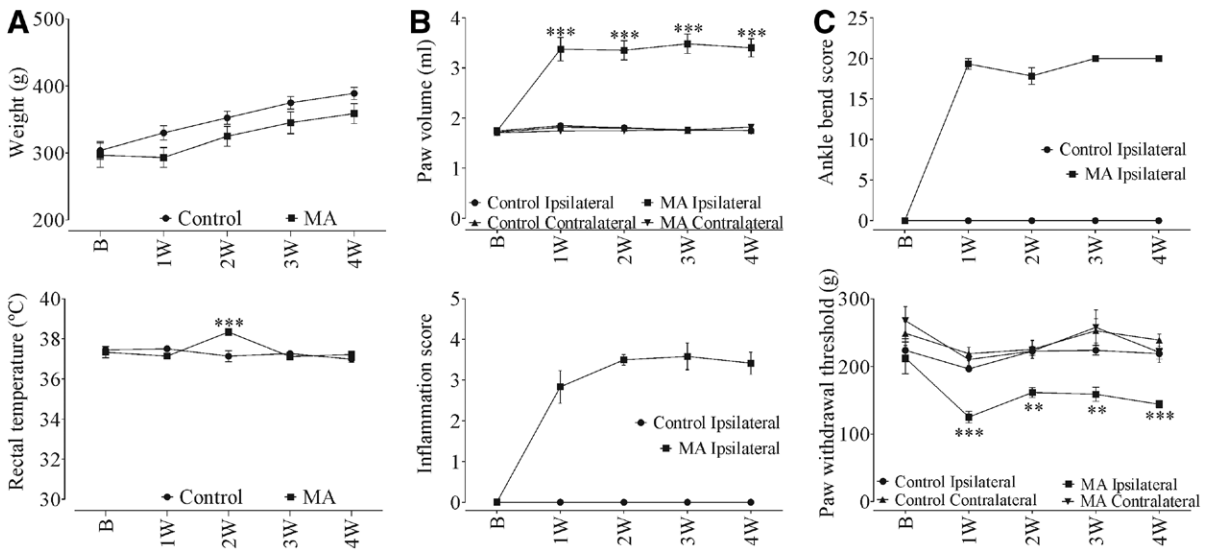

Fig. 1. Time course of the development of inflammation and nociceptive behavior in control and monoarthritic rats. (*A*) General parameters of health: weight and temperature. (*B*) Parameters of inflammation: paw volume and inflammation score. (*C*) Parameters of nociception: allodynia (ankle-bend score) and hyperalgesia (paw withdrawal threshold). All values are expressed as the mean ± SEM: $^{**}P < 0.01$, $^{***}P < 0.001$ monoarthritis (MA) *versus* corresponding controls, repeated-measures ANOVA followed by Student–Neuman–Keuls *post hoc* test for each time point. n = 6 per experimental group. B = baseline; W = week.

serum albumin–TBST. After thorough washing, these primary antibodies were detected by incubating for 1 h at room temperature with IRDye 800CW goat anti-rabbit (green) or IRDye 680LT goat anti-mouse (red) secondary antibodies (1:10,000; LI-COR®, Lincoln, NE). After three final washes with TBST, the antibody binding was detected by using a LI-COR Odyssey® two-channel quantitative fluorescence imaging system (LI-COR®). Digital images of Western blots were analyzed by densitometry using the ImageJ free access software (National Institutes of Health, Bethesda, MD). The data were expressed as $pERK_{1/2}$ expression levels relative to those of total $ERK_{1/2}$, as no significant differences in the loading control (tubulin) were observed. As no differences in $pERK_{1/2}$ expression were observed between the ipsilateral and contralateral sides for the PGi, LC, or PFC, these values were combined and averaged.

Topical Diclofenac Administration

To induce analgesia, 10 mg of sodium diclofenac (equivalent to 1 g of commercial Voltaren Gel®; Novartis, Basel,

MA					*P* Value (Repetead-measures ANOVA)			
Basal (f)	First Week (g)	Second Week (h)	Third Week (i)	Foruth Week (j)	Arthritis	Time	Interaction	Student–Newman–Keuls *Post Hoc* Test
296.7 ± 18.1 (6)	293.2 ± 14.9 (6)	324.8 ± 14.8 (6)	345.2 ± 16.3 (6)	358.7 ± 14.9 (6)	0.1812	0.0000***	0.0178*	ns
37.3 ± 0.3 (6)	37.1 ± 0.1 (6)	38.3 ± 0.2 (6)	37.1 ± 0.2 (6)	37.2 ± 0.2 (6)	0.1562	0.0191*	0.0014**	c/h (*P* = 0.0008)
1.7 ± 0.0 (6)	3.4 ± 0.2 (6)	3.4 ± 0.2 (6)	3.5 ± 0.2 (6)	3.4 ± 0.2 (6)	0.0000***	0.0000***	0.0000***	b/g (*P* = 0.0001); c/h (*P* = 0.0001); d/i (*P* = 0.0001); e/j (*P* = 0.0001)
1.7 ± 0.0 (6)	1.7 ± 0.0 (6)	1.7 ± 0.0 (6)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	0.6992	0.0994	0.1439	ns
0.0 ± 0.0 (6)	2.8 ± 0.4 (6)	3.5 ± 0.1 (6)	3.6 ± 0.3 (6)	3.4 ± 0.3 (6)	—	—	—	—
0.0 ± 0.0 (6)	19.3 ± 0.7 (6)	17.8 ± 1.0 (6)	20.0 ± 0.0 (6)	20.0 ± 0.0 (6)	—	—	—	—
212.5 ± 23.4 (6)	125.0 ± 8.6 (6)	161.3 ± 7.4 (6)	158.8 ± 10.5 (6)	143.8 ± 5.3 (6)	0.0000***	0.0004***	0.5470	b/g (*P* = 0.0005); c/h (*P* = 0.0032); d/i (*P* = 0.0039); e/j (*P* = 0.0004)
267.5 ± 20.7 (6)	210.0 ± 9.3 (6)	222.5 ± 6.6 (6)	257.5 ± 26.0 (6)	220.8 ± 15.0 (6)	0.9179	0.0244*	0.8047	ns

Switzerland) was applied topically twice daily for 3 to 5 days to the ipsilateral paw of control and monoarthritic rats until analgesia was achieved, beginning 21 days after CFA injection. Pure Vaseline (Acofarmaderm, Acofarma S.A., Barcelona, Spain) was applied to the control and monoarthritic rats and served as a control of diclofenac application. A plastic Elizabeth collar was fixed around the neck of each animal to prevent ingestion of the cream/vaseline.[24] The effect of diclofenac on the signs of inflammation and nociceptive behavior was analyzed after the last topical application (see Inflammation Assessment and Nociceptive Behavioral Assessment sections). As indicators of paw inflammation, photographs and footprints of the hind paw were taken to evaluate the shape and area of paw. At the end of the experiment, Western blot procedures were performed to evaluate the effect of diclofenac in the pattern of $ERK_{1/2}$ activation in the SC, PGi, LC, and PFC. Next, topical application of diclofenac/vaseline was repeated in another set of animals to evaluate the effect of nonsteroidal anti-inflammatory drug analgesia on pain-induced affective changes following the protocols described in the Anxiety- and Depression-like Behavior section.

To study the possible site of action of diclofenac, an additional group of animals was organized to receive local topical treatment of diclofenac in the contralateral paw (right paw) following the same protocol as described in the first paragraph of this section. Thus, the following extra experimental groups consisted of control and monoarthritic animals receiving vaseline (Cont + VAS and MA + VAS) and a group of monoarthritic animals receiving diclofenac treatment (MA + DIC). Before and after vaseline/diclofenac administration, baseline measures were taken for the assessment of the paw volume, ankle-bend score, and paw-pinch test values. Afterwards, Western blot procedures were performed to evaluate the effect of contralateral paw administration in the pattern of $ERK_{1/2}$ activation in the SC, PGi, LC, and PFC.

Statistical Analysis

All data are presented as the means ± SEM and all the results were analyzed using STATISTICA 10.0 (StatSoft, Tulsa, OK) or GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA) using either a Student *t* test (unpaired or paired, two-tailed) or a one-way or two-way ANOVA with or without repeated measures, followed by the appropriate *post hoc* tests (Student–Newman–Keuls or Dunnett tests). The independent variables were monoarthritis (between-groups) and treatment (between-groups). The level of significance was set at a *P* value of less than 0.05. Detailed information regarding statistical results is shown in tables 1-4.

Results

Monoarthritis as a Model of Chronic Inflammatory Pain

The injection of the vehicle alone (control group) did not provoke any inflammatory reaction in rats and these animals exhibited a normal behavior. By contrast, stable monoarthritis was induced by CFA injection, with marked inflammatory signs within several hours of induction that persisted for 4 weeks. Monoarthritic rats displayed: (1) normal body weight gain; (2) normal body temperature, except in the second week when a significant increase was observed ($P < 0.001$; monoarthritis *vs.* control for week 2 by ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 1A); (3) a significant increase in the ipsilateral paw volume evident each week ($P < 0.001$ for each time point; monoarthritis *vs.* control by ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 1B); and (4) a higher inflammation score in the ipsilateral paw that persisted until the fourth week (fig. 1B). Significantly, none of these features were observed in the contralateral paw of these monoarthritic rats. When the movement-induced nociceptive behavior was evaluated, CFA injection provoked higher ankle-bend scores (allodynia) throughout the experimental period (fig. 1C) and monoarthritic rats exhibited significantly stronger mechanical hyperalgesia in the ipsilateral paw ($P < 0.001$ for weeks 1 and 4, $P < 0.01$ for weeks 2 and 3; monoarthritis *vs.* control by ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 1C). No painful reactions were observed in the control rats or in the contralateral paw of monoarthritic rats. Descriptive statistics is shown in table 1.

Monoarthritis-induced Anxiety- and Depression-like Behavior

To determine whether chronic pain associated with joint inflammation induced anxiogenic-like behavior, the rats were subjected to the marble-burying and EZM tests at several time points during the development of monoarthritis (fig. 2A). Unlike MA4D rats, more marbles were buried by MA14D ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test) and MA28D ($P < 0.01$ by one-way ANOVA followed by Dunnett *post hoc* test) rats compared with the marbles buried by their corresponding controls, indicative of anxiety-like behavior.

In the EZM maze test, there was no difference in the percentage of time spent in the open arms between control rats and MA4D or MA14D rats. However, MA28D rats spent significantly lesser in the open arms than by their corresponding controls ($P < 0.001$ by one-way ANOVA followed by Dunnett *post hoc* test), again indicative of the development of anxiety-like behavior. No changes in locomotor activity were detected between groups in the EZM, ruling out a motor component in the effects observed.

The FST was performed to evaluate the possible development of depressive-like behaviors (fig. 2B), in which MA28D but not MA4D or MA14D rats exhibited significantly higher immobility scores compared with the scores of their corresponding controls ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test). Moreover, this effect was accompanied by a significant decrease in climbing behavior ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test), but there were no significant changes in swimming

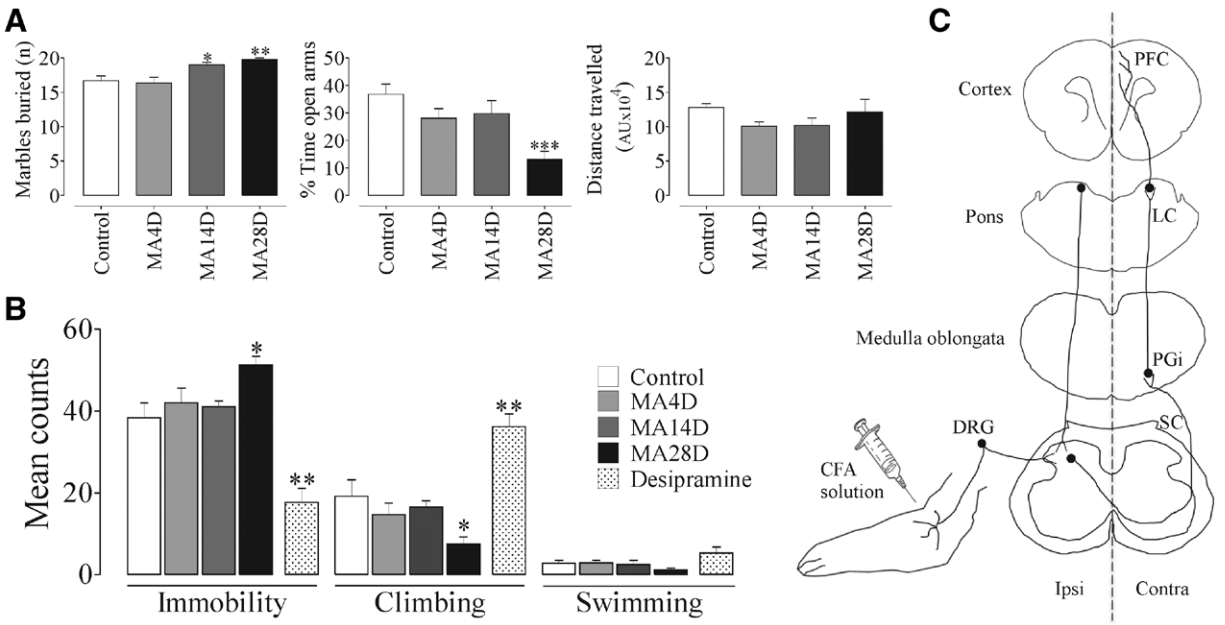

Fig. 2. Anxiety- (*A*) and depressive- (*B*) like behavioral responses to monoarthritis and schematic representation (*C*) of the paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway. (*A*) Increased anxiety was observed in MA28D rats in the marble-burying test (*left*), as indicated by an increase in burying behavior relative to the controls. Similarly, MA28D rats spent more time in closed areas of an elevated zero maze (*center*), instead of exploring the open areas, a hallmark of anxiety-like behavior. Measurement of the total distance travelled indicated that there were no changes in locomotor activity in the elevated zero maze (*right panel*). (*B*) The modified forced swimming test was used as an index of depressive-like behavior/pro-depressive activity, indicated by an increase in the immobility time of MA28D rats with respect to controls. Naive rats treated with the antidepressant desipramine were used as positive controls of antidepressant activity in all the tests. Values are expressed as the mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 monoarthritis *versus* control, one-way ANOVA followed by Dunnett *post hoc* test. n = 5 to 6 per experimental group; ***P* < 0.01, desipramine *versus* control; unpaired Student *t* test (two-tailed). (*C*) Scheme illustrating the PGi–LC–PFC nociceptive pathway, originating in the inflamed paw, passing through the dorsal root ganglion neurons (DRGs) to spinal cord (SC) neurons, where it crosses the *medial line* and finally ascends to the supraspinal structures. AU = arbitrary units; CFA = complete Freund's adjuvant; MA = monoarthritis.

behavior. As expected, desipramine treatment diminished the rat's immobility ($P < 0.01$ *vs.* corresponding control, Student *t* test) and significantly increased their climbing behavior ($P < 0.01$ *vs.* corresponding control, Student *t* test). Descriptive statistics is shown in table 2.

Monoarthritis-induced $ERK_{1/2}$ Activation in the SC–PGi–LC–PFC Pathway

The expression of $pERK_{1/2}$ was evaluated as an indicator of neuronal activity in the SC, PGi, LC, and PFC in the early, middle, and late phases of monoarthritis development (fig. 3). In the ipsilateral side of the SC, significant increases in the expression of $pERK_{1/2}$ were observed at 14 ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test) and 28 ($P < 0.001$ by one-way ANOVA followed by Dunnett *post hoc* test) days after monoarthritis induction when comparing with that in control groups. No significant changes were observed at 4 days of monoarthritis disease. In addition, no significant changes in $pERK_{1/2}$ were observed at 4 or 14 days after monoarthritis induction in any of the brain areas analyzed. By contrast, significantly more $pERK_{1/2}$ was observed in the PGi ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test), LC ($P < 0.01$ by one-way ANOVA followed by Dunnett *post hoc* test), and PFC ($P < 0.05$ by one-way ANOVA followed by Dunnett *post hoc* test) at 28 days after monoarthritis induction compared with that in the corresponding controls. Descriptive statistics is shown in table 2.

Effect of Topical Administration of Diclofenac on Nociceptive Behavior

As expected, topical application of diclofenac to the ipsilateral paw decreased the inflammation and increased the pain threshold of that paw, without producing any significant change in body weight or body temperature (fig. 4). Although a small edema was observed in monoarthritic rats that received diclofenac ($P < 0.01$, control + diclofenac *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test), this was substantially smaller than that seen in those that received vaseline ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test: fig. 4B). A similar effect on the inflammation score was observed (fig. 4B) and the ankle-bend score of monoarthritic rats that received a topical application of diclofenac was significantly lower ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls

Table 2. Descriptive Statistics and Exact *P* Values for the One-way ANOVA

	Control (a)	MA4D (b)	MA14D (c)	MA28D (d)	Desipramine (e)	*P* Value (One-way ANOVA) Arthritis	*P* Value (Student *t* Test, Unpaired, Two-tailed)	Dunnett *Post hoc* Test
Marbles buried (n)	16.7 ± 0.7 (6)	16.3 ± 0.8 (6)	19.0 ± 0.4 (6)	19.8 ± 0.2 (5)	—	0.0016**	—	a/c (*P* = 0.0344); a/d (*P* = 0.0065)
Time in open arms (%)	36.8 ± 3.7 (6)	28.1 ± 3.4 (6)	29.8 ± 4.7 (6)	13.2 ± 2.9 (6)	—	0.0020**	—	a/d (*P* = 0.0007)
Distance travelled (AU × 10^4)	12.8 ± 0.6 (6)	10.1 ± 1.1 (6)	10.1 ± 1.1 (6)	12.1 ± 1.9 (6)	—	0.2732	—	ns
Immobility (mean counts)	38.3 ± 3.7 (6)	42.0 ± 3.6 (6)	41.0 ± 1.4 (6)	51.3 ± 2.0 (6)	17.7 + 3.5 (6)	0.0228*	a/e (*P* = 0.0021)	a/d (*P* = 0.0112)
Climbing (mean counts)	19.2 ± 4.0 (6)	14.7 ± 2.8 (6)	16.5 ± 1.5 (6)	7.5 ± 1.7 (6)	36.2 + 3.1 (6)	0.0371*	a/e (*P* = 0.0072)	a/d (*P* = 0.0167)
Swimming (mean counts)	3.5 ± 0.8 (6)	3.3 ± 0.8 (6)	2.5 ± 1.1 (6)	1.2 ± 0.4 (6)	6.2 + 1.3 (6)	0.1858	ns	ns
$pERK_{1/2}$ (SC)	1.0 ± 0.0 (8)	2.3 ± 0.5 (12)	3.1 ± 0.7 (11)	5.1 ± 0.8 (8)	—	0.0009***	—	a/c (*P* = 0.0314); a/d (*P* = 0.0003)
$pERK_{1/2}$ (PGi)	0.9 ± 0.0 (6)	1.3 ± 0.3 (6)	0.8 ± 0.3 (6)	1.9 ± 0.4 (6)	—	0.0328*	—	a/d (*P* = 0.0386)
$pERK_{1/2}$ (LC)	1.0 ± 0.3 (6)	1.6 ± 0.3 (6)	1.6 ± 0.8 (6)	2.9 ± 0.6 (6)	—	0.0114*	—	a/d (*P* = 0.0043)
$pERK_{1/2}$ (PFC)	1.0 ± 0.0 (6)	0.9 ± 0.2 (5)	1.0 ± 0.3 (6)	1.8 ± 0.1 (6)	—	0.0092**	—	a/d (*P* = 0.0207)

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). For desipramine-positive control, a Student test was performed to directly compare with the control group. In the *post hoc* column, a/c (*P* = 0.0344) means that MA14D was statistically different from control rats with a *P* value of 0.0344.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

AU = arbitrary units; LC = locus coeruleus; MA = monoarthritis; ns = no significancies; pERK1/2 = phosphorylated extracellular signal–regulated kinases 1 and 2; PFC = prefrontal cortex; PGi = paragigantocellularis; SC = spinal cord.

post hoc test), reflecting a higher nociceptive threshold, although this score remained significantly higher than that observed in control animals ($P < 0.001$ for the inflammation score and $P < 0.01$ for the ankle-bend score, control + diclofenac *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test: fig. 4C). Diclofenac treatment also increased the force supported by the ipsilateral paw of monoarthritic rats ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test), completely abolishing mechanical hyperalgesia (fig. 4C). Although not quantified, photographs and footprints revealed a clear improvement in paw posture and weight loading in the affected paw after diclofenac treatment (fig. 5). Descriptive statistics is shown in table 3.

Effect of Diclofenac on Anxiety- and Depression-like Behaviors

Diclofenac administration had significant effects on monoarthritis-induced anxiety- and depression-like behaviors. In the EZM, monoarthritis-induced anxiety-like behavior was reversed by diclofenac treatment ($P < 0.05$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 5A), yet no changes in locomotor activity were produced in this paradigm (fig. 5A). Similarly, monoarthritis-induced depression-like behavior was successfully reversed by topical diclofenac administration, as revealed by the significant differences in immobility time between groups ($P < 0.05$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 5B). The climbing behavior displayed by the monoarthritic rats receiving vaseline showed a tendency to decrease compared with the respective control group ($P = 0.056$ control + vaseline *vs.* monoarthritis + vaseline, by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 5B). Descriptive statistics is shown in table 3.

Effect of Diclofenac on $pERK_{1/2}$ Expression in the SC, PGi, LC, and PFC

Local inflammation produced a significant increase in the expression of $pERK_{1/2}$ levels in the ipsilateral SC as witnessed in monoarthritic rats treated with vaseline as compared with that in the corresponding controls ($P < 0.001$, monoarthritis + vaseline *vs.* control + vaseline by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test). Analgesia through application of diclofenac to the inflamed paw significantly reduced $pERK_{1/2}$ levels ($P < 0.001$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac by two-way ANOVA followed by Student–Newman–Keuls *post hoc*

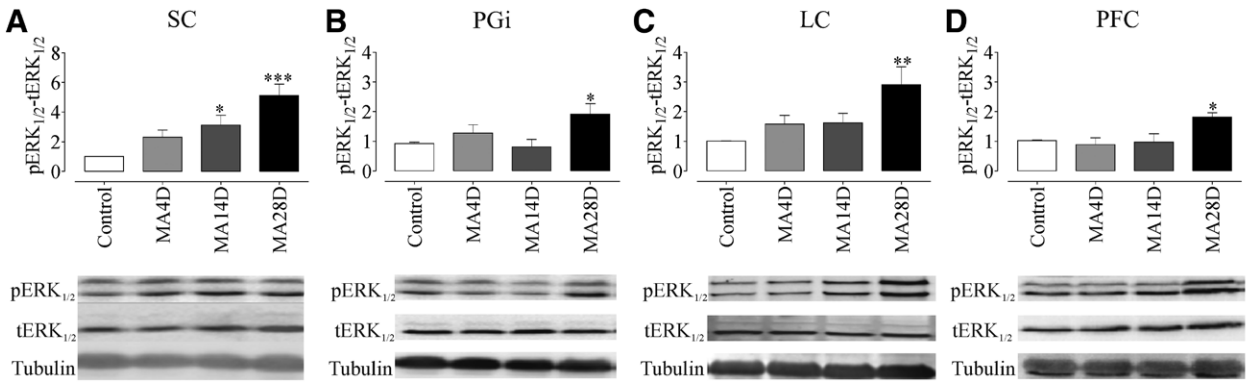

Fig. 3. pERK$_{1/2}$ expression in the spinal cord (SC)–paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway in response to chronic monoarthritis (MA). (*A*) In the SC, significant changes were observed in MA14D and MA28D rats. In addition, increased ERK$_{1/2}$ phosphorylation was observed in MA28D rats in the PGi (*B*), LC (*C*), and PFC (*D*). Below the graphs are images of the blots showing pERK$_{1/2}$ (44–42 kDa), tERK$_{1/2}$ (44–42 kDa), and tubulin (50 kDa) expression for each structure from each experimental group. Values are expressed as mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, MA *versus* corresponding controls, one-way ANOVA followed by Dunnett test. *Each column* represents the mean pERK$_{1/2}$ levels from three assays performed on samples from independent groups of 2–4 animals. These levels were normalized to the corresponding total ERK$_{1/2}$ values, as no significant changes in tubulin levels were observed. pERK$_{1/2}$/tERK$_{1/2}$ = phosphorylated/total extracellular signal–regulated kinases 1 and 2, respectively.

test; fig. 6, A and B) and the significant increase in pERK$_{1/2}$ levels in the PGi–LC–PFC pathway of monoarthritic rats ($P < 0.05$, monoarthritis + vaseline *vs.* control + vaseline in the PGi; $P < 0.01$ monoarthritis + vaseline *vs.* control + vaseline in the LC and PFC by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 6, A and B) was also successfully restored to control levels after topical treatment with diclofenac ($P < 0.05$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac in the PGi and PFC; $P < 0.01$, monoarthritis + vaseline *vs.* monoarthritis + diclofenac in the LC by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test; fig. 6, A and B). Descriptive statistics is shown in table 3.

Effect of Diclofenac Administration in the Contralateral Paw

The results point to the absence of an effect of the contralateral administration of diclofenac (fig. 7) on the paw volume

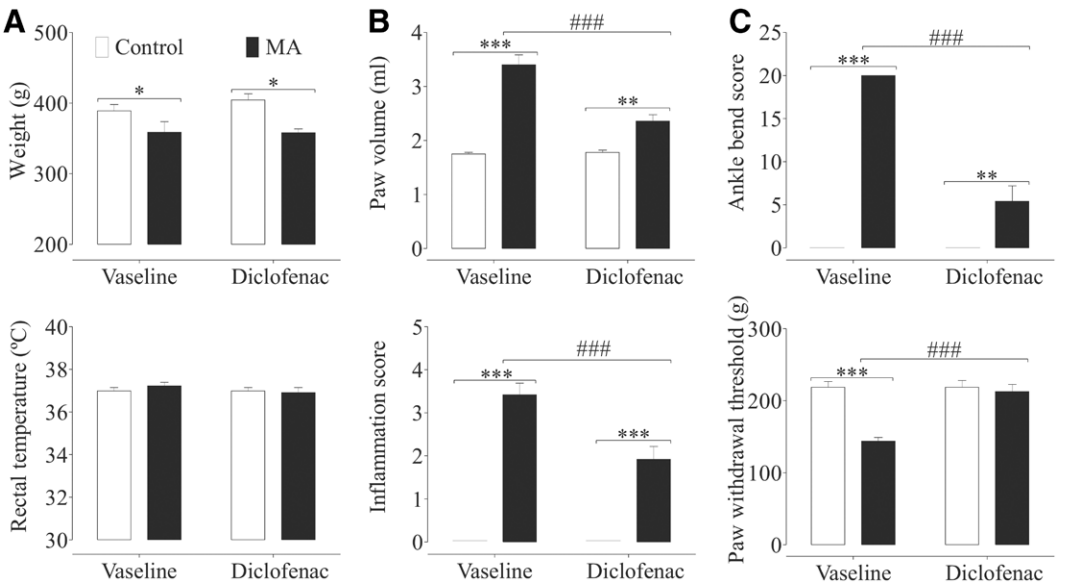

Fig. 4. Effect of topical diclofenac application to the ipsilateral paw on inflammation and nociceptive behavior. (*A*) General parameters of health (weight and temperature) were not significantly altered by diclofenac treatment. (*B*) Paw volume and the inflammation score in monoarthritic rats were significantly attenuated by diclofenac treatment. (*C*) The ankle-bend score was significantly lower in diclofenac-treated monoarthritic rats and the paw withdrawal threshold was restored to control levels. Values are expressed as the mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, monoarthritis (MA) *versus* corresponding controls; ###*P* < 0.001, MA + vaseline *versus* MA + diclofenac, two-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. n = 6 animals per experimental group.

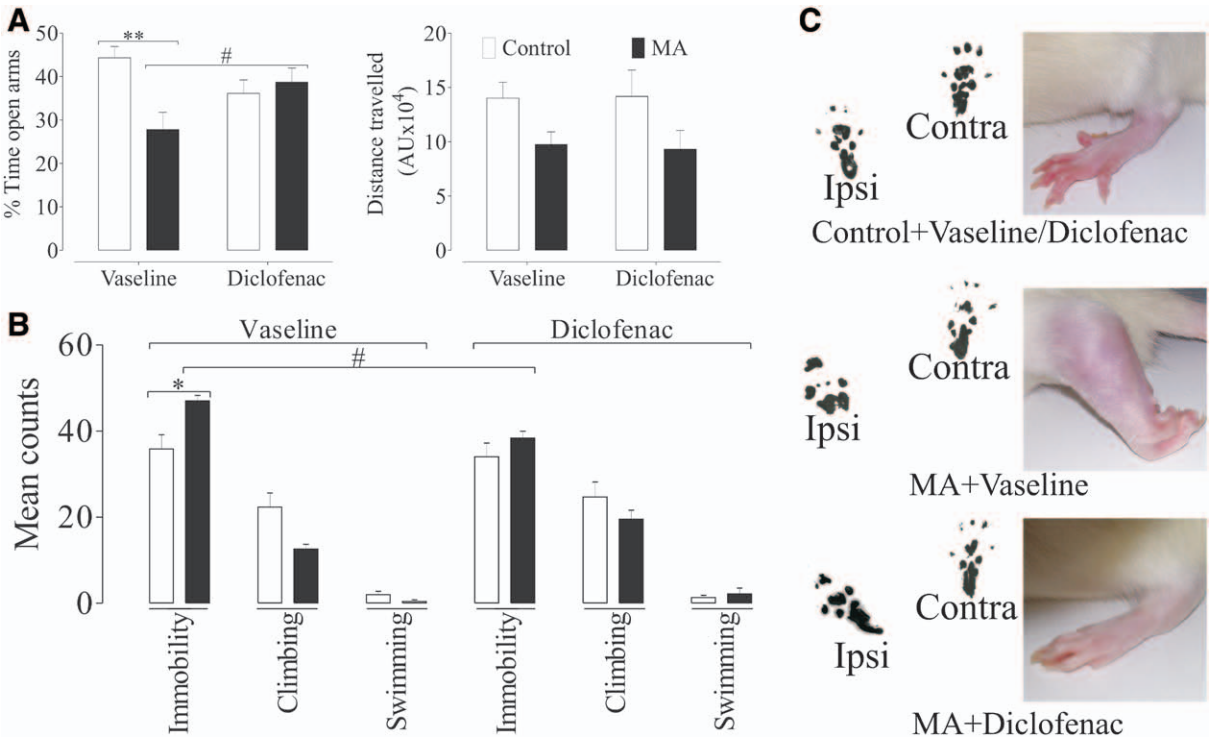

Fig. 5. Effect of topical diclofenac application to the ipsilateral paw on anxiety- and depression-like behaviors induced by chronic monoarthritis (MA). (*A*) Diclofenac treatment abolished the anxiety-like behavior produced by chronic MA, as witnessed by an increase in the time spent in the open areas of the elevated zero maze. There were no significant differences between experimental groups in the total distance travelled in the elevated zero maze. (*B*) Topical diclofenac application also eliminated MA-induced depressive-like behavior, as witnessed by the decreased immobility in the forced swimming test. (*C*) Hind paw photographs and footprints revealed a clear improvement in the guarding posture in diclofenac-treated monoarthritic rats (Ipsi = ipsilateral paw; Contra = contralateral paw). Note the paw position of monoarthritic rats treated with vaseline or diclofenac. Values are expressed as mean ± SEM: **P* < 0.05 and ***P* < 0.01, MA *vs.* corresponding control; #*P* < 0.05, MA + vaseline *versus* MA + diclofenac; two-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. n = 5 to 6 animals per experimental group. AU = arbitrary units.

(fig. 7A), and paw-pinch threshold (fig. 7C) of the inflamed paw (ipsilateral paw), as can be observed by the lack of significant changes between the values obtained before the treatment and after the treatment, in the group of monoarthritic animals receiving diclofenac (paired Student *t* test). However, in the ankle-bend score (fig. 7B), contralateral treatment with diclofenac caused a decrease in the values obtained after the treatment when comparing with those obtained before the treatment ($P < 0.01$; paired Student *t* test; fig. 7B). Descriptive statistics is shown in table 4.

In addition, quantification of the immunoblots (fig. 8) showed that the increased $ERK_{1/2}$ activation observed in MA + VAS group was maintained in the MA + DIC group in the SC ($P < 0.01$ for MA + VAS and MA + DIC *vs.* control, unpaired Student *t* test), PGi ($P < 0.01$ for MA + VAS *vs.* control and $P < 0.05$ for MA + DIC *vs.* control unpaired Student *t* test), LC ($P < 0.05$ for MA + VAS *vs.* control and $P < 0.01$ for MA + DIC *vs.* control, unpaired Student *t* test), and PFC ($P < 0.05$ for MA + VAS and MA + DIC *vs.* control, unpaired Student *t* test). Descriptive statistics is shown in table 4.

Discussion

The current findings demonstrate that chronic joint inflammation interferes with sensitivity to noxious stimulation and with physiological movement of the inflamed joint, as well as profoundly affecting emotional states when the painful condition is prolonged. Furthermore, we demonstrated that chronic pain enhances $ERK_{1/2}$ activation in certain central nervous system regions, specifically in the SC, PGi, LC, and PFC. Promoting analgesia through the topical application of anti-inflammatory cream successfully attenuated nociception, as well the anxiety- and depressive-like behaviors observed. Interestingly, this effect was accompanied by the normalization $ERK_{1/2}$ activation in the SC, PGi, LC, and PFC.

We initially analyzed here the nociceptive behavior produced by joint inflammatory pain with the use of the monoarthritis model. As expected, unilateral arthritic inflammation induced constant allodynia and hyperalgesia in the ipsilateral paw at all time points studied. The induction of monoarthritis was associated with the development of anxiety-like symptoms in the EZM and marble-burying tests. The EZM is based on the natural aversion of rodents to bright

Table 3. Descriptive Statistics and Exact *P* Values for the Two-way Analysis

	Control		MA		*P* Value (Two-way ANOVA)			Student–Newman–Keuls *Post hoc* Test	
	Vaseline (a)	Diclofenac (b)	Vaseline (c)	Diclofenac (d)	Arthritis	Treatment	Interaction		
Weight (g)	388.8 ± 9.1 (6)	404.3 ± 8.8 (6)	358.7 ± 14.9 (6)	358.0 ± 5.5 (6)	0.0012**	0.4736	0.4353	a/c (*P* = 0.0486); b/d (*P* = 0.0200)	
Temperature (°C)	36.9 ± 0.2 (6)	36.9 ± 0.2 (6)	37.2 ± 0.2 (6)	36.9 ± 0.2 (6)	0.6943	0.4098	0.4098	ns	
Paw volume Ip (ml)	1.8 ± 0.0 (6)	1.8 ± 0.0 (6)	3.4 ± 0.2 (6)	2.4 ± 0.1 (6)	0.0000***	0.0002***	0.0001***	a/c (*P* = 0.0002); b/d (*P* = 0.0016); c/d (*P* = 0.0002)	
Inflammation score Ip	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	3.4 ± 0.3 (6)	1.9 ± 0.3 (6)	0.0000***	0.0014**	0.0014**	a/c (*P* = 0.0001); b/d (*P* = 0.0001); c/d (*P* = 0.0002)	
Ankle bend score Ip	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	20.0 ± 0.0 (6)	5.4 ± 1.8 (6)	0.0000***	0.0000***	0.0000***	a/c (*P* = 0.0001); b/d (*P* = 0.0011); c/d (*P* = 0.0002)	
Paw withdrawal Ip (g)	218.6 ± 7.8 (6)	218.6 ± 9.4 (6)	143.8 ± 5.3 (6)	212.6 ± 10.0 (6)	0.0001***	0.0005***	0.0005***	a/c (*P* = 0.0002); c/d (*P* = 0.0002)	Ipsilateral treatment
Time in open arms (%)	44.3 ± 2.6 (6)	36.1 ± 3.1 (6)	27.8 ± 4.0 (6)	38.7 ± 3.2 (6)	0.0465*	0.6783	0.0084**	a/c (*P* = 0.0017); c/d (*P* = 0.0132)	
Distance travelled (AU × 10^4)	14.0 ± 1.5 (6)	14.2 ± 2.4 (6)	9.8 ± 1.2 (6)	9.3 ± 1.7 (6)	0.0170*	0.9356	0.8669	ns	
Immobility (mean counts)	35.8 ± 3.3 (6)	34.0 ± 3.2 (6)	47.0 ± 1.3 (5)	38.3 ± 1.6 (6)	0.0077**	0.0578	0.2045	a/c (*P* = 0.0180); c/d (*P* = 0.0294)	
Climbing (mean counts)	22.3 ± 3.2 (6)	24.5 ± 3.5 (6)	12.6 ± 1.1 (5)	19.5 ± 2.1 (6)	0.0145	0.1120	0.4201	ns	
Swimming (mean counts)	2.0 ± 0.8 (6)	1.3 ± 0.5 (6)	0.4 ± 0.4 (5)	2.2 ± 1.3 (6)	0.6629	0.5328	0.1760	ns	
$pERK_{1/2}$ (SC)	1.0 ± 0.0 (6)	1.7 ± 0.4 (5)	5.0 ± 0.5 (5)	2.5 ± 0.5 (5)	0.0000***	0.0444*	0.0010**	a/c (*P* = 0.0002); c/d (*P* = 0.0006)	
$pERK_{1/2}$ (PGi)	1.1 ± 0.1 (6)	1.2 ± 0.4 (6)	3.0 ± 0.9 (6)	1.0 ± 0.1 (6)	0.0730	0.0984	0.0472*	a/c (*P* = 0.0338); c/d (*P* = 0.0466)	
$pERK_{1/2}$ (LC)	1.0 ± 0.0 (6)	1.0 ± 0.1 (6)	1.9 ± 0.3 (6)	1.2 ± 0.1 (6)	0.0083**	0.0287*	0.0800	a/c (*P* = 0.0082); c/d (*P* = 0.0096)	
$pERK_{1/2}$ (PFC)	1.0 ± 0.0 (6)	1.6 ± 0.3 (6)	3.1 ± 0.6 (6)	1.4 ± 0.3 (6)	0.0225*	0.1730	0.0076**	a/c (*P* = 0.0052); c/d (*P* = 0.0150)	

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). In the *post hoc* column, a/c (*P* = 0.0486) means that MA + vaseline group was statistically different from control + vaseline group with a *P* value of 0.0486.

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

AU = arbitrary units; Ip = ipsilateral; LC = locus coeruleus; MA = monoarthritis; ns = no significancies; $pERK_{1/2}$ = phosphorylated extracellular signal–regulated kinases 1 and 2; PFC = prefrontal cortex; PGi = paragigantocellularis; SC = spinal cord.

and exposed places, whereas marble-burying behavior gauges the level of anxiety-like behavior of a rodent on encountering unfamiliar and bright objects. In both tests, anxiety-like behavior was evident at late disease stages, and in the case of the marble-burying test, the anxiety-like behavior was already evident at 14 days after monoarthritis induction. We also evaluated the time course of depression-like behavior by using the FST. This test induces "behavioral despair" in animals and abandoning the struggle to escape a stressful environment may resemble the psychological concept of "entrapment" described in clinical populations.[23] MA28D rats displayed greater immobility and milder escape behavior (climbing) in the FST test. This reduction in climbing behavior was not accompanied by changes in swimming behavior, suggesting noradrenergic dysfunction. This is mainly due to the fact that noradrenaline-selective reuptake inhibitors produce significant increases in this behavior in the FST[23] indicating that a decrease in the climbing behavior suggests a dysfunction in the noradrenergic system. More studies are underway to clarify whether this impairment is really occurring. These data are in consistent with previous findings,[25–27] and with clinical data obtained from patients with osteoarthritis,[28] indicating that chronic inflammatory pain conditions profoundly affect emotional states, as also described previously in other rheumatic diseases.[29] Moreover, the time course for development of anxiety and then depression may indicate a certain chronological development of pain-related mood disorders, which was already observed in other pain models.[30] Overall, this implies that the affective consequences of chronic inflammatory pain evolve over time, probably mediated by long-term molecular and neural plastic changes at different brain areas.

Because $ERK_{1/2}$ have been proposed as promising target molecules in the regulation of both pain[31,32] and affective disorders,[33–35] we investigated whether monoarthritis-induced affective disorders were associated with altered $ERK_{1/2}$ activation. In the SC, $pERK_{1/2}$ was not significantly increased

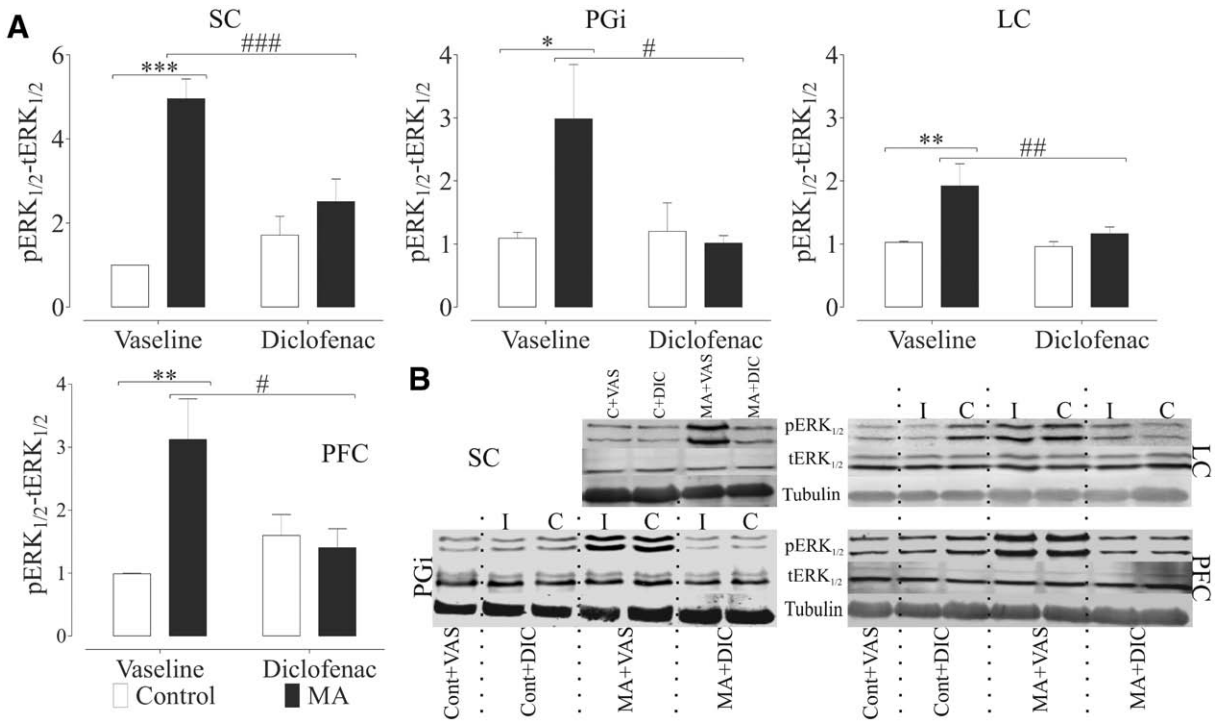

Fig. 6. Effect of topical diclofenac application to the ipsilateral paw on the $pERK_{1/2}$ in the spinal cord (SC)–paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway. (*A*) Graphs depict the changes in the $pERK_{1/2}$ expression in response to chronic monoarthritis (MA). Diclofenac administration significantly reduced $pERK_{1/2}$ levels on the ipsilateral side of the SC and it reversed the increase in $pERK_{1/2}$ observed in the PGi, LC, and PFC. (*B*) Images of the blots showing $pERK_{1/2}$ (44–42 kDa), $tERK_{1/2}$ (44–42 kDa), and tubulin (50 kDa) expression for each structure from each experimental group. Values are expressed as the mean ± SEM: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, control *versus* MA; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, MA + vaseline *versus* MA + diclofenac; two-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. *Each column* represents the mean $pERK_{1/2}$ levels of three assays performed on samples from independent groups of 2–4 animals. These levels were normalized to the corresponding total $ERK_{1/2}$ values, as no significant changes in tubulin levels were observed. C = contralateral; Cont = control; DIC = diclofenac; I = ipsilateral; $pERK_{1/2}$/$tERK_{1/2}$ = phosphorylated/total extracellular signal–regulated kinases 1 and 2, respectively; VAS = vaseline.

at 4 days after monoarthritis induction but was significantly increased at both 14 and 28 days after monoarthritis. Indeed, other authors showed that 2 and 4 days of inflammation were not accompanied by increased $ERK_{1/2}$ activation in the SC.[36] The significant increase of $pERK_{1/2}$ observed at 14 and 28 days may be related with increased metabolic activity which was already demonstrated by Schadrack *et al.*,[37] at least for the 14 days of monoarthritic time point. The onset of affective symptoms was accompanied by increased $pERK_{1/2}$ levels in the PGi, LC, and PFC of monoarthritic rats, in agreement with previous reports of increased $pERK_{1/2}$ expression in the rat PGi 7 days after the induction of neuropathic

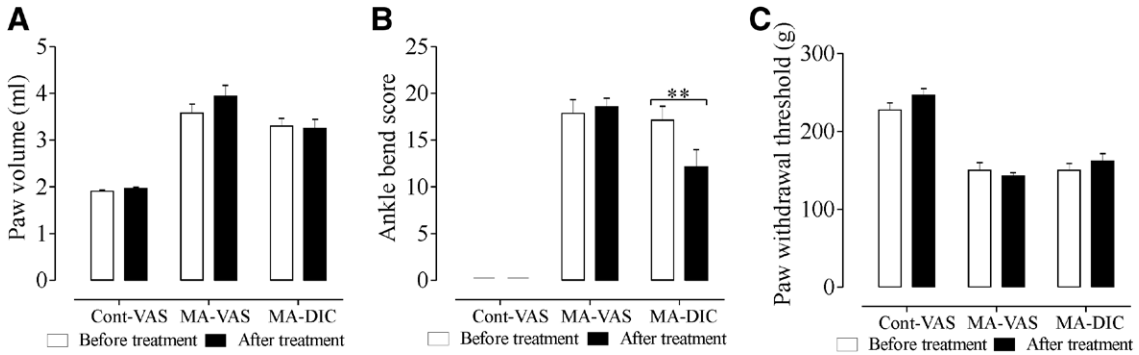

Fig. 7. Effect of the topical administration of vaseline/diclofenac to the contralateral paw on the paw volume (*A*), ankle-bend score (*B*), and paw withdrawal threshold (*C*). Valued expressed as mean ± SEM. Comparisons between the values obtained before and after the treatment, for each experimental group, were performed by using a paired Student *t* test. ***P* < 0.01. Cont = control; DIC = diclofenac; MA = monoarthritis; VAS = vaseline.

Table 4. Descriptive Statistics and Exact *P* Values for the Student *t* Test

	Before Treatment			After Treatment				
	Control	MA		Control	MA			
	Vaseline (a)	Vaseline (c)	Diclofenac (e)	Vaseline (b)	Vaseline (d)	Diclofenac (f)	*P* Value (Paired Student *t* Test)	
Paw volume Ip (ml)	1.9 ± 0.0 (6)	3.6 ± 0.2 (7)	3.3 ± 0.2 (7)	2.0 ± 0.0 (6)	3.9 ± 0.2 (7)	3.2 ± 0.2 (7)	ns	
Ankle-bend score Ip	0.0 ± 0.0 (6)	17.9 ± 1.5 (7)	17.1 ± 1.5 (7)	0.0 ± 0.0 (6)	18.6 ± 0.9 (7)	12.1 ± 1.8 (7)	e/f (*P* = 0.0038)	
Paw withdrawal Ip (g)	227.5 ± 8.8 (6)	150.0 ± 9.8 (7)	150.0 ± 8.7 (7)	246.3 ± 8.8 (7)	142.5 ± 4.3 (7)	161.8 ± 9.4 (7)	ns	
							P Value (Unpaired Student *t* Test)	Contralateral treatment
$pERK_{1/2}$ (SC)		—		1.0 ± 0.0 (6)	3.9 ± 0.9 (6)	2.9 ± 0.5 (6)	b/d (*P* = 0.0072); b/f (*P* = 0.0023)	
$pERK_{1/2}$ (PGi)		—		1.0 ± 0.0 (6)	1.3 ± 0.1 (6)	1.9 ± 0.3 (6)	b/d (*P* = 0.0039); b/f (*P* = 0.0198)	
$pERK_{1/2}$ (LC)		—		1.0 ± 0.0 (6)	1.4 ± 0.1 (6)	2.2 ± 0.3 (6)	b/d (*P* = 0.0174); b/f (*P* = 0.0013)	
$pERK_{1/2}$ (PFC)		—		1.0 ± 0.0 (6)	3.6 ± 1.1 (6)	2.5 ± 0.5 (6)	b/d (*P* = 0.0157); b/f (*P* = 0.0427)	

Values expressed as mean ± SEM for each experimental group followed by the sample size (n). e/f (*P* = 0.0038) means that MA diclofenac before treatment was statistically different from MA diclofenac after treatment with a *P* value of 0.0038.

Ip = ipsilateral; LC = locus coeruleus; MA = monoarthritis; ns = no significancies; pERK1/2 = phosphorylated extracellular signal–regulated kinases 1 and 2; PFC = prefrontal cortex; PGi = paragigantocellularis; SC = spinal cord.

pain.[38,39] We observed increased $ERK_{1/2}$ activation in the LC after chronic monoarthritis, possibly due to increased excitatory input from the PGi. Interestingly, CFA injection has been shown to induce a sharp increase in LC $pERK_{1/2}$ after 5 min that disappears 7 h later,[40] and indeed, we observed no changes in $pERK_{1/2}$ in the early stages after CFA administration. By contrast, $pERK_{1/2}$ expression was consistently increased 28 days postadministration, coinciding with altered nociceptive behavior and the onset of anxiety and, especially, depressive states. These findings suggest that the LC is involved in both acute pain and the subsequent development of pain-induced affective disorders. Finally, $ERK_{1/2}$ activation was also enhanced in the PFC region 28 days after CFA administration. The PFC is one of the most important projection targets of LC noradrenergic terminals, and increased $pERK_{1/2}$ expression in this area has been correlated with anxiety- and depressive-like behaviors.[9,10,13,35] Another interesting observation was that the pattern of $ERK_{1/2}$ activation at supraspinal level was bilateral, which means that unilateral inflammation of the ankle produced both ipsilateral and contralateral phosphorylation of $ERK_{1/2}$ in the PGi–LC–PFC pathway. This was already shown in other pain studies,[38,39] and particularly, such lack of lateralization of $ERK_{1/2}$ phosphorylation was common in studies regarding

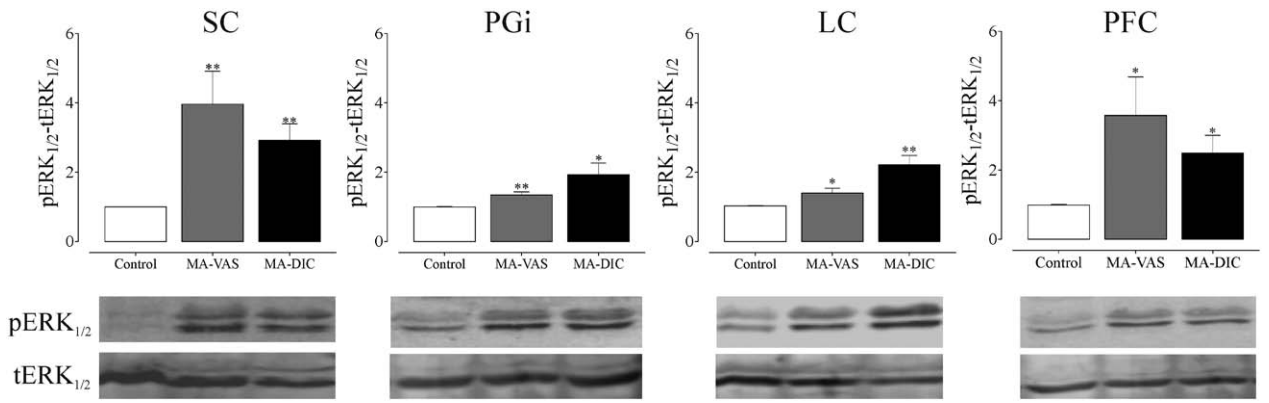

Fig. 8. Effect of the topical administration of vaseline/diclofenac to the contralateral paw in the pattern of $ERK_{1/2}$ activation in the spinal cord (SC)–paragigantocellularis (PGi)–locus coeruleus (LC)–prefrontal cortex (PFC) pathway. Below the graphs, there is a representation of the immunoblots showing the differences between experimental groups. Values expressed as mean ± SEM. **P* < 0.05 and ***P* < 0.01 by unpaired Student *t* test *versus* the control group. Cont = control; DIC = diclofenac; MA = monoarthritis; $pERK_{1/2}$/$tERK_{1/2}$ = phosphorylated/total extracellular signal–regulated kinases 1 and 2, respectively; VAS = vaseline.

pain-related anxiety paradigms.[9,10,13] Taken together, these findings suggest that alterations in the levels of $pERK_{1/2}$ may be one of the molecular mechanisms that underlie the onset of chronic pain–induced affective disorders.

One of the most remarkable findings of the current study was that the blockade of nociceptive inputs affected monoarthritis-induced anxiety- and depression-like behavior. As indicated elsewhere, administration of sodium diclofenac ointment attenuated inflammation and pain,[41,42] as well as reducing $pERK_{1/2}$ levels in the lumbar SC. Significantly, the induction of analgesia was accompanied by the disappearance of anxiety- and depression-like behaviors, suggesting that sensorial pain inputs are the source of affective alterations in chronic monoarthritis. Importantly, the increased $ERK_{1/2}$ activation in the PGi–LC–PFC pathway on monoarthritis induction was successfully restored to control levels by diclofenac treatment, indicating that the increased $ERK_{1/2}$ activity in these regions is a consequence of the nociceptive inputs. Based on its increased expression only when anxiety- and depression-like behaviors are present, we propose that sustained altered pERK1/2 expression in the PGi–LC–PFC pathway in chronic monoarthritis is probably more closely related to the development of pain-related affective disorders than with nociception itself. However, further studies in other areas widely involved in facilitating or inhibiting nociception, such as the periaqueductal gray, rostral ventromedial medulla, and dorsal reticular nucleus, will be necessary to confirm this hypothesis.

To study the possible site of action of diclofenac, we evaluated the effect of administering vaseline/diclofenac in the contralateral paw. Behavioral studies showed that diclofenac administered into the contralateral paw modify neither the paw volume nor the hyperalgesia level, but a slight reduction was observed in the ankle-bend score. Nevertheless, when studying the effect of contralateral application of diclofenac on the expression of $pERK_{1/2}$ in the PGi, LC, and PFC, no significant changes were observed with respect to its control group of monoarthritic rats receiving vaseline on the contralateral paw. Hence, these data suggest that topical application has a low systemic effect that would explain the small effect in the ankle-bend test. Indeed, previous data have shown that local administration (cream, gel, or dermal patch) produces a very low systemic effect when compared with that in oral administration.[43–45] However and of relevance for our study, $pERK_{1/2}$ expression is not modified in any of the brain areas studied; so, it seems unlikely that a central effect produced may be involved in all the pain-related features restored by diclofenac treatment. Overall data suggest that diclofenac might be peripherally acting and that the blockade of the nociceptive inputs originated in the inflamed paw is able to restore $pERK_{1/2}$ expression levels in the brain areas studied. However, it is important to note that further studies will be necessary to discern the peripheral and/or central effect of diclofenac in the reversal of all the monoarthritis-related features.

Although the translation of findings from animal models to humans must always be approached with caution, we believe that our findings have interesting parallels in the clinical setting. Thus, we propose that achieving effective pain relief reverses the molecular changes induced by chronic pain. This hypothesis is consistent with the data from patients with osteoarthritis in whom successful arthroplasty reverses thalamic atrophy.[46] Furthermore, the current findings suggest that effective analgesia also benefits other symptoms (anxio-depressive behaviors) that, although not directly related to sensorial pain, have been identified as major contributors to a worse patient outcome. Finally, it is important to note that neuroelasticity (*i.e.*, reversal of an effect on removal of the stimulus) is observed at the onset of anxio-depressive symptoms. It is possible that a critical window exists after which inducing such reversal will be more difficult, or no longer feasible, due to additional changes in neuronal architecture. These findings have important implications for the ongoing debate regarding the optimal therapeutic approaches to treating patients with arthritis (pharmacological *vs.* surgical strategies).

The delayed onset of monoarthritis-induced affective pathologies suggests that peripheral pain inputs induce some reorganization in the central nervous system. Our study demonstrates that affective behavioral changes are accompanied by $ERK_{1/2}$ activation in the PGi–LC–PFC pathway. Moreover, these findings indicate that successful analgesia can reverse sensorial and affective pain-induced changes.

Acknowledgments

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Competing Interests

The authors declare no competing interests.

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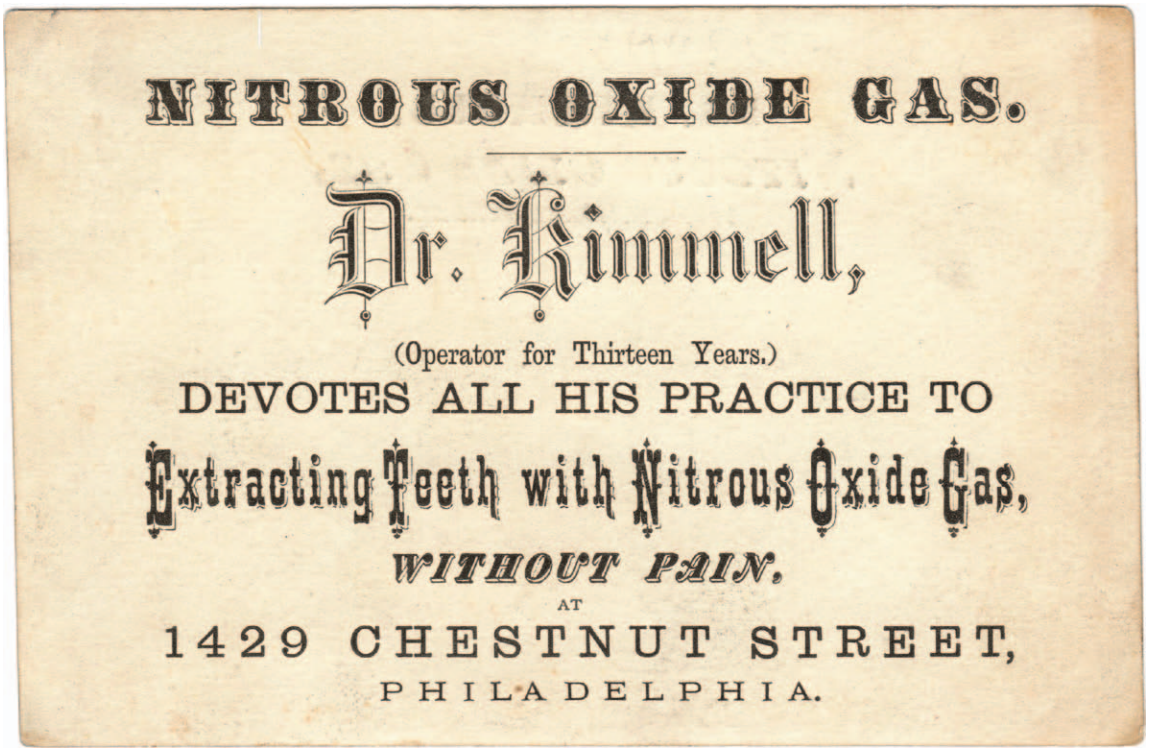
ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Kimmell's Practice Devoted to Extracting Teeth Using Nitrous Oxide



Unlike the obverse (see Anesthesiology Reflections, this issue, p. 1369), the reverse of dentist Samuel Kimmell's trade card made no mention of the Centennial Exposition of 1876. However, Dr. Kimmell advertised that he devoted "ALL HIS PRACTICE TO Extracting Teeth with Nitrous Oxide Gas, Without Pain…." Because he also noted that he had worked as an "Operator for Thirteen Years," Dr. Kimmell may have begun using nitrous oxide fairly shortly after Gardner Q. Colton revived dental use of nitrous oxide anesthesia in 1863. This trade card is part of the Wood Library-Museum's Ben Z. Swanson Collection. (Copyright © the American Society of Anesthesiologists, Inc.)

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4.3. Publication III

Activation of extracellular signal-regulated kinases (ERK 1/2) in the Locus Coeruleus contributes to pain-related anxiety in monoarthritic rats. Submitted to Biological Psychiatry.

Activation of extracellular signal-regulated kinases (ERK 1/2) in the Locus Coeruleus contributes to pain-related anxiety in rats

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Abstract

Background: Chronic pain exponentially increases the risk of suffering emotional disturbances, such as those characterized by persistent anxiety, although the mechanisms underlying this co-morbidity are unknown. Prolonged arthritis can produce anxiety-like behavior in rats, along with enhanced phosphorylation of the extracellular signal-regulated kinase 1/2 (pERK1/2) in the Locus Coeruleus (LC). Therefore, we propose that ERK1/2 activation in the LC plays a crucial role in pain-related anxiety.

Methods: As a model of painful arthritis, monoarthritis (MA) was induced in rats by injection of complete Freund´s adjuvant. We first evaluated the behavioral attributes of pain and anxiety, as well as LC function, at an early (1 week) and late-phase (4 weeks, MA4W) of the disease. Subsequently, we evaluated the effect of pERK1/2 blockade in MA4W.

Results: Pain was evident in monoarthritic rats 1 week after induction, although anxiety did not appear until 4 weeks later. This late-phase of the disease was accompanied by diminished tonic LC activity, which was coupled to exacerbated evoked LC responses to noxious stimulation of the inflamed and the healthy paw. When ERK1/2 activation was dampened by intra-LC SL327 administration, the exaggerated evoked response of MA4W rats was blocked. Importantly, SL327 did not change pain hypersensitivity but rather, it reversed both the anxiogenic-like behavior and the increase in pERK1/2 in the anterior cingulate cortex (ACC) of MA4W rats.

Conclusions: Prolonged monoarthritis produced anxiety-like behavior, ERK1/2 activation and impaired electrical activity in the LC. Blockade of pERK1/2 in the LC reduced pERK1/2 expression in the ACC and relieved anxiety.

Keywords: Locus coeruleus, anxiety, pain, arthritis, ERK1/2, anterior cingulate cortex

Introduction

Chronic pain (CP) is now considered an independent disease, with repercussions that reach far beyond hypersensitivity. Many studies have emphasized that CP exponentially increases the risk of suffering emotional disturbances, such as those characterized by persistent anxiety and low mood, generating a vicious circle that magnifies and exacerbates the painful experience (1-3). In the case of arthritis, pain is produced joints inflammation and it is estimated that more than 30% of the arthritic population in the U.S. suffer anxiety (4). While this co-morbidity is clinically well established, the underlying mechanisms remain unclear. Similarly to what was found by other authors (5-6), we previously reported that prolonged experimental arthritis produced anxiety-like behaviors in rats (7), mimicking the situation

in patients and providing us with an useful experimental tool to assess what causes the anxiety that develops some time after pain begins. By studying phosphorylated isoforms of extracellular signal-regulated kinase 1/2 (pERK1/2) as a marker of plasticity, we have found that long-term arthritis enhances pERK1/2 expression in the Locus Coeruleus (LC) and its projection area, the prefrontal cortex (7), strongly suggesting that this pathway is implicated in the effects of arthritis.

The noradrenergic-LC system is a bilateral pontine nucleus that modulates pain perception through an intricate network of projections. It is widely known that noxious or inflammatory stimuli activate LC descending innervations to the spinal cord to promote the feedback inhibition of pain (8-10). However, there is little information available about the ascending LC projections to the prefrontal areas where the emotional consequences of CP might be modulated (11-12) (Figure 1A). Indeed, the LC is the only source of noradrenaline in the anterior cingulate cortex (ACC), a key structure for pain-related negative emotions (13-14). Therefore, the LC may modulate the sensorial and affective features of CP (7, 15-16).

In the present study we have used an animal model of chronic inflammatory pain (monoarthritis, MA) to evaluate the characteristics of LC discharge following noxious stimulation at an early (non-anxious phenotype) and late (anxious phenotype) phase of disease development. Furthermore, as ERK1/2 activity has been implicated in long-lasting synaptic plasticity in several areas involved in pain processing (e.g., the spinal cord and ACC: 17-18), we also studied the behavioral and electrophysiological effects of the pharmacological inhibition of ERK1/2 activation at the LC level.

Materials and Methods

Animals and Experimental Design

Two hundred male Harlan Sprague-Dawley rats (200-300g, University of Cádiz - ES110120000210) were used in this study (22 ± 1 ºC, 12 h light/dark cycles, lights on at 08:00 AM, food and water *ad libitum*). All the experimental work was carried out in compliance with the European Communities Council Directive 2010/63/EC, Spanish Law (RD 1201/2005) and the ethical guidelines for investigation of experimental pain in animals (19). The experimental protocols were approved by the Institutional Ethical Committee.

Behavior (Group 1) and electrophysiological LC activity (Group 2) were evaluated in control rats, and one (MA1W) or four weeks (MA4W) after inducing inflammation. The effect of intra-LC administration of a pERK1/2 inhibitor (SL327) on LC electrophysiological activity and behavior was evaluated in control and MA4W rats (Groups 3 and 4 respectively). Furthermore, immunohistochemistry studies were performed in parallel groups (Group 5: Figure 1B).

Monoarthritis induction and behavioral assessment

MA was induced under isoflurane (Abbott, Spain) anesthesia (4% to induce, 2% to maintain) by injection into the left tibiotarsal joint (ipsilateral) of a complete Freund's adjuvant (CFA, 50 µL) solution containing 30 mg of desiccated *Mycobacterium butyricum* (Difco-Laboratories, USA) diluted in the vehicle solution (3mL paraffin oil, 2mL saline and 500µL Tween® 80: 20). Control rats were injected with the vehicle solution. Animals that developed polyarthritis were excluded from the study.

The paw-pinch test was used to evaluate mechanical hyperalgesia (21). Briefly, increasing pressure was gradually applied to the dorsal side of the paw using a graded motor-driven device (Ugo Basile, Italy), beginning with 30 g of pressure. A reduction in the pressure that provokes withdrawal indicates mechanical hyperalgesia. Two measures were taken in both the ipsi- and contralateral paws with a 5 minute of interval between each.

Anxiety-like behavior was evaluated in the elevated zero maze (EZM) (7). It consists of a black circular platform divided into four quadrants of equal length: two opposing open quadrants with 1 cm high clear curbs to prevent falls; and two opposing closed quadrants with black walls. A 5 min trial under the same lighting conditions began by placing the animal in the center of a closed quadrant. Spontaneous Motor Activity Recording and Tracking (SMART, Panlab S.L. Spain) software was used to analyze the percentage of time the rat spent in the open arms and the total distance travelled by each rat. Increases in the time spent in the closed areas are correlated with anxiety-like behavior.

LC Electrophysiology

As the ascending pain pathway is mainly contralateral (22), single-unit extracellular recordings of LC neurons contralateral to the injected articulation were obtained (Supplement 1, Figure 1A, 15, 23). When a single LC unit was isolated, the spontaneous basal discharge (baseline) and the sensory-evoked response of LC neurons was studied (Figure 2A). Sensory-evoked LC responses (n=5) were induced by manually applying mechanical pressure for 1s to the ipsilateral or contralateral paw (paw compression, PC) using a surgical forceps. The sensory evoked-response, the duration (D) of the response and the

suppression period (SP) were quantified after each PC (Figure 2B). The post-compression tonic activity was measured over an interval of 70 seconds, 30 seconds after each PC. Changes in sensory-evoked discharge and post-compression discharge were expressed as a percentage of the baseline for each neuron and averaged across recordings.

In another set of experiments, a calibrated pipette alongside the recording micropipette (24) and containing a solution of the inhibitor of ERK1/2 phosphorylation, SL327 (4.17 mM: S4069, Sigma-Aldrich, St Louis, MO), was used to explore the effect on LC activity (Figure 1A – Group 3).

Intra-LC drug administration
Five days before completing the 4 weeks of MA, a guide cannula was implanted into the contralateral (right side) LC at the following coordinates relative to lambda: anteroposterior=-3.7 mm, mediolateral= -1.1 mm, and dorsoventral -6.2 mm, below the brain surface in anaesthetized rats (Supplement 1 and (25)). Following at least 5 days recovery post-surgery, animals received SL327 (0.7 µg) dissolved in DMSO at a final concentration of 4.17 mM. The behavioral tests were performed within 10-25 minutes of drug administration.

Immunohistochemistry
Immunohistochemistry was performed on free-floating sections of the LC and ACC as described in Supplement 1 (26-27). Labeling was visualized on an optical microscope (Olympus BX60, Spain) equipped with a U-MNU filter system (Olympus DP71, Spain) and quantified using Image J software (National Institutes of Health, Bethesda, Maryland).

Statistical analysis
All the data are presented as the means ± SEM and the results were all analyzed using STATISTICA 10.0 (StatSoft, OK, USA) or GraphPad Prism 5 software (GraphPad Software, CA, USA), using either Student´s t-test (unpaired or paired, two-tailed), or a one-way or two-way analysis of variance (ANOVA) followed by the appropriate post-hoc tests (Bonferroni or Dunnett's tests). The independent variables were monoarthritis (between-groups), paw or drug (between-groups: Table S3). Fisher's exact test was used to evaluate the incidence of burst activity. Significance was accepted at $p<0.05$.

Results
Nociceptive and anxiety-like behavior
In MA rats, the ipsilateral paw withdrawal threshold is significantly lower than that of control rats ($p<0.001$ for MA1W and MA4W: Figure 1C), indicative of mechanical hyperalgesia. In addition, MA4W rats spent significantly less time in the open arms of the EZM than control rats, indicative of anxiety-like behavior ($p<0.001$: Figure 1D), although no such changes were observed with MA1W rats. Moreover, no significant changes were observed in the total distance travelled in the EZM, indicating that locomotor activity was not impaired in the MA rats (Figure 1E).

Spontaneous activity of LC neurons
Data from 57, 36 and 37 neurons were recorded from 19 control, 11 MA1W and 17 MA4W rats, respectively. The basal spontaneous LC activity was significantly lower in MA4W rats than in controls ($p<0.01$: Figure 1F), and there tended to be less burst activity in the MA rats (both 1W and 4W, $p=0.059$ and $p=0.056$ vs control, respectively: Figure 1G). However, the number of spikes per burst did not differ among the experimental groups (Figure 1H).

Activity of LC neurons evoked by ipsilateral paw compression
The electrophysiological profile of the sensory-evoked response was assessed during ipsilateral paw compression (Figure 2A and 2B, and Table S1). The application of 5 sequential stimulations did not significantly alter any parameter studied of the sensory responses evoked in any group (S1 vs. S5 for control, MA1W and MA4W, $p>0.05$). However, the evoked activity in MA4W rats was significantly stronger than that of control and MA1W rats, reaching significance in the last stimulation ($p<0.05$ vs. control: Figure 2C and 2K-2M). This reinforcement was particularly evident when analyzing the averaged data of all 5 stimulations, which reflected the significant increase in this parameter in MA4W rats ($p<0.001$ vs. controls: Figure 2D). This analysis also revealed a significantly weaker evoked response in MA1W rats than in the control rats ($p<0.05$: Figure 2D). Furthermore, the duration of the response also increased in MA4W, particularly following stimulations S3 ($p<0.01$), S4 ($p<0.05$) and S5 ($p<0.001$ vs. control: Figure 2E). Likewise, a significant increase in duration was observed in MA4W rats when the averaged data of all stimulations was compared with the controls ($p<0.001$: Figure 2F). Finally, no significant differences were observed in the suppression period between the experimental groups (Figure 2G and 2H).

We also explored the tonic LC rate after each noxious stimulus, expressed as the percentage change from the basal firing rate (post-compression tonic activity: Figure 2B). In the control group, a slight decrease of 12.2% in tonic

activity was observed with each application of mechanical stimulation, while the MA1W group hardly changed its spontaneous firing pattern in response to sequential stimulation. By contrast, an increase in tonic activity of about 16.5% was observed with sequential paw compressions in MA4W rats, with significant differences observed between the MA4W and control rats following each paw compression ($p<0.05$ for S1; $p<0.01$ for S1 and S5; $p<0.001$ for S3 and S4; Figure 2I). Moreover, there was a significant increase in the averaged post-compression tonic activity in MA1W and MA4W rats compared with the controls ($p<0.05$ and $p<0.001$, respectively: Figure 2J).

LC neuron activity evoked by contralateral paw compression

After 5 successive ipsilateral paw compressions, one compression was applied to the non-inflamed contralateral paw (Figure 2A). As expected, no sensory response of the LC was evoked when a noxious stimulus was applied to the contralateral paw of control rats (Figure 2N and 2O). However, when the same procedure was applied to monoarthritic rats after either one or four weeks of inflammation, a sensory-evoked response was observed that was particularly evident in MA4W rats (Figure 2O). Indeed, burst activity was stronger in MA1W rats ($p=0.052$) and significantly higher in MA4W rats ($p<0.05$) than in control rats (Table S1: Figure 2N). No significant differences were detected between the MA1W and MA4W rats.

TH and pERK1/2 expression in the LC

Since increased use of noradrenaline is accompanied by TH upregulation (28), we evaluated the TH expression in the LC. As expected, TH immunolabeling was observed in the cytoplasm and dendrites of noradrenergic neurons. At the caudal level, the number of neurons expressing TH per section increased significantly in MA4W rats compared with control animals ($p<0.01$ and $p<0.05$ for ipsi- and contralateral sides, respectively: Figure 3A and 3G). Similar results were obtained when considering the entire rostro-caudal extension (total portion, $p<0.05$ for ipsi- and contralateral sides: Figure 3C), although no differences were observed in the rostral domain (Figure 3B) in MA1W rats or when comparing the ipsi- and contralateral sides. This increased TH expression in the LC of MA4W animals was confirmed in western blots (Figure S1F). Similar results were obtained when pERK1/2 was evaluated in the LC, where immunolabeling was detected in the cytoplasm, nucleus and in the dendrites, as well as in a large number of fibers surrounding the LC neurons (26). Again, no differences were observed between the ipsi- and contralateral LC. In MA4W rats in the density of pERK1/2 labeling increased significantly in the caudal portion of the LC compared with control animals (Ipsilateral, $p<0.001$; Contralateral, $p<0.001$: Figure 3D and 3H), as well as in the rostral domain (Ipsilateral, $p<0.01$; Contralateral, $p<0.001$: Figure 3E and 3I), and along the entire rostro-caudal extension (Ipsilateral, $p<0.001$; Contralateral, $p<0.001$: Figure 3F). These data suggest that prolonged inflammatory pain produces greater activation of the ERK cascade in the LC (MA4W). Moreover, a slight increase in pERK1/2 expression was detected in the contralateral LC of MA1W rats when the entire rostro-caudal extension was considered ($p<0.05$: Figure 3F).

Electrophysiological effect of intra-LC SL327 microinjection

Next, we studied the effect of SL327 on the LC discharge evoked by ipsilateral paw compression in control and MA4W rats (Group 3, Figure 1B). After noxious stimulations S1, S2, S3 and S4, a dose of SL327 (8.35 - 33.39 pmol) was administered into the LC and the evoked response was measured. SL327 administration did not significantly modify the responses provoked by S1 administration in control animals (evoked response, duration, suppression period and post-compression tonic activity: S1 vs. S2-S5, $p>0.05$: Figure 4A-D), yet SL327 produced a dose-dependent decrease in the evoked discharge after each noxious stimulus in MA4W rats, which was particularly evident at S5 (S1 vs S2-S4, $p>0.05$; S1 vs. S5, $p<0.01$: Figure 4A). Additionally, we found that the evoked discharge produced in MA4W and control rats receiving SL327 differed significantly after S4 and S5 (control vs. MA4W for S4 and S5, $p<0.05$ and $p<0.01$, respectively). However, the duration of the response in control and MA4W rats was not modified by microinjection of SL327 ($p>0.05$). Overall, these results suggested that SL327 decreased the duration of the response that had been significantly increased in MA4W rats (Figure 4B). Regarding the effect of SL327 in suppressing LC neurons after ipsilateral paw compression, a dose-dependent increase was observed in MA4W that was statistically different from the basal S1 value once 33.39 pmol had been administered (S5: S1 vs. S5, $p<0.01$: Figure 4C), and when compared with control animals receiving the same dose (control vs. MA4W, $p<0.001$: Figure 4C). Finally, microinjection of SL327 produced a significant decrease in the post-compression tonic activity in MA4W compared with the basal S1 value (S1 vs. S3-S5, $p<0.001$: Figure 4D), and with the control animals receiving the same dose (control vs. MA4W, $p<0.05$ for S3, S4 and S5: Figure 4D, detailed information can

also be found in Table S2). Representative oscillography traces can be found in Figure 4E.

Effect of intra-LC SL327 administration on nociception, anxiety and pERK1/2 expression in the ACC

Having verified that SL327 (4.17 mM) can revert the pERK1/2 in the LC to control levels (Figure S1D, Supplementary material 1), we assessed the behavioral effects of this inhibitor. The significant decrease in the pain threshold observed in the ipsilateral paw of MA4W rats before microinjection of SL327 (Pre-drug: $p<0.05$ vs. contralateral paw: Figure 5A) was still evident after administering the inhibitor (SL327 vs contralateral paw, $p<0.01$: Figure 5A). Hence, pERK1/2 inhibition appeared to have no effect on the ipsilateral and contralateral paw withdrawal threshold in MA4W rats. However, when their anxiety-like behavior was assessed, microinjection of SL327 reverted the shorter times spent by MA4W rats in the open arms ($p<0.05$, MA4W saline vs. MA4W SL327: Figure 5B), indicating an anxiolytic effect. No changes were observed in control animals receiving SL327 and moreover, no significant changes were observed in the total distance travelled (Figure 5C).

Subsequently, we explored the effect of intra-LC SL327 administration on pERK1/2 expression in the ACC (Figure 5D). A bilateral increase in the number of pERK1/2 immunoreactive cells was found in layers II-III of the ACC of MA4W animals compared to their respective controls, both at the rostral and caudal levels of the nucleus ($p<0.05$, MA4W vehicle vs. control vehicle: Figure 5D and 5F). Interestingly, microinjection of SL327 into the LC of MA4W rats successfully reverted the number of cells expressing pERK1/2 in layers II-III of the ACC to control levels (Figure 5D and 5F), while no significant changes were observed in the control animals that received SL327. The pERK1/2 immunoreactivity in neurons was confirmed by the co-localization with NeuN (Figure S1G).

Discussion

This study shows that pain-related anxiety in monoarthritic rats is accompanied by altered electrophysiological activity in the LC and an increase in pERK1/2 in both the LC and ACC. Furthermore, pharmacological blockade of pERK1/2 at the LC level reversed these alterations. Therefore, this study provides direct evidence of how alterations in the LC provoke ERK1/2 activation in the ACC, leading to pain-related anxiety.

In agreement with our previous data, we show that the pain threshold decreases significantly in MA rats in the first week of inflammation, although these animals do not display emotional changes (7). Hence, MA1W rats serve as a control for pain in the absence of emotional changes given that the anxiogenic phenotype was established after 4 weeks of MA. Electrophysiological LC studies in the early (MA1W) and late phase (MA4W) of the monoarthritis showed a significant decrease in the firing rate in MA4W rats and a tendency towards weaker burst activity in MA1W and MA4W rats. In addition, significant increases in TH expression, the rate-limiting enzyme for the synthesis of noradrenaline, were only evident in MA4W rats, suggesting an increase in the demand for this neurotransmitter. This is consistent with the altered climbing behavior in the forced swimming test observed previously in MA4W rats (7), which could reflect a diminished availability of noradrenaline in the face of a stressful situations (29-30). Indeed, the electrophysiological recordings of the tonic activity of the contralateral LC neurons in monoarthritic conditions may reflect a deficiency in noradrenergic transmission.

We also evaluated the sensory-evoked activity of LC neurons by applying several noxious stimulations to the paw. As expected and in agreement with previous studies, contralateral LC neurons are potently activated by noxious stimulation of the hind paw in control and MA animals. This is consistent with the activation of the contralateral spinoreticular tract (Figure 1A) (31). However, a greater evoked discharge rate was found in prolonged (MA4W) but not in earlier (MA1W) monoarthritis, suggesting an exaggerated response to nociceptive inputs in the late-phase. An analogous increase in the evoked response and in TH expression was observed in chronic neuropathic rats under social stress (16) and in a rat model of post-traumatic stress (32), suggesting that prolonged monoarthritis may produce similar changes in the LC as those observed in stressful conditions. Thus, the increased responsiveness in prolonged monoarthritis might enhance pain transmission upon noxious events and have repercussions on signaling to the corticolimbic areas. LC activation releases moderate amounts of noradrenaline to coordinate arousal, attention and vigilance (33-34). However, hyperactivation of LC circuits may lead to excessive noradrenaline release, an effect that could provoke anxiogenic-like behavior (11).

Interestingly, paw compression of the healthy paw (contralateral) evoked an LC response especially in long-term arthritic animals (MA4W). Hence, the plasticity associated with CP in cortical and sub-cortical areas might fully or partially reflect a disturbance to the ascending sensory pathways. This is a very interesting because it agrees with the bilateral changes found when exploring TH,

pERK1/2 and the noradrenaline transporter in animal models of CP where the injury is produced on one side of the body (7, 15, 35). Nevertheless, as the contralateral paw was stimulated after several stimulations of the injured paw, this effect may be secondary to the stimulation of the injured paw. Nevertheless, we conclude that the ipsilateral LC is electrophysiologically activated in CP conditions, i.e. transmitting information to higher structures, when in healthy conditions it remains silent.

Consistent with our previous western blot studies (7), here we also show an enhancement of pERK1/2 in MA4W by immunohistochemistry. We also explored the effect of the pharmacological blockade of ERK1/2 phosphorylation (SL327 microinjections) on contralateral LC activity, which reverted the alterations to sensory-evoked parameters and post-compression activity after prolonged inflammation to control levels or even beyond. Importantly, this effect only occurred in pathological conditions and SL327 did not produce any significant change in the control rats. We also assessed whether SL327 administration to the contralateral LC had a repercussion on the manifestation of anxiety and pain in MA4W rats, evaluating these effects on mechanical hyperalgesia and anxiety-like behavior. Interestingly, while anxiety behavior reverted to control levels, the mechanical hyperalgesia remained unchanged. Furthermore, the corticotropin-releasing factor (CRF) is a widely known anxiety-related neuropeptide that modulates LC activity (36) and we have recently shown that the microinjection of a CRF antagonist into the contralateral LC reverted both the anxiogenic-like behavior and the pERK1/2 levels in the LC but it did not modify pain threshold (37). This suggests that enhanced ERK1/2 phosphorylation linked with CRF signaling in the contralateral LC at prolonged times of the monoarthritis disease is more related with the emergence of "affective-behaviors". Furthermore, these data demonstrate that pain-related anxiety is dissociated from the perception of the sensory component of pain. This result sheds light on the link between pain hypersensitivity and secondary mental effects. In this sense, it was recently shown that gabapentin administration produces analgesia but it does not rescue the impaired attention produced by neuropathic pain (38-39). Hence, pain-related affective and cognitive impairment does not appear to be due to the inherent stress provoked by the painful experience but rather, it may be attributed to a permanent alteration to a specific circuit.

We also explored the effect of intra-LC SL327 administration on pERK1/2 activation in the ACC. The ACC is one of the largest components of the limbic system and it is known to play an important role in the anxiety- and depressive-like behaviors resulting from pain. The ACC critically modulates pain-related anxiety in several animal models (visceral pain, 18; post-operative pain, 40; neuropathic pain, 13), and intraplantar formalin injection produces persistent and bilateral ERK activation in laminae II-III of the ACC that is required to induce pain-related negative effects (27). This is consistent with the bilateral increase of pERK1/2 observed in laminae II-III of the ACC of MA4W rats. The reduced pERK1/2 activation in the ACC provoked by intra-LC administration of SL327 is consistent with the reduction of anxiety-like behavior in MA4W rats. Furthermore, this effect was found on both sides of lamina II-III of the cortex, suggesting that the effect of LC neurons on the ACC is not direct, as most of the LC projections to the ACC are ipsilateral (14). As such, circuits involving the thalamus (41) or the amygdala should be explored or at least cortico-cortical projections from one hemisphere to the other. It should be noted that for the first time, our studies show that the LC-ACC pathway is critical for pain-induced anxiety. Furthermore, these results agree with the neuroanatomical overlapping hypothesis, which postulates that a brain region (LC) involved in the processing of both pain and affective information may be disrupted by CP and that this may lead to an alteration in the processing of the affective information, thereby triggering a secondary effect.

In an animal model of chronic inflammatory pain (monoarthritis), our data shows that rats displayed a sharp decrease in their sensorial threshold within 1 week of induction, although anxiety-like behavior did not appear until much later (4 weeks). This late phase temporally coincided with a decrease of the tonic LC activity but also, with an exacerbated evoked LC response to noxious stimulation of the inflamed paw. These changes were accompanied by enhanced TH and pERK1/2 expression in the LC. Moreover, when ERK1/2 activation was blocked pharmacologically in the contralateral LC, the exaggerated electrophysiological evoked LC response of MA rats was impaired. Importantly, ERK1/2 blockade did not modify pain hypersensitivity but rather, it reversed the anxiogenic-like behavior, as well as normalizing pERK1/2 expression in the ACC. Thus, we provide direct evidence of how a sub-cortical structure decreases ERK1/2 activation in the ACC, leading to relief from pain-related anxiety.

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Figure Legends

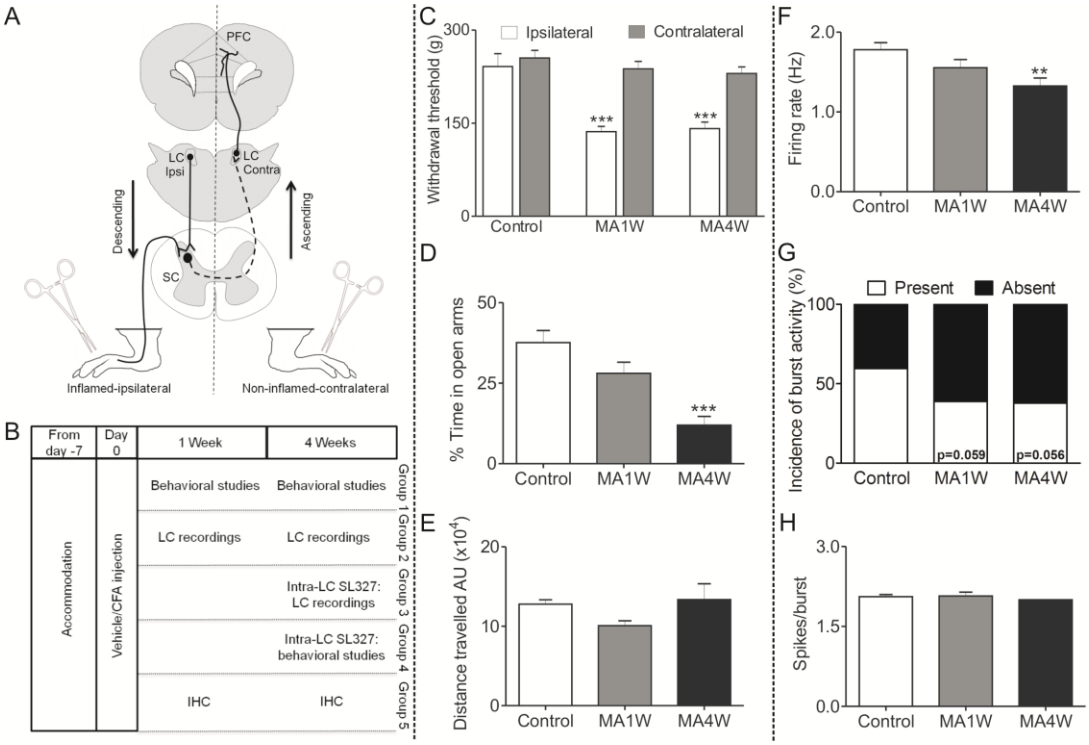

Figure 1: A) Schematic representation of the classical anatomical pathways involved in pain transmission from the paw to the supraspinal centers, passing through the spinal cord, the LC and reaching the PFC (ascending pathway, contralateral) as well as the direct projection originated in the LC to the spinal cord (descending pathway, ipsilateral). B) Schematic representation of the experimental design. C) Mechanical hyperalgesia was confirmed by a decrease in the withdrawal threshold of the ipsilateral paw of MA1W and MA4W rats when compared to the ipsilateral paw of control rats: ***p<0.001 by two-way ANOVA followed by Bonferroni post-hoc test. D) The percentage time spent in the open arms of the elevated zero maze (EZM) decreased significantly in MA4W rats, indicating anxiety-like behavior: ***p<0.001 by one-way ANOVA followed by Dunnett's post-hoc test. E) The total distance travelled in the EZM was no different between the experimental groups. F) The spontaneous firing discharge of LC neurons decreased significantly in MA4W rats: **p<0.01 by one-way ANOVA followed by Dunnett's post-hoc test. G) There was a tendency towards a decrease in the incidence of spontaneous burst activity in MA rats, although the number of spikes/burst was maintained (H). AU, Arbitrary units; CFA, complete Freund's adjuvant; Contra, Contralateral; IHC, immunohistochemistry; Ipsi, Ipsilateral; LC, Locus Coeruleus; MA, monoarthritis; PFC, Prefrontal cortex; SC, spinal cord.

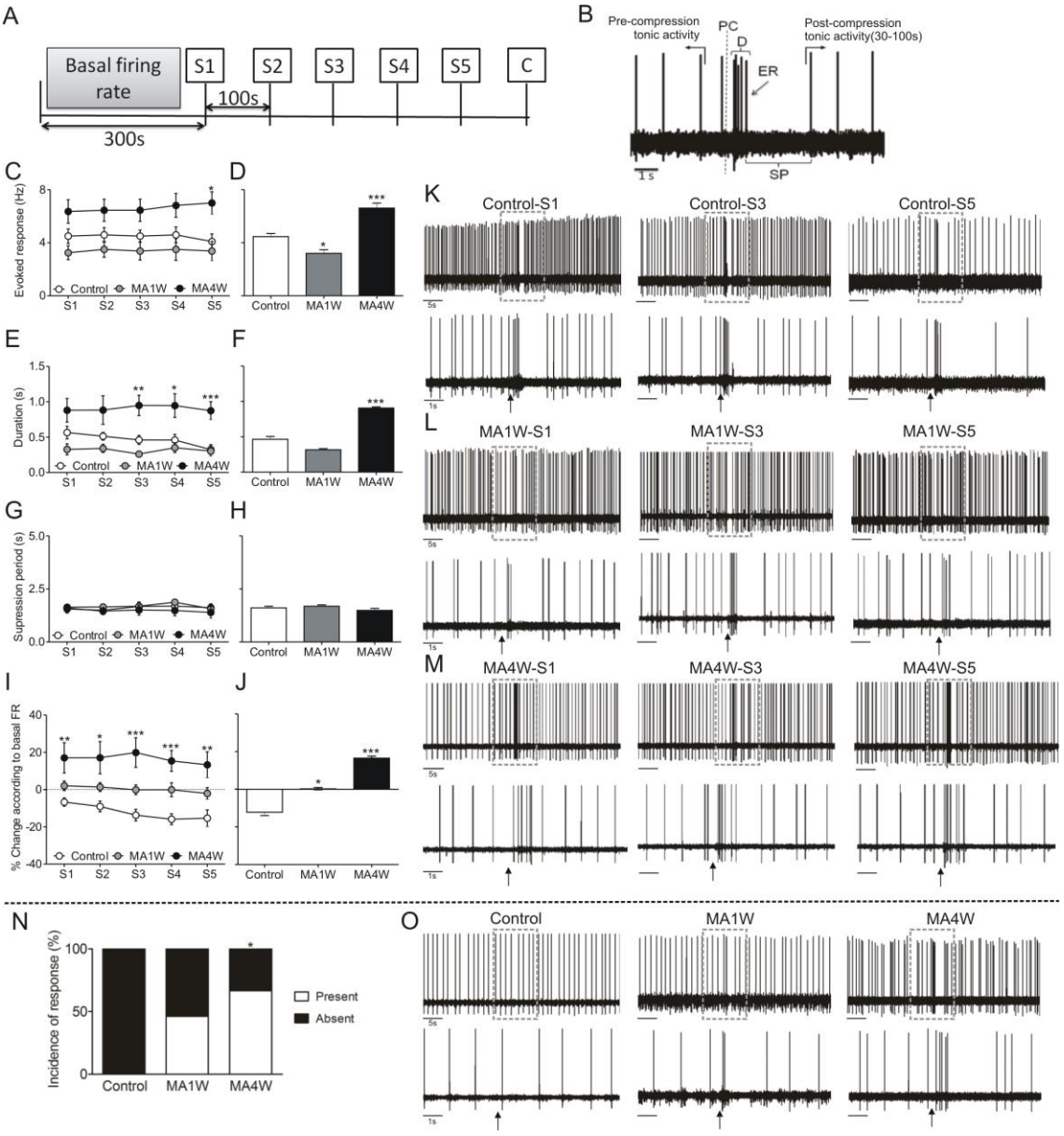

Figure 2: Sensory-evoked responses of LC neurons to sequential ipsilateral paw compression followed by contralateral paw compression in control, MA1W and MA4W rats. A) Schematic representation of the protocol used for electrophysiological recordings of the phasic activity of LC neurons. B) Representative oscillography trace of a typical LC neuron's response to paw compression. C) The evoked response, was not significantly altered during the five paw compressions (S1-S5), although it was significantly higher in the S5 of MA4W animals that in the controls. D) The overall data showed that this response decreased significantly in MA1W rats and that it increased significantly in MA4W rats. E) After S3, S4 and S5, the duration of the response increased significantly in MA4W rats when compared with the controls. F) The overall data showed that the duration of the response increased significantly in MA4W rats. G-H) The period of suppression was no different between the experimental groups. I) The percentage change in the tonic activity relative to the basal values after each paw compression was significantly altered in MA4W rats when compared with the control group. J) These changes were also observed when analyzing the overall data for this parameter. In addition, the MA1W rats also revealed a significant increase when compared with the control group. The statistics represented in the graphs C, E, G and I are *p<0.05, **p<0.01 and ***p<0.001 by One-way ANOVA for each stimulation followed by Dunnett's post-hoc test. The statistics represented in the graphs D, F, H and J are *p<0.05, **p<0.01 and ***p<0.001 by one-way ANOVA followed by Dunnett's post-hoc test. K-M) Oscillography traces representing the LC response in S1, S3 and S5 in control, MA1W and MA4W rats. N) The incidence of evoked burst activity increased in the MA animals. O) Oscillography traces representing the LC response to contralateral stimulation in control, MA1W and MA4W rats. C, contralateral stimulation; D, duration; ER, evoked

response; FR, firing rate; MA, monoarthritis; PC, paw compression; S1-S5, stimulation 1-5; SP, suppression period.

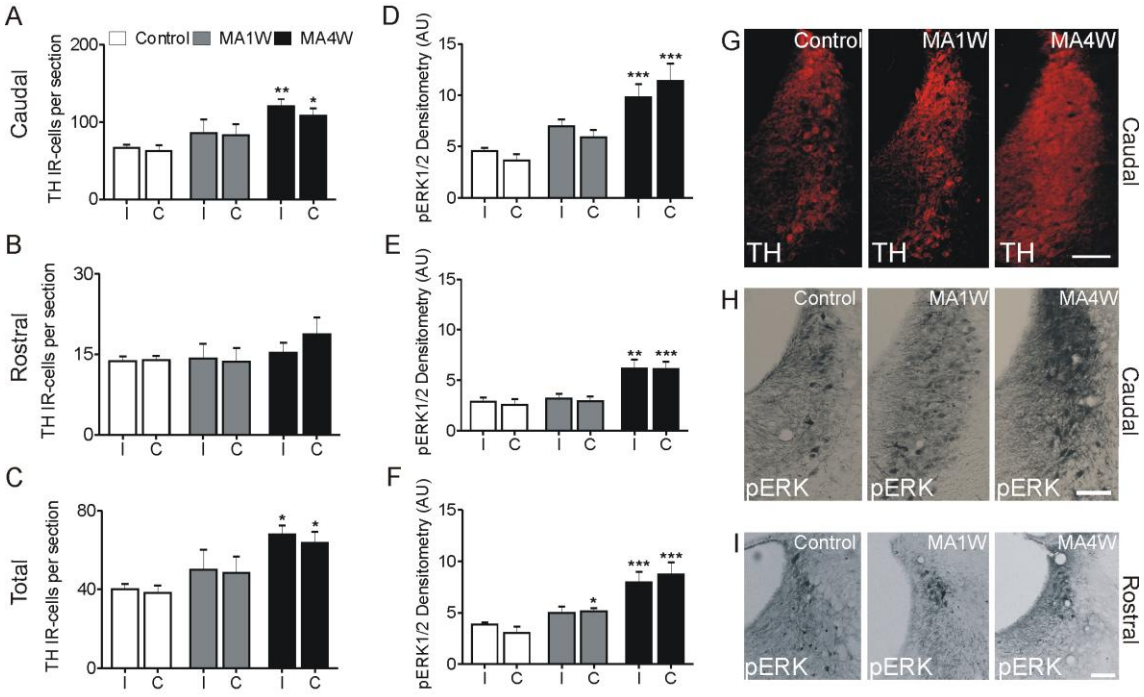

Figure 3: The number of TH immunoreactivity cells (A-C) and the mean values for pERK1/2 (D-F) in the caudal, rostral and total portions of the LC, and representative photomicrographs showing TH (red, G) and pERK1/2 labeling (gray, H and I) in the LC: *p<0.05, **p<0.01 and ***p<0.001 by two-way ANOVA followed by Bonferroni post-hoc test. Scale bar=100µm. AU, Arbitrary units; C, contralateral; I, ipsilateral; MA, monoarthritis; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; TH, tyrosine hydroxylase.

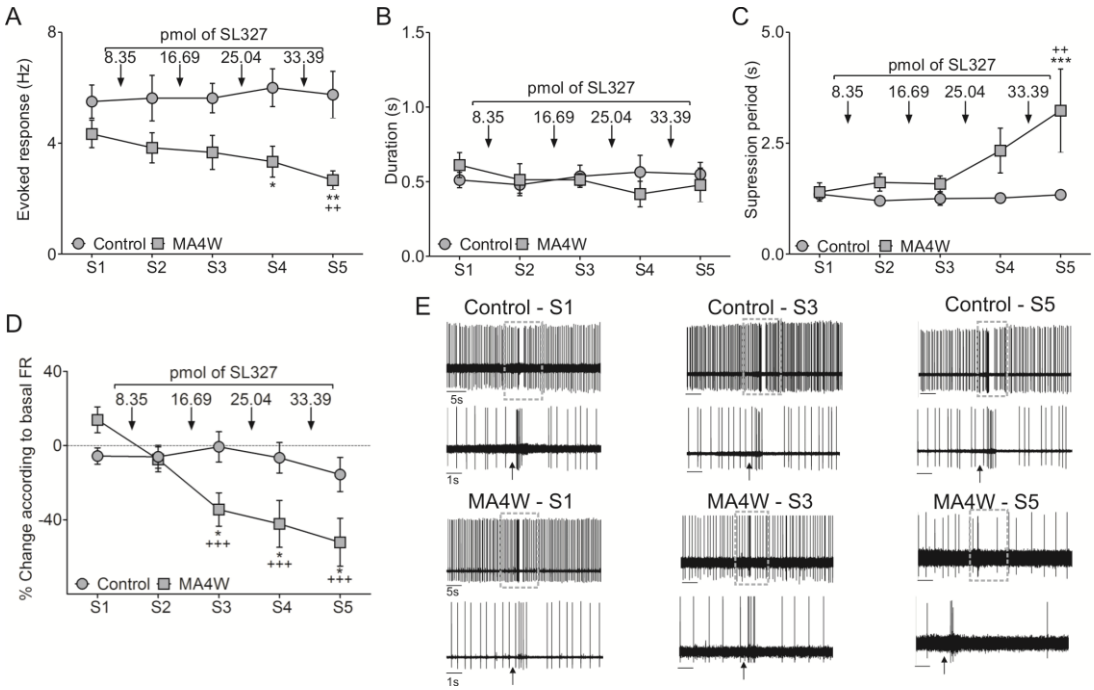

Figure 4: Effect of SL327 microinjection into the LC on electrophysiological parameters. A) The microinjection of SL327 significantly decreased the sensory-evoked response of MA4W rats. B) No significant effects were observed on the duration of the response. C) SL327 microinjection significantly increased the period of suppression. D) SL327 microinjection significantly decreased the change in the tonic activity relative to the basal values: +p<0.05, ++p<0.01 and +++p<0.001 by one-way ANOVA followed by Dunnett's post-hoc test comparing S2-S5 vs. S1; *p<0.05, **p<0.01 and ***p<0.001 by repeated measures followed by Bonferroni post-hoc test comparing control vs. MA4W. E-H) Representative oscillography traces for S1 and S5 in control and MA4W rats. I-J) Representative examples of the firing of LC neurons upon intra-LC administration of SL327 to control and MA4W rats. FR, firing rate; MA, monoarthritis; S1-S5, stimulation 1-5.

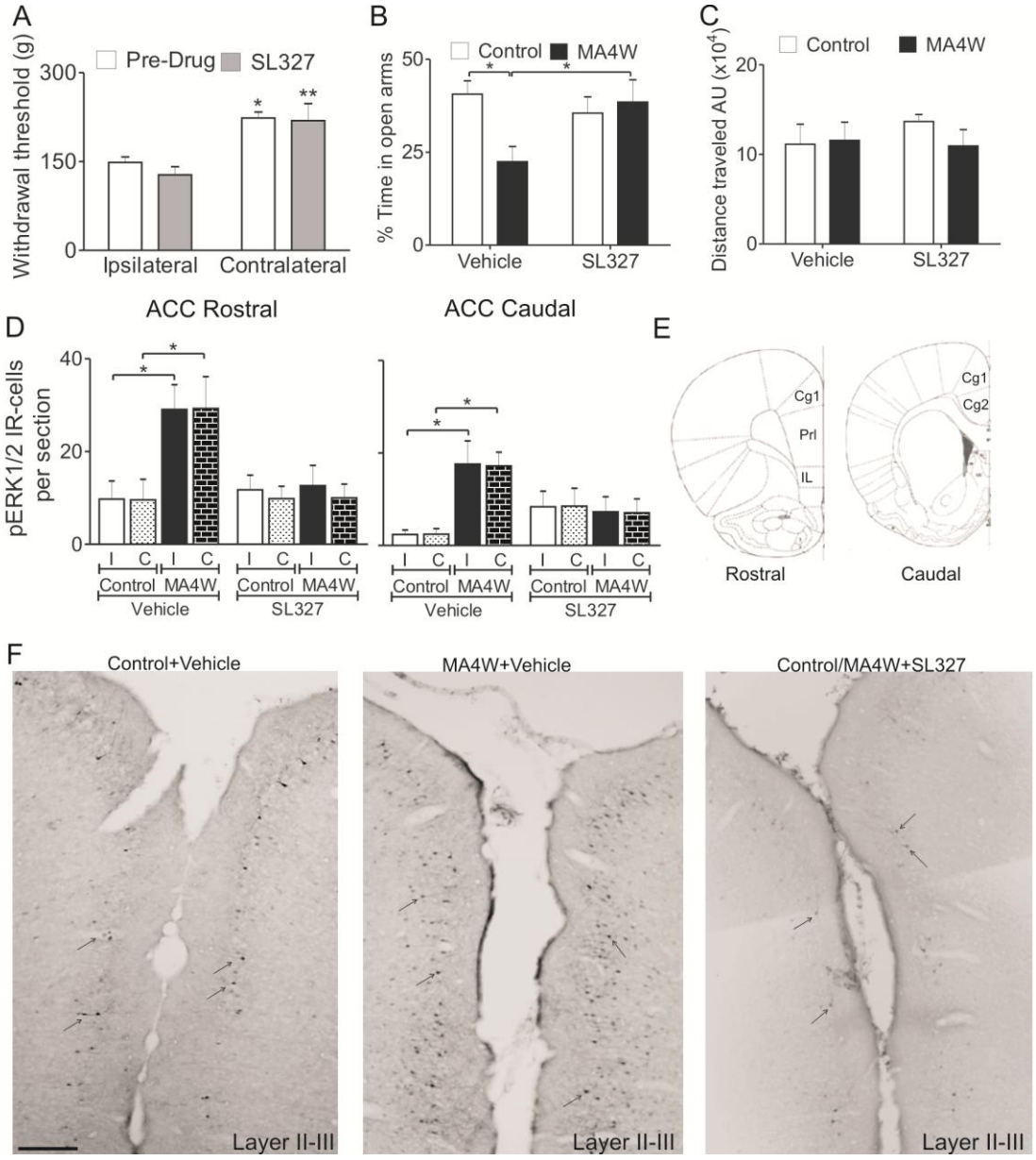

Figure 5: Effect of SL327 microinjection into the LC on the behavior (A-C) and on pERK1/2 expression in the anterior cingulate cortex (ACC: D-F). A) SL327 microinjection into the LC had no effect on the paw withdrawal threshold but it did reverse the anxiety-like behavior in MA4W rats (B), without affecting locomotor activity (C). D) There were significantly fewer cells expressing pERK1/2 in the rostral and caudal ACC of MA4W rats receiving SL327 when comparing with MA4W rats that received the vehicle alone: *$p<0.05$ by two-way ANOVA followed by Bonferroni post-hoc test. E) Representative coronal rat brain sections showing the localization of the rostral and caudal ACC. F) Representative photomicrographs showing pERK1/2 labeling in the layers II-III of the ACC of control and MA4W rats that received an intra-LC microinjection of vehicle or SL327. Scale bar = 100µm. ACC, anterior cingulate cortex; C, contralateral; Cg1, cingulated cortex area 1; Cg2, cingulated cortex area 2; IL, infralimbic cortex; I, ipsilateral; MA, monoarthritis; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; PrL, prelimbic cortex; S1-S5, stimulation 1-5.

Supplementary material

Electrophysiological recordings of the LC neurons

As the ascending pain pathway is mainly contralateral (1), single-unit extracellular recordings of LC neurons from the brain hemisphere contralateral to the injected articulation were obtained. Anaesthesia was induced by a single intraperitoneal injection of chloral hydrate (400 mg/kg) and maintained using a perfusion pump (60 mg/kg/h) (2). Rats were placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA), with the nose down to obtain a 15° angle (Figure S1A) and body temperature was maintained at 37°C with a heated pad. The recording electrode was lowered into the LC contralateral to the inflamed hind paw (anteroposterior: -3.7 mm, mediolateral: -1.1 mm relative to lambda; 5.0-7.0 mm from brain surface after dura removal; (3)). Well-established criteria described elsewhere (4) were used to identify LC neurons: long-duration action potential (>2 ms), spontaneous firing at a regular rhythm, a slow firing rate between 0.5 and 5 Hz, and characteristic spikes with a long-lasting positive-negative waveform (Figure S1C). The signal was amplified with a high-input impedance amplifier and discriminated spikes were fed into a PC and processed using CED Micro 1401 and Spike2 computer software (Cambridge Electronic Design, Cambridge, UK).

When a single LC unit was isolated (Figure 2A), the spontaneous basal discharge (baseline) was recorded for, at least, 2 min and the following parameters were calculated: firing rate (Hz), the incidence of burst activity, and the number of spikes per burst. A LC cell was considered to exhibit burst firing when it displayed at least two spikes with an initial interspike interval below 80 ms and with subsequent interspike intervals higher or equal to 160 ms (5). The sensory-evoked response of LC neurons was studied after stable basal spontaneous firing by manually applying mechanical pressure to the ipsilateral or contralateral paw (paw compression: PC: Figure 2B) for 1s using a surgical forceps. This sensory-evoked discharge was followed by a suppression period (SP) (6-9). This type of PC does show neither sensitization nor habituation with repeated trials in naive animals (10). For each recorded neuron, PC was applied 5 times with 100s of interval (Figure 1B). In order to prevent paw damage, no more than 4 neurons were studied per rat. The sensory evoked-response, the duration (D) of the response and the SP were quantified after PC. The post-compression tonic activity was measured in an interval of 70 seconds, thirty seconds after each PC. Changes in sensory-evoked discharge and post-compression discharge were expressed as a percentage of the baseline for each neuron and averaged across recordings. At the end of the experiment, a Pontamina Sky Blue mark was deposited by applying a 5 μA cathodic current through the recording electrode and subsequently the site of recording was histologically verified (Figure S1B). Only measurements from cells within the LC area were included in this study.

A calibrated pipette glued adjacent to a recording micropipette (11) with a solution containing 4.17 mM of SL327, an ERK1/2 phosphorylation inhibitor (S4069; Sigma-Aldrich, St Louis, MO), was used to explore the effect of pERK1/2 inhibition on electrophysiological LC activity in another set of experiments (Figure 1A – Group 3). This compound was dissolved in a vehicle solution of 50% dimethyl sulfoxide (DMSO) and 50% of Dulbecco's buffered saline containing: NaCl 136.9 mM, KCl 2.7mM, NaH2PO4 8.1mM, KH2PO4 1.5mM, MgCl2 0.5mM, and CaCl2 0.9 (pH = 7.40). Pulses of pressure (50-150ms) were applied with a Picospritzer™ II (General Valve Corporation, Fairfield, NJ, USA) to inject the compound. The injected volume was measured by monitoring the meniscus movement in the pipette which was previously calibrated so that each pulse corresponded to the injection of 2 nl of solution. The vehicle solution did not alter the LC electrical activity in control or MA rats.

Intra-LC drug administration

Five days before completing the 4 weeks of MA, rats were anaesthetized with intraperitoneal injection of ketamine (100mg/kg) and xylazine (20mg/kg), and placed in a stereotaxic instrument with the head tilted at a 15º angle to the horizontal plane (nose down). A guide cannula (22 gauge, 15mm length) was implanted in the contralateral (right side) LC at the following coordinates relative to lambda: anteroposterior=-3.7 mm, mediolateral= -1.1 mm, and dorsoventral -6.2 mm below the brain surface (3). After positioning, the guide cannula was fixed to the skull and anchored to stainless screws with polyacrylic cement. A stainless steel wire was inserted into the guide cannula to prevent occlusion. Following at least five days of postsurgical recovery, animals were gently immobilized with a cloth and the steel wire was cut. Microinjection was performed by lowering a removable injector with 1mm longer than the guide tip connected to a 10μL Hamilton syringe (7). Animals received 0.7 μg of SL327 dissolved in DMSO to obtain a 4.17mM solution. The injection volume was 0.5μL. DMSO was used as a vehicle,

since no behavioral changes were observed when compared to rats receiving saline. The microinjection lasted for 30 seconds and the injector remained inserted for more 60 seconds to allow drug spreading. The behavioral tests (paw pressure and EZM tests) were performed within 10-25 minutes after drug administration. Random animals in all groups were selected for histological confirmation of the localization of the implanted cannula (Figure S1E).

Immunohistochemistry

Control, MA1W and MA4W rats were anaesthetized with 8% chloral hydrate (400 mg/kg) and were immediately perfused through the ascending aorta with 250 mL of oxygenated Tyrode's solution followed by 750 mL of paraformaldehyde 4% in phosphate buffer (PB), 0.1M pH 7.2. Brains were removed and processed for free-floating immunohistochemistry. Sections of 30µm of thickness containing the LC region were immunorreacted against TH (raised in sheep, 1:2000; Abcam, UK) or against the phosphorylated isoforms of ERK1/2 (raised in rabbit, pERK1/2; 1:500; Acris antibodies, Germany), as described elsewhere (12). Immunodetection was achieved upon incubation with the correspondent secondary antibody and, in the case of the pERK1/2 protein, an ABC kit followed by incubation in a 3,3'-diaminobenzidine solution was used. Sections were mounted on gelatin-coated glass slides, and cover-slipped with a PBS-Glycerol based medium. In parallel, sections containing the ACC region were similarly immunorreacted against the phosphorylated isoforms of pERK1/2 and revealed as mentioned above. Additionally, several sections containing the ACC region were double immunorreacted against pERK1/2 and NeuN (Neuronal nuclear antigen, raised in mouse; 1:500; Millipore, Chemicon, CA, USA). This double immunoreaction was revealed using the correspondent fluorescent secondary antibodies: alexa® 488 (green) for pERK1/2, alexa® 568 (red) for NeuN. The histological delimitation of the LC and the ACC areas was made according to The Rat Brain Atlas of Paxinos and Watson (3).

Positive cells to TH were quantified by counting the number of cellular bodies labelled in all sections containing each region analyzed (either the caudal, the rostral or the total), per animal, by using an Olympus BX60 microscope coupled to a digital camera (Olympus DP71), and an image software (Cell F 2.4). The average number of immunoreactive (IR)-cells/section was used for statistical purposes. Similar procedures were performed for quantification of the number of positive cells to pERK1/2 in the ACC, which was based on what was described elsewhere (13). The expression of pERK1/2 in the LC was quantified by densitometry as described before (12).

SL327 dosage adjustment

A preliminary study was performed to verify the dosage of SL327 required for both electrophysiological and behavioral procedures as the volumes necessary in each technique are quite different. Previous reports where it was administered, by intracerebroventricular injection, 2ul of a solution of 1nmol/ul of SL327, which corresponds to 0.67µg of SL327, were used as reference for dosage adjustment (14). Thus, we studied if a similar amount was able to reduce the significant increase of ERK1/2 activation observed in the LC of MA4W rats. For that propose, we microinjected in the LC, a DMSO-based solution containing 0.7ug or 1.4ug of SL327 and the animals were sacrificed for western blot procedures 15 minutes after microinjection, as described in the following section. The results obtained are depicted in Figure S1 and allowed the establishment of the dose of SL327 to be used in the behavioral assessment. However, in the electrophysiological recordings, where a single neuron is recorded at each time, an identical formulation was used but the volumes administered at each time were adjusted.

Western blotting

Fresh tissue samples from the LC were collected from two sets of rats. One set was used for TH quantification and included control, MA1W and MA4W rats divided by ipsi- and contralateral sides. The other set included control and MA4W rats receiving DMSO, 0.7µg or 1.4µg of SL327. All the samples were processed for Western blotting and after the tissue was lysed, an aliquot (50 µg) was separated on a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (PVDF; BioRad, Hercules, CA). After washing in Tris-buffered saline containing 0.1% Tween-20, the blots were blocked with 5% Bovine Serum Albumin (Sigma, St Louis, MO) in TBST. The blots containing the samples for TH quantification were incubated overnight at 4ºC with rabbit anti-TH (1:5,000; Acris antibodies, Herford, Germany) and mouse anti-tubulin (1:250,000; Sigma-Aldrich, St Louis, MO). The blots containing the samples for pERK1/2 quantification were incubated overnight at 4ºC with rabbit anti-phospho-ERK1/2 (1:5,000; Acris Antibodies, Herford, Germany), and mouse-anti ERK1/2 (1:2,000; Cell Signaling Technology, Danvers, MA) antibodies diluted in 5% Bovine Serum Albumin-TBST. After thorough washing, these primary antibodies were detected by incubating for 1 hour at room temperature with IRDye 800CW goat anti-rabbit (green) or IRDye 680LT goat anti-mouse (red) secondary antibodies

(1:10,000; LI-COR®, Lincoln, NE). After 3 final washes with TBST, the antibody binding was detected using a LI-COR Odyssey® two-channel quantitative fluorescence imaging system (LI-COR®, Lincoln, NE). Digital images of Western-blots were analyzed by densitometry using the ImageJ free access software. The data were expressed as pERK1/2 expression levels relative to those of total ERK1/2. These values were combined and averaged.

Antibody specificity control

In this study, we did not perform any antibody specificity control as the antibodies used are well-known for their specificity and widely used in previous studies (7, 12, 15-18).

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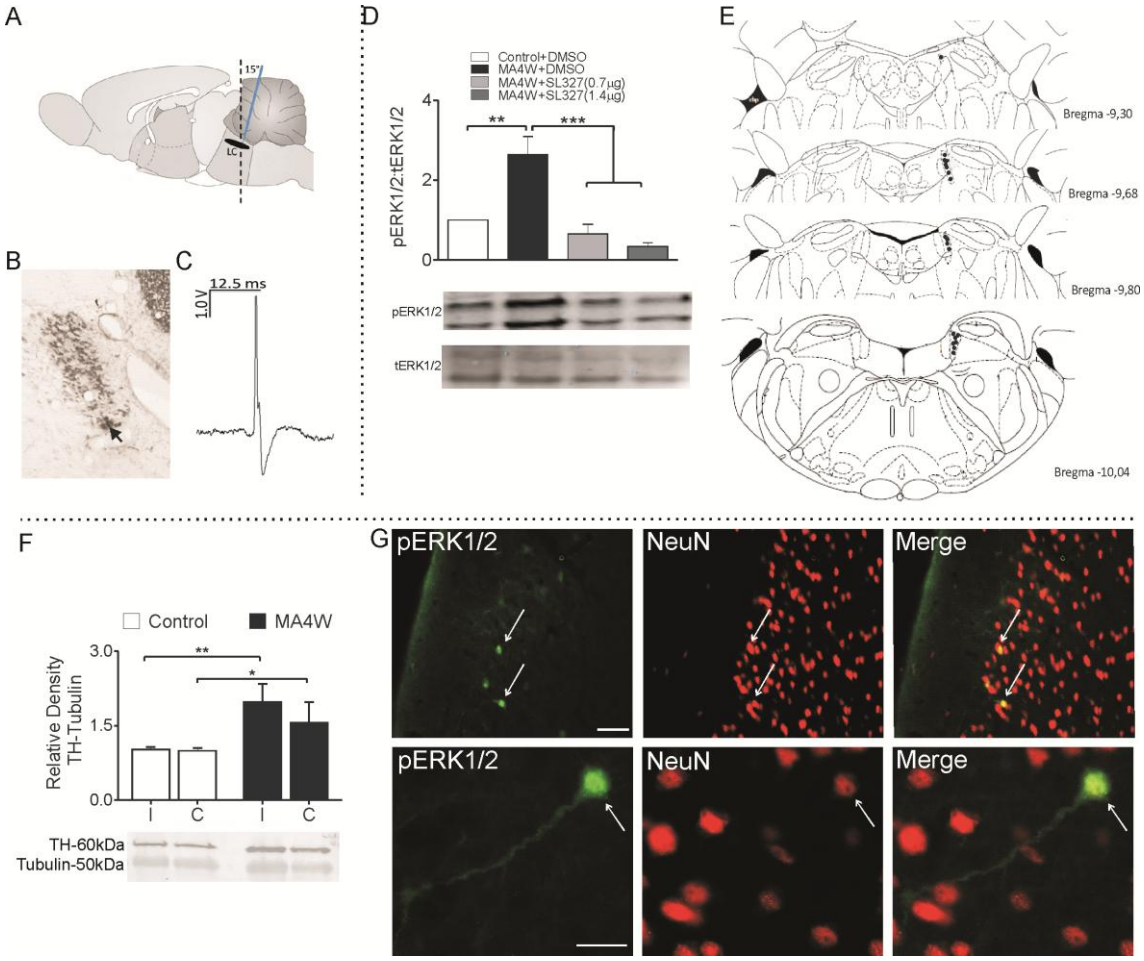

Figure S1 – Supplementary material. A) Schematic representation of the recording electrode's position. B) Pontamine spot (arrow) certifying that the recording electrode was well positioned in the LC. C) LC typical spike shape. D) Expression of pERK1/2 (42-44 kDa) in the LC in response to MA or SL327 (0.7 ug or 1.4 ug) and corresponding images of the western blots for each experimental group (below the graph). These levels were normalized to the corresponding total ERK1/2 values as no significant changes in tubulin levels were observed: **p<0.01 and ***p<0.001 by one-way ANOVA followed by Dunnett's post-hoc test. E) Histological representation of the unilateral injection sites and cannula placement within the LC. F) TH expression evaluated in western blots increased significantly in MA4W rats: p<0.05 by two-way ANOVA followed by Bonferroni post-hoc test. Below the graphs are the corresponding images of the western blots for each experimental group. G) Photomicrographs showing the co-localization of pERK1/2 immunolabeling with NeuN positive neurons in the ACC. The lower row shows higher magnification images. Scale bar = 50µm. C, contralateral; I, ipsilateral; LC. Locus Coeruleus; MA, monoarthritis; NeuN, Neuronal nuclear antigen; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; tERK1/2, total extracellular signal-regulated kinase 1/2; TH, tyrosine hydroxylase.

Table S1 - Effect of five sequential compressions to the ipsilateral paw followed by one compression to the contralateral paw in the phasic activity parameters

		S1	S2	S3	S4	S5	C
I (%)	Control	100.00 (10/10)	100.00 (10/10)	100.00 (10/10)	100.00 (10/10)	100.00 (10/10)	0.00 (0/7)
	MA1W	100.00 (8/8)	100.00 (8/8)	100.00 (8/8)	100.00 (8/8)	100.00 (8/8)	46.20 (6/13)
	MA4W	100.00 (11/11)	100.00 (11/11)	100.00 (11/11)	100.00 (11/11)	100.00 11/11)	66.70 (6/9)*
ER (Hz)	Control	4.50±0.54	4.60±0.54	4.50±0.45	4.60±0.60	4.10±0.57	-
	MA1W	3.25±0.53	3.50±0.60	3.38±0.68	3.50±0.82	3.38±0.73	2.17±0.17
	MA4W	6.36±0.89	6.46±0.84	6.46±0.84	6.82±0.90	7.00±0.84	2.83±0.54
Duration (s)	Control	0.57±0.09	0.51±0.05	0.46±0.06	0.46±0.08	0.33±0.07	-
	MA1W	0.33±0.08	0.34±0.06	0.26±0.04	0.35±0.07	0.30±0.07	0.15±0.02
	MA4W	0.88±0.17	0.88±0.20	0.95±0.14	0.95±0.17	0.88±0.13	0.22±0.07
SP (s)	Control	1.56±0.19	1.49±0.17	1.68±0.19	1.69±0.19	1.62±0.18	-
	MA1W	1.64±0.15	1.65±0.14	1.69±0.20	1.88±0.14	1.57±0.16	1.73±0.13
	MA4W	1.60±0.16	1.46±0.17	1.51±0.24	1.48±0.24	1.39±0.28	1.64±0.30
% Change	Control	-6.70+2.22	-9.17+3.23	-13.73+3.10	-15.92+2.93	-15.36+4.44	-
	MA1W	2.04+2.56	1.36+2.36	-0.13+2.58	-0.09+3.73	-2.08+3.12	-
	MA4W	17.04±8.10	17.10±8.67	19.88±7.94	-2.08±3.12	13.29±6.94	-

Table S2 - Effect of SL327 on the ipsilateral paw compression response

		S1 (Basal)	S2 (8.35pmol)	S3 (16.69pmol)	S4 (25.04pmol)	S5 (33.39pmol)
I (%)	Control	100.00 (8/8)	100.00 (8/8)	100.00 (8/8)	100.00 (8/8)	100.00 (8/8)
	MA4W	100.00 (6/6)	100.00 (6/6)	100.00 (6/6)	100.00 (6/6)	100.00 (6/6)
ER (Hz)	Control	5.50±0.60	5.63±0.82	5.63±0.53	6.00±0.68	5.75±0.84
	MA4W	4.33±0.49	3.83±0.54	3.67±0.62	3.33±0.56	2.67±0.33
Duration (s)	Control	0.51±0.05	0.48±0.06	0.54±0.08	0.57±0.11	0.55±0.08
	MA4W	0.61±0.08	0.51±0.11	0.51±0.04	0.42±0.09	0.48±0.11
SP (s)	Control	1.35±0.12	1.20±0.09	1.25±0.15	1.26±0.08	1.34±0.12
	MA4W	1.40±0.21	1.62±0.20	1.58±0.18	2.33±0.50	3.23±0.94
% Change	Control	-5.63±4.39	-6.03±6.17	0.63±8.20	-6.55±8.26	-15.56±9.18
	MA4W	13.92±6.97	-7.43±6.73	-34.44±8.9	-42.14±12.62	-52.10±12.95

Table S3- Statistics of the analysis of variance (ANOVA).

		Without SL327			With intra-LC SL327			
	S	Factor	Factor	Interaction	S (dose)	Factor	Factor	Interaction
Behavior								
Paw Withdrawal (g)		$F_{a(2,30)}=15.21^{***}$	$F_{p(2,30)}=41.22^{***}$	$F_{i(2,30)}=6.67^{**}$		$F_{t(1,8)}=0.78$	$F_{p(1,8)}=17.51^{**}$	$F_{i(1,8)}=0.32$
% Time in open arms		$F_{a(2,16)}=16.25^{***}$				$F_{a(1,20)}=2.70$	$F_{d(1,20)}=1.39$	$F_{i(1,20)}=5.25^{*}$
Distance Travelled (AUx10^4)		$F_{a(2,16)}=1.61$				$F_{a(1,20)}=0.39$	$F_{d(1,20)}=0.28$	$F_{i(1,20)}=0.76$
Electrophysiology								
Tonic activity								
Firing rate (Hz)		$F_{a(2,127)}=5.76^{**}$						
Spikes/burst		$F_{a(2,60)}=0.49$						
Phasic activity		One-Way						
Evoked response (Hz)	S1	$F_{a(2,26)}=4.74^{*}$			S1 (Basal)	$F_{a(1,48)}=6.44^{*}$	$F_{t(4,48)}=1.31$	$F_{i(4,48)}=2.74^{*}$
	S2	$F_{a(2,26)}=4.53^{*}$			S2 (8.35)			
	S3	$F_{a(2,26)}=5.02^{*}$			S3 (16.69)			
	S4	$F_{a(2,26)}=4.50^{*}$			S4 (25.04)			
	S5	$F_{a(2,26)}=6.96^{**}$			S5 (33.39)			
	T	$F_{a(2,142)}=30.66^{***}$						
	RM	$F_{a(2,104)}=5.83^{**}$	$F_{s(4,104)}=0.33$	$F_{i(8,104)}=0.63$				
Duration (s)	S1	$F_{a(2,26)}=4.63^{*}$			S1 (Basal)	$F_{a(1,48)}=0.05$	$F_{t(4,48)}=0.48$	$F_{i(4,48)}=1.39$
	S2	$F_{a(2,26)}=3.90^{*}$			S2 (8.35)			
	S3	$F_{a(2,26)}=11.84^{***}$			S3 (16.69)			
	S4	$F_{a(2,26)}=6.65^{**}$			S4 (25.04)			
	S5	$F_{a(2,26)}=11.19^{***}$			S5 (33.39)			
	T	$F_{a(2,142)}=35.45^{***}$						
	RM	$F_{a(2,104)}=11.99^{***}$	$F_{s(4,104)}=0.50$	$F_{i(8,104)}=0.42$				
Suppression period (s)	S1	$F_{a(2,26)}=0.06$			S1 (Basal)	$F_{a(1,48)}=4.90^{*}$	$F_{t(4,48)}=5.92^{***}$	$F_{i(4,48)}=5.28^{**}$
	S2	$F_{a(2,26)}=0.37$			S2 (8.35)			
	S3	$F_{a(2,26)}=0.22$			S3 (16.69)			
	S4	$F_{a(2,26)}=0.88$			S4 (25.04)			
	S5	$F_{a(2,26)}=0.30$			S5 (33.39)			
	T	$F_{a(2,142)}=1.32$						
	RM	$F_{a(2,104)}=0.61$	$F_{s(4,104)}=0.43$	$F_{i(8,104)}=0.23$				
% change according to basal firing rate	S1	$F_{a(2,26)}=4.78^{*}$			S1 (Basal)	$F_{a(1,80)}=3.15$	$F_{t(4,80)}=12.21^{***}$	$F_{i(4,80)}=8.96^{***}$
	S2	$F_{a(2,26)}=4.74^{*}$			S2 (8.35)			
	S3	$F_{a(2,26)}=8.72^{**}$			S3 (16.69)			
	S4	$F_{a(2,26)}=12.63^{***}$			S4 (25.04)			

		S5	$F_{a(2,26)}$=6.75**			S5 (33.39)			
		T	$F_{a(2,142)}$=36.05***						
		RM	$F_{a(2,104)}$ =8.39**	$F_{s(4,104)=}$3.51**	$F_{i(8,104)}$=1.09				
			Factor	Factor	Interaction		Factor	Factor	Interaction
Immunohistochemistry									
TH in LC									
	Caudal		$F_{a(2,34)}$=9.21***	$F_{p(1,34)}$=0.46	$F_{i(2,34)}$=0.09				
	Rostral		$F_{a(2,34)}$=1.33	$F_{p(1,34)}$=0.36	$F_{i(2,34)}$=0.47				
	Total		$F_{a(2,34)}$=8.12**	$F_{p(1,34)}$=0.25	$F_{i(2,34)}$=0.02				
pERK1/2 in LC									
	Caudal		$F_{a(2,32)}$=25.58***	$F_{p(1,32)}$=0.04	$F_{i(2,32)}$=1.28				
	Rostral		$F_{a(2,32)}$=18.14***	$F_{p(1,32)}$=0.17	$F_{i(2,32)}$=0.03				
	Total		$F_{a(2,32)}$=27.29***	$F_{p(1,32)}$=0.00	$F_{i(2,32)}$=0.76				
pERK1/2 in ACC									
	Caudal								
						Ipsilateral	$F_{a(1,12)}$=3.56	$F_{d(1,12)}$=0.33	$F_{i(1,12)}$=4.75*
						Contralateral	$F_{a(1,12)}$=4.36	$F_{d(1,12)}$=0.41	$F_{i(1,12)}$=6.39*
	Rostral								
						Ipsilateral	$F_{a(1,13)}$=5.46*	$F_{d(1,13)}$=2.79	$F_{i(1,13)}$=4.54
						Contralateral	$F_{a(1,13)}$=5.08*	$F_{d(1,13)}$=4.63	$F_{i(1,13)}$=4.90*

The F values with their associated degrees of freedom were expressed as F_a (df of arthritis, residual), F_s (df of stimulation, residual), F_d (df of drug SL327, residual), F_p (df of paw, residual), F_t (df of time, residual), or F_i (df of interaction, residual). Significance was represented as *$p<0.05$, **$p<0.01$ and ***$p<0.001$

4.4. Publication IV

Corticotropin-Releasing Factor acts through the ERK1/2 Signaling Cascade in Locus coeruleus Neurons to Mediate Pain-Induced Anxiety

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Corticotropin-Releasing Factor Mediates Pain-Induced Anxiety through the ERK1/2 Signaling Cascade in Locus Coeruleus Neurons

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ABSTRACT

Background: The corticotropin-releasing factor (CRF) is a stress-related neuropeptide that modulates Locus Coeruleus (LC) activity. As LC has been involved in pain and stress-related patologies, we tested whether the pain-induced anxiety is a result of the CRF released in the LC.

Methods: Complete Freund's adjuvant (CFA)-induced monoarthritis (MA) was used as inflammatory chronic pain model. α-helical CRF receptor antagonist was microinjected into the contralateral LC of four weeks MA animals. The nociceptive and anxiety-like behaviors, as well as phosphorylated extracellular signal-regulated kinases 1/2 (pERK1/2) and CRF receptors expression were quantified in the Paraventricular Nucleus (PVN) and LC.

Results: MA rats manifested anxiety and increased pERK1/2 levels in the LC and PVN, although the expression of CRF receptors was unaltered. α-helical CRF antagonist administration reversed both the anxiogenic-like behavior and the pERK1/2 levels in the LC.

Conclusions: Pain-induced anxiety is mediated by CRF neurotransmission in the LC through ERK1/2 signaling cascade.

Keywords: Anxiety; Corticotropin-releasing factor; Locus Coeruleus; Pain; pERK1/2.

INTRODUCTION

Corticotropin-releasing factor (CRF) is a neuropeptide released from neurons in the paraventricular nucleus (PVN) of the hypothalamus that activates stress-related hypophysial structures (Bale and Vale, 2004). Extrahypophysial CRF operates as a neurotransmitter in several brain areas, influencing different actions related to the stress response (Valentino and Wehby, 1988). Also, it may exacerbate many chronic diseases, in particular, those involving severe pain like arthritis (Zautra et al., 2007), a prevalent inflammatory condition. Equally, the emergence of anxiety due to persistent pain is a negative factor commonly reported by arthritic patients (Gyurcsik et al., 2014), representing in itself a stressful situation (Hummel et al., 2010) and inducing similar effects to other stressors (Vierck et al., 2010). Indeed, it is estimated that up to 20% of patients with arthritis will develop depression and/or anxiety (Covic et al., 2012). Although inflammatory pain is a stressor that may modulate the hypothalamic-pituitary-adrenal (HPA) axis (Bomholt et al., 2004), the neurobiological features and behavioral repercussion of such association remain poorly understood.

CRF acts at different sites in important regulatory pain structures, directly implicating this molecule in pain modulation (Lariviere and Melzack, 2000). In particular, the Locus Coeruleus (LC), the major noradrenaline source in the brain, is one important target for CRF neurotransmission (Valentino and Van Bockstaele, 2008). Besides its important role in modulating ascending and descending pain pathways, the LC also represents a convergent nucleus that is correlated with adaptive responses to stress (Valentino and Van Bockstaele, 2008).

Thus, the activity of CRF in the LC could influence chronic inflammatory pain and it would not be unreasonable to hypothesize that the onset of emotional changes produced by pain might be the result of stress-induced CRF release.

The extracellular signal-regulated kinases 1/2 (ERK1/2) cascade is a strong candidate to mediate the effects of CRF in pain and it is known to participate in CRF receptor signaling in neuronal cells (Hauger et al., 2006). In addition, CRF administration into the LC region promotes c-Fos and ERK1/2 activation in the prefrontal cortex (Snyder et al., 2012). Accordingly, we demonstrated that animals suffering chronic inflammation also display an anxio-depressive phenotype, with an enhancement of ERK1/2 activation in the prefrontal cortex (Borges et al., 2014).

To evaluate whether CRF neurotransmission in the LC triggers the development of anxiety in chronic inflammation (e.g., a model of rheumatoid arthritis), an antagonist of the CRF receptors was microinjected into the LC and the nociceptive and anxiety behavior, as well as the activation of ERK1/2 in the LC, were evaluated.

METHODS

Animals

Harlan Sprague-Dawley male rats (250-300g) were provided by the Experimental Unit of the University of Cádiz (registration No. ES110120000210). Animals were housed 2-4 per cage with *ad libitum* access to food and water, and kept under controlled conditions of lighting (12h light/dark cycle), temperature (22 ºC) and humidity (45-60%). The protocols followed the European Communities Council Directive of 22 September 2010 (2010/63/EC), Spanish Law (RD 1201/2005) and the ethical guidelines for investigation of experimental pain in animals (Zimmermann, 1983), and they were reviewed and approved by the Institutional Ethical Committee for animal care and use.

Monoarthritis model of inflammatory pain

Monoarthritis (MA) was induced as described previously (Butler et al., 1992), injecting the left tibiotarsal joint of rats anaesthetized with isoflurane (4% to induce and 2% to maintain: Abbott, Spain) with 50 µL of complete Freund's adjuvant (CFA) solution, containing 30 mg of desiccated *Mycobacterium butyricum* (Difco Laboratories, USA), paraffin oil (3 mL), saline (2 mL) and Tween®80 (500 µL). Animals that developed polyarthritis were excluded. The control rats were injected with the vehicle solution (paraffin oil, saline and Tween®80). The experimental design is represented in Supplementary Fig. S1*a*.

Surgery and intra-LC drug administration

Animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg), and placed in a stereotaxic apparatus with the head tilted at an angle of 15º to the horizontal plane. A guide cannula (22 gauge, 15 mm length) was implanted into the contralateral LC (lambda: AP=-3.2 mm, ML -1.1 mm, and DV -6.2 mm; Fig. 1*a* and Supplementary Fig. S1*b*) and was fixed with skull screws and dental cement. A stainless steel wire was inserted into the guide cannula to prevent occlusion. Five days after recovery, animals were immobilized and the steel wire was cut. Microinjection was performed by inserting an injector cannula (30 gauge) that was 1 mm longer than the guide cannula (i.e.: 16 mm). Animals received 28 ng or 34 ng of α-helical $CRF_{(9-41)}$ (αCRF) dissolved in sterile water (0.5 µL; Sigma Aldrich, Ref. C246), which blocks the CRF I and II receptors, inhibiting the endogenous CRF activity. Sterile water was used as a vehicle. Behavioral tests or sacrifice for western blot procedures were performed 10-25 minutes after drug/vehicle administration (see Supplementary Material and Fig. S1*a*). As no differences between doses were observed in terms of pERK1/2 expression, the behavioral effects were analyzed in animals receiving the 28 ng αCRF. Behavior was assessed in groups of 6 animals and random animals were selected for histological verification of the cannula implantation.

Health parameters, nociceptive and anxiety-like behavior

The body weight (g) and rectal temperature (°C) were recorded weekly and nociceptive mechanical allodynia (automated von Frey test) and hyperalgesia (paw-pinch test) were assessed as described in the Supplementary Material. Anxiety-like behavior was evaluated in the elevated zero maze (EZM) test, which consisted in a black circular platform divided into four quadrants, with two opposing open quadrants with 1 cm high clear curbs to prevent falls and two opposing closed quadrants with 27 cm high black walls. A 5 min trial under the same lighting conditions began with the animal placed in the centre of a closed quadrant. The SMART software was used to analyze the time spent in the open arms and the total distance travelled by each rat. Increases in the time spent in the closed areas were correlated with anxiety-like behavior (Borges et al., 2014).

Immunohistochemistry

Another set of control and MA rats (4 weeks) was used for quantification of the expression of pERK1/2 in the PVN and CRFI/II receptors in the LC (Supplementary Material).

Statistical analysis

All data is represented as mean ± SEM and were analyzed using STATISTICA 10.0 or GraphPad Prism 5 software, using either an unpaired Student *t* test (two-tailed) or one-way, two-way or repeated measures analysis of variance (ANOVA), followed by the appropriate post-hoc tests. The level of significance was considered as $p<0.05$.

RESULTS

Effect of MA on health parameters and nociceptive responses

The behavior of control animals was normal, with no signs of an inflammatory reaction. CFA injection produced a stable MA, with the signs of inflammation restricted to the injected joint and evident a few hours after induction, persisting into the fourth week. The weight gain of MA rats was significantly lower than that of the control animals 1 and 3 weeks after CFA injection (repeated measures, Bonferroni; $p<0.05$: Fig. 1*b*), while their body temperature remained normal during the experiment (Fig. 1*c*). Regarding the pain threshold, a significant decrease in the withdrawal threshold of the ipsilateral paw was evident when compared with the contralateral paw, indicative of mechanical hyperalgesia (repeated measures, Bonferroni test; $p<0.05$ for week 1, 2 and 4, $p<0.01$ for week 3: Fig. 1*d*). Additionally, there was a significant decrease in the paw withdrawal threshold to von Frey stimulation by the ipsilateral-inflamed paw of MA rats when compared with the contralateral paw, indicative of mechanical allodynia (repeated measures, Bonferroni test; $p<0.001$: Fig. 1*e*).

Effect of intra-LC microinjection of an α-helical CRF receptor antagonist on the pERK1/2 levels in the LC

The administration of the α-helical CRF receptor antagonist in the LC normalized the pERK1/2 values in MA4W rats at both doses of the compound used (one-way ANOVA, Dunnett's test;; $p>0.05$ MA4W 28ng and MA4W 34ng vs control; Fig. 1*f*), suggesting that CRF acts through the ERK1/2 signaling cascade in LC neurons.

Effect of intra-LC microinjection of an α-helical receptor antagonist on MA-induced pain

In the paw withdrawal threshold, a significant increase in pain sensitivity was observed when the ipsilateral paw was compared with the contralateral paw in the MA4W rats before (pre-drug, two-way ANOVA, Bonferroni test; $p<0.001$) and after microinjection of the αCRF into the LC (two-way ANOVA, Bonferroni test; $p<0.01$: Fig. 1*g*). Thus, the intra-LC administration of the α-helical CRF receptor antagonist (28 ng) had no significant effect on the paw withdrawal threshold of MA4W rats ($p>0.05$; Fig. 1*g*). In the force threshold, microinjection of the αCRF receptor antagonist into the LC had no effect on the ipsilateral sensitivity to innocuous stimulation. Indeed, the significant decrease in the force supported by the ipsilateral paw of MA4W rats when compared to the contralateral paw was present before and after administration of the drug (two-way ANOVA, Bonferroni test; $p<0.001$: Fig. 1*h*).

Effect of intra-LC microinjection of an α-helical CRF receptor antagonist on MA-induced anxiety.

In the EZM, MA4W rats that received the vehicle alone (MA4W-vehicle) spent significantly less time in the open arms than control animals, indicative of anxiety-like behavior (two-way ANOVA, Bonferroni test; $p<0.05$: Fig. 1*i*). By contrast, those animals that received an intra-LC microinjection of the αCRF receptor antagonist (MA4W-αCRF) spent significantly longer time in the open arms when compared to the MA4W-vehicle animals (two-way ANOVA, Bonferroni test; $p<0.05$: Fig. 1*i*). No effect of microinjecting the αCRF receptor antagonist into the LC was observed in the control rats. Moreover, no differences were observed in the total distance travelled in the EZM (Fig. 1*j*), ruling out any influence of locomotor impairment on the experimental results. Regarding the number of entries into the open arms, the MA4W-vehicle rats appeared to enter these arms less frequently than the control animals that received the vehicle alone, although this difference was not significant. No such difference was observed in the MA4W-αCRF rats (Fig. 1*k*).

Expression of CRF receptors in the LC and pERK1/2 in the PVN

When the expression of the CRF I and II receptors was studied in the LC, no differences were observed between the control and MA4W rats (Fig. 2*a*, *b* and *c*). Most of the neurons expressing CRFI/II receptors also expressed TH, indicating their noradrenergic nature and demonstrating that the CRFI/II receptors were expressed by neurons in the LC area (Fig. 2*d*).

The expression of pERK1/2 was also studied in the PVN of the hypothalamus in order to determine the activity of this structure as a readout of CRF stimulation in the central nervous system. Interestingly, an increase in pERK1/2 was observed in MA4W rats compared with control rats (unpaired Student's *t*-test; $p<0.001$: Fig. *2e-g).*

DISCUSSION

This study shows that the action of CRF on LC neurons is involved in the development of anxiety-like symptoms associated with prolonged inflammatory pain. As expected, four weeks after CFA injection, rats displayed signs of pain and

anxiety, consistent with previous reports (Borges et al., 2014). We also observed a significant increase in ERK1/2 phosphorylation in the LC, in accordance with previous data (Borges et al., 2014), and this increased ERK1/2 activation in the LC seems to be related with the development of anxiety-like behaviors in chronic inflammatory conditions. This raises the question as to what produces this increase in ERK1/2 activation in the LC when painful conditions develop. CRF is a molecule linked with the endocrine and behavioral response to stress (Bale and Vale, 2004), and the role of CRF in different pain conditions has been studied (Lariviere and Melzack, 2000), although not its effects after prolonged times of inflammation (e.g., 4 weeks). Here, we studied the PVN nucleus, a CRF-producing structure, and we found that pERK1/2 levels increase in MA4W rats when compared with control rats, suggesting that PVN hyperactivation occurs in association with chronic inflammatory pain. As the PVN and LC have reciprocal excitatory connections (Perez et al., 2006), we hypothesized that this would underpin the ERK1/2 activation in the LC of MA4W rats. Indeed, the LC is rich in CRF receptors (Reyes et al., 2006; Mousa et al., 2007) and it has already been shown that CRF activates LC neurons (Valentino and Foote, 1988). Here, we found no significant differences in CRFI/II receptor expression in the LC of control and MA4W rats, which indicates that while enhanced neurotransmission might originate in the PVN when chronic inflammatory pain is established, it is not accompanied by changes in the expression of the CRFI/II receptors in the LC. The co-localization of CRFI/II receptors with the TH protein, as described previously (Reyes et al., 2006), confirmed the specificity of this labeling.

To better understand how CRF neurotransmission influences the role of the LC in nociception and anxiety behavior, an antagonist blocking the CRF receptors was microinjected into the contralateral LC. This strategy was adopted in order to study the ascending pain pathway passing through the LC given its important projections to corticolimbic areas (Fig. 1*a*). The dose of the α-helical CRF antagonist used was based on previous studies (Mousa et al., 2007), and at both 28ng and 34ng this antagonist successfully dampened pERK1/2 expression in the LC of MA4W rats. Thus, the effects of the lower dose alone (28 ng) were evaluated on behavior. This procedure had no effect on pain sensitivity in the ipsilateral/inflamed or contralateral paws of MA4W rats.

In contrast, microinjection of the α-helical CRF receptor antagonist reverses the anxiety-like behavior observed in MA4W rats without interfering with locomotor activity. Indeed, the decrease in the time MA4W rats spent in the open arms was no longer observed when they received this antagonist. These results suggest that the increased CRF neurotransmission in chronic inflammatory conditions enhances the LC driven activation of corticolimbic areas, which may be responsible for the development of anxiety. Indeed, it has already been shown that CRF infused into the LC increases anxiety, a behavioral effect of CRF associated with increased noradrenergic neurotransmission in LC terminal areas like the amygdala and hypothalamus (Butler et al., 1990; Weiss et al., 1994). Moreover, the α-helical CRF receptor antagonist prevents the development of anxiety induced by a neuropeptide Y receptor antagonist, while not producing any significant change when administered in a non-anxious state (Kask et al., 1997; Donatti and Leite-Panissi, 2011). Similarly, we did not find a significant effect of the α-CRF antagonist in control non-inflamed animals. Overall, the effects in the ipsilateral paw and on anxiety-like behavior in MA rats are consistent with studies showing that administration of the α-helical CRF receptor antagonist to the basolateral or central nuclei of the amygdala has no effect on the nociceptive threshold but that it reduced innate fear behavior (Donatti and Leite-Panissi, 2013). Nevertheless, the lack of changes in nociception might be related to the use of a broad-spectrum CRF antagonist that blocks, nonspecifically, the signaling of CRF1 and CRF2 receptors. Indeed, when NBI27914, a specific CRF1 receptor antagonist, was microinjected into the amygdala, the withdrawal thresholds of the arthritic rats, as well and the anxiety-like behavior, were reversed (Ji et al., 2007).

Concluding, CRF signaling through the ERK1/2 cascade in the LC appears to be an important mechanism directly related with anxious behavior associated with chronic inflammatory conditions.

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Statement of Interest
None to declare

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FIGURES AND LEGENDS

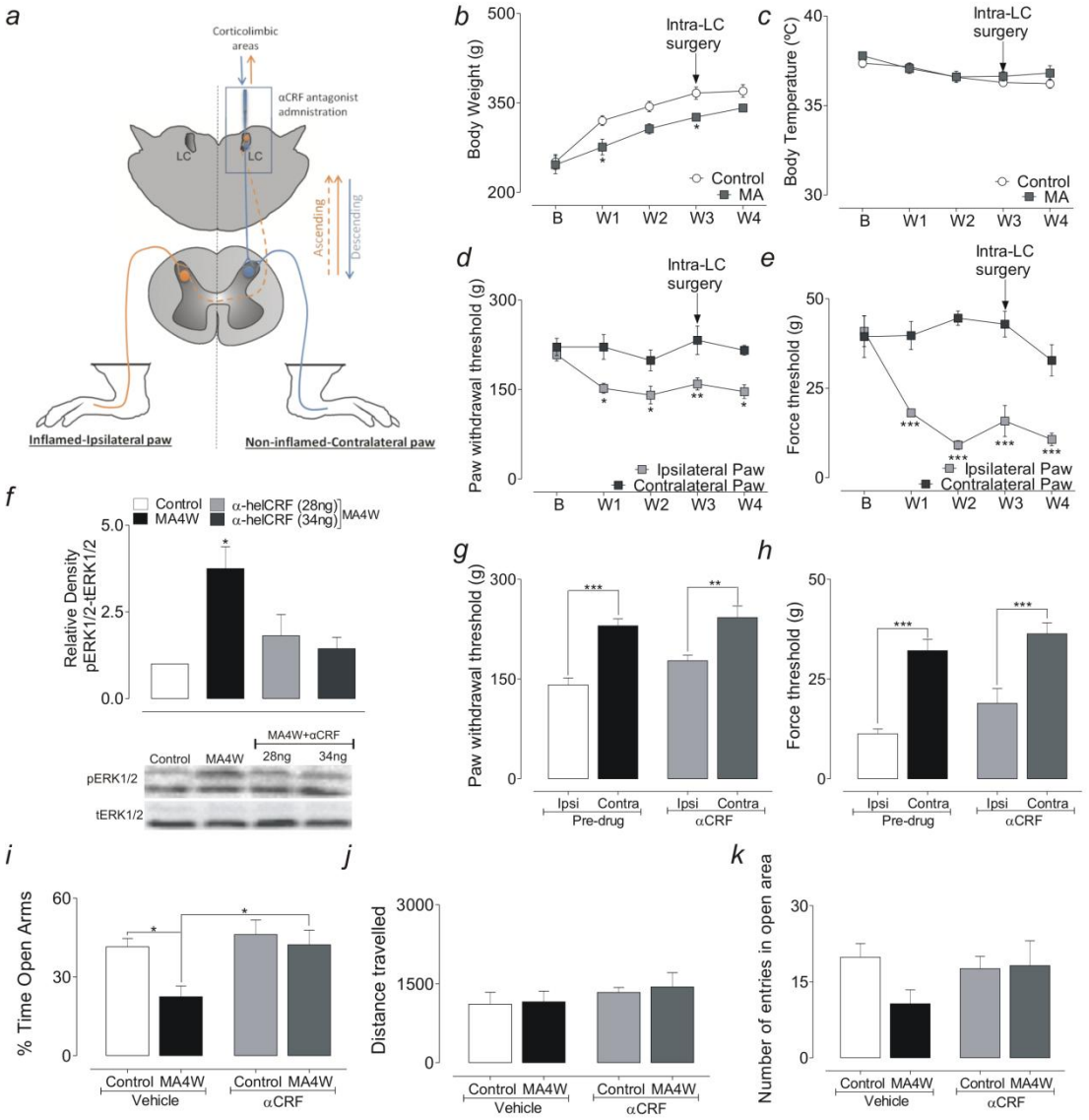

Fig. 1: *a*) Schematic representation of the anatomical pathways implicated. Briefly, the contralateral LC indirectly receives inputs from the inflamed paw (red dashed line; ascending pathways) and, subsequently, the information is sent to corticolimbic areas. Additionally, the LC sends direct projections to the spinal cord (blue straight line; descending pathways). *b*) Body weight of the control and MA rats. *c*) Body rectal temperature of control and MA rats. *d*) Mechanical hyperalgesia represented by a significant decrease in the paw withdrawal threshold of the ipsilateral paw of MA rats. *e*) Mechanical allodynia represented by a significant decrease in the force threshold of the ipsilateral paw of MA rats. *p<0.05, **p<0.01 and ***p<0.001; repeated measures followed by a Bonferroni post-hoc test comparing control vs MA for the same week (*b* and *c*) or comparing the ipsilateral vs contralateral paw for the same week (*d* and *e*). *f*) Graph depicting the expression of pERK1/2 in the LC after intra-LC administration of the αCRF receptor antagonist, showing that the significant increase of pERK1/2 in MA4W animals was no longer observed when this antagonist was administered: *p<0.05 (one-way ANOVA followed by Dunnett's post-hoc test). *g*) Graph showing that the local administration of the αCRF antagonist had no significant effect on mechanical hyperalgesia in MA4W rats. *h*) Graph showing that local administration of the α-helical CRF antagonist had no significant effect on mechanical allodynia in the ipsilateral paw of MA rats. **p<0.01 and ***p<0.001 (two-way ANOVA followed by Bonferroni post-hoc test). *i*) Graph showing that the time spent in the open arms decreased in MA4W rats receiving the vehicle alone but this effect was successfully reversed by administration of the αCRF antagonist. *p<0.05 (two-way ANOVA followed by Bonferroni post-hoc test). *j*) Graph showing that local administration of the α-helical CRF antagonist had no significant effect on the total distance travelled in the elevated zero maze. *k*) Graph

showing that local administration of the α-helical CRF antagonist reversed the decrease in the number of entries into the open arms observed in MA4W rats receiving the vehicle alone. B=Baseline; LC=Locus Coeruleus; αCRF=antagonist of the corticotropin-releasing factor receptor I and II; W=Week; MA=Monoarthritis.

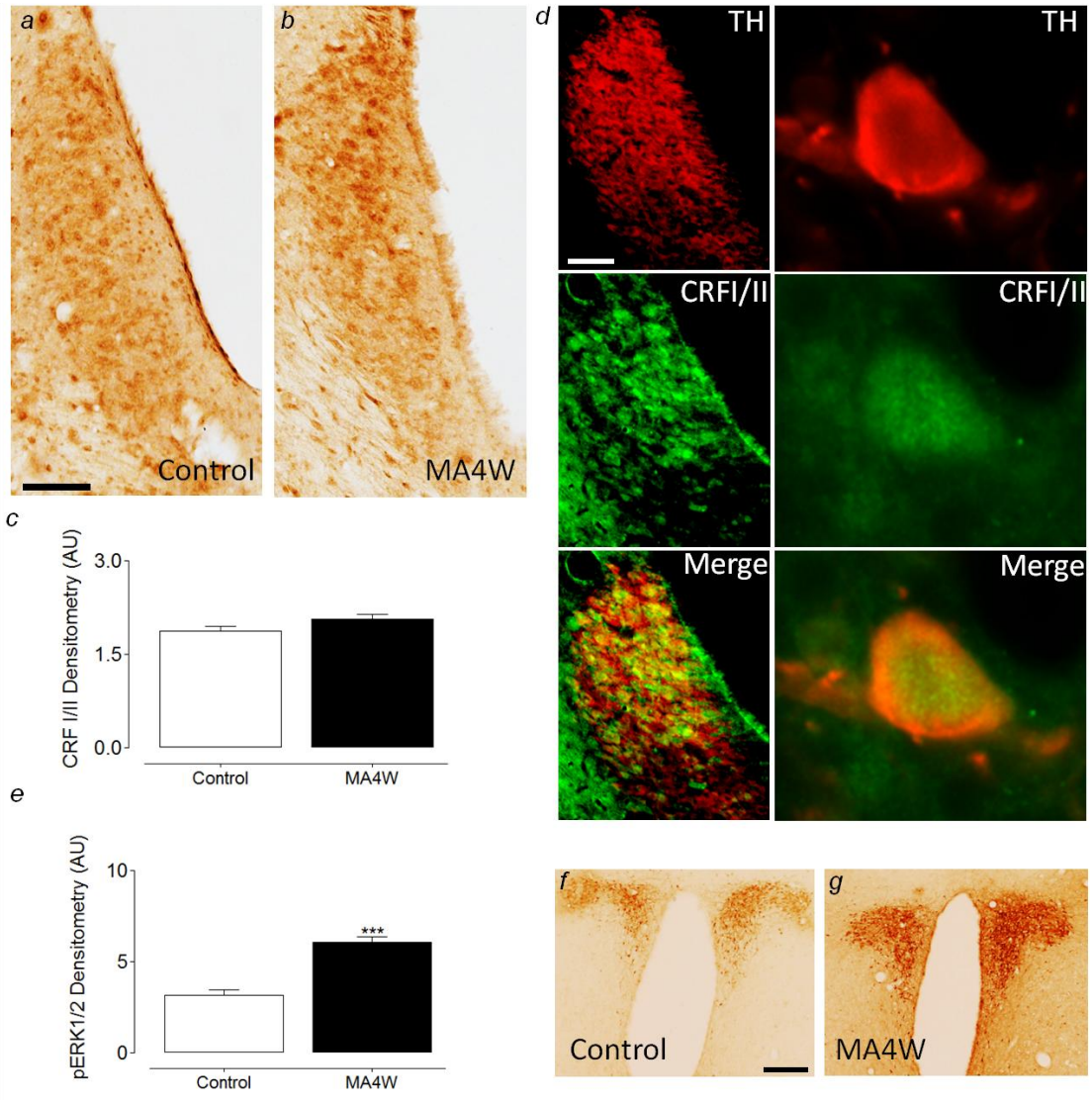

Fig. 2: Expression of CRFI/II receptors in the LC of control and MA4W rats. *a*) and *b*) Photomicrographs showing the expression of CRFI/II receptors in control and MA4W rats, respectively. *c*) Graph showing that there were no significant changes between control and MA4W rats in terms of CRFI/II receptor expression in the LC. *d*) Immunofluorescence photomicrographs showing that almost all the neurons expressing CRFI/II receptors (green) are noradrenergic neurons, since they co-localize with TH immunolabeling. *e*) Graph showing the increase in pERK1/2 expression in the PVN nucleus of MA4W rats: ***p<0.001 (unpaired two-tailed Student's t-test). *f*) and *g*) Photomicrographs of pERK1/2 expression in control and MA4W rats, respectively. Scale bar=100µm. AU=Arbitrary units; W=Week; MA=Monoarthritis. W=Week; MA=Monoarthritis.

Supplementary material

Nociception

Mechanical allodynia was evaluated using an electronic version of the von Frey test (Dynamic Plantar Aesthesiometer, Ugo Basile, Italy), applying an increasing vertical force (from 0 to 50 g) to both the ipsi- and contralateral paws over a period of 20 s. In addition, the presence of mechanical hyperalgesia was tested using the paw-pinch test (Randall and Selitto, 1957). Increasing pressure (from 30g of pressure) was gradually applied to the dorsal side of the paw using a graded motor-driven device (Ugo Basile, Italy). A 250g cut-off was used to prevent damage to the paw. In both tests, three measures were taken on each paw at 5 min intervals and the average value was used.

Western blotting

Fresh tissue samples from the contralateral LC of a preliminary group including control, MA4W-vehicle and MA4W-α-CRF rats (28 ng or 36 ng) were obtained after sacrificing them by decapitation, 10-25 minutes after administration. After the tissue was lysed, an aliquot of the sample (50 µg) was separated in a 10% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (PVDF; BioRad, Spain). After washing in TBST (Tris-buffered saline containing 0.1% Tween-20), the membranes were blocked with 5% Bovine Serum Albumin (BSA; Sigma, Spain) in TBST and probed overnight at 4 ºC with a rabbit anti-phospho-ERK1/2 (1:5,000; Neuromics, USA) and a mouse-anti ERK1/2 (1:2,000; Cell Signaling; Izasa, Spain) antibody diluted in 5% BSA-TBST. After thorough washing, these primary antibodies were detected by incubating for 1 hour at room temperature with IRDye 800CW goat anti-rabbit (green) or IRDye 680LT goat anti-mouse (red) secondary antibodies (1:10,000; Bonsai Advanced Technologies, Spain). After 3 final washes with TBST, antibody binding was detected using a LI-COR Odyssey® two-channel quantitative fluorescence imaging system (Bonsai Advanced Technologies, Spain). Digital images of the Western blots were analyzed by densitometry using the Image J free access software and the data were expressed as the pERK1/2 expression relative to the total ERK1/2. Three assays were performed on LC samples from 3 rats per group.

Immunohistochemistry (IHC)

Control and MA rats 4 weeks after CFA injection (MA4W; N=5 per group) were anesthetized with 8% chloral hydrate (400mg/kg) and they were transcardially perfused through the ascending aorta with 250 mL of oxygenated Tyrode's solution followed by 750 mL of paraformaldehyde 4% in phosphate buffer (PB, 0.1M [pH 7.2]). Brains were removed and processed for free-floating immunohistochemistry. One in five sequential transverse brain sections (30 µm) containing the PVN from each rat were washed, blocked and incubated with a rabbit antiserum against the phosphorylated ERK1 and ERK2 isoforms (pERK1/2; 1:1000; 48 hours at 4-8ºC: Neuromics, USA). Immunodetection was achieved with a biotinylated donkey anti-rabbit antiserum (1:500; 1 hour; Jackson ImmunoResearch, USA), followed by an ABC solution (1:200, 1 hour; ABC Elite kit, Vector Laboratories, UK) and a colorimetric reaction with 3,3-diaminobenzidine tetrahydrochloride (DAB; 10 min) in 0.05M Tris-HCl buffer containing 0.003% hydrogen peroxide (Cruz et al., 2005). Sections were then washed in PBS, mounted on gelatin-coated glass slides, cleared in xylene, cover-slipped with DPX and analyzed by light microscopy. Furthermore, immunohistochemistry to detect CRF receptors I/II in LC sections was performed following a similar protocol with an anti-CRFI/II rabbit antiserum (1:50; Santa Cruz), a biotinylated donkey anti-rabbit antibody and streptavidin 488 (1:1000, Invitrogen). Additionally, to co-localize the CRF I/II receptors with tyrosine hydroxylase (TH) positive neurons, a sheep anti-TH antibody was used (1:4000; abcam, UK), which was detected with an Alexa 568 conjugated donkey anti-sheep antibody (1:1000, Invitrogen). The specificity of the antibodies was controlled by omitting the primary antibodies in various sets of immunoreactions.

Quantification of the expression of pERK1/2 in the PVN and CRF I/II receptors in the LC was performed by densitometry as described elsewhere (Borges et al., 2013). As no differences were observed in ipsilateral side when comparing with the contralateral side, the values obtained in each area were averaged and used for statistical purposes.

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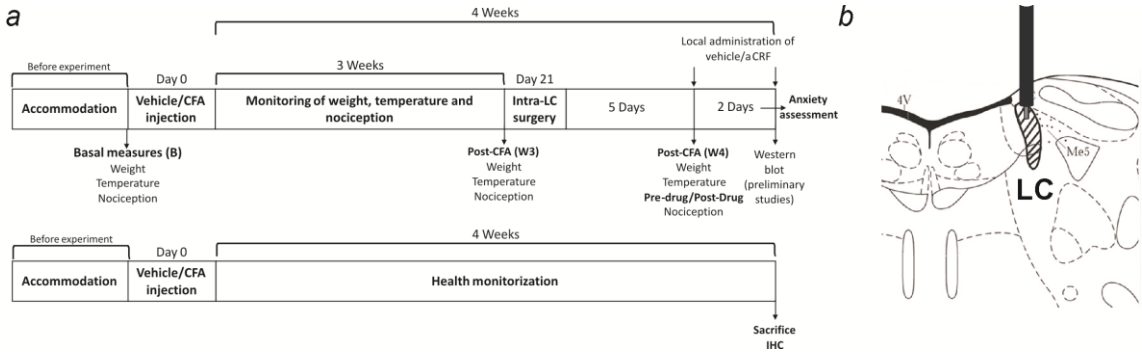

Fig. S1: *a*) Schematic representation of the experimental protocol followed in this study and *b*) Schematic representation of the local cannula implantation.

Discussion

5. Discussion

The present thesis focuses on the study of the activity of the Locus coeruleus-Noradrenergic (LC-NA) system in chronic painful conditions, particularly in chronic inflammatory conditions. Indeed, during the development of this work it was becoming clear that chronic painful conditions lead to a deep biochemical and electrophysiological deregulation of the activity of LC neurons with repercussions on the sensory and emotional components of pain.

The CCI and MA models were used to simulate the neuropathic and chronic inflammatory conditions, respectively. We observed that the unilateral constriction of the sciatic nerve resulted in a stable mononeuropathy accompanied by increased pain sensitivity, such as allodynia and hyperalgesia (**Publication I**), in accordance with what was expected for the CCI model (Bennett and Xie 1988). Similarly, we observed that the unilateral injection of the CFA solution into the rats' tibio-tarsal joint, to induce MA, was able to cause reasonably evident inflammatory signs accompanied by an increased pain sensitivity of the affected paw. Moreover, the inflammation remained stable during the period of experimentation (maximum 4 weeks) and the rats showed normal body weight gain, normal body temperature and normal locomotor activity (**Publication II**). Such observations were in accordance with what was expected for this experimental model (Butler et al. 1992).

In the CCI model of neuropathic pain we evaluated the expression of pERK1/2 and the effect, on this expression, of an acute mechanical noxious stimulation

(**Publication I**). At the time these experiments were performed, few data was available regarding the pattern of pERK1/2 expression in supraspinal structures following acute or chronic painful conditions, particularly in noradrenergic structures. Using seven days SHAM and CCI rats (CCI7D), we showed that, in neuropathic pain conditions, pERK1/2 expression was increased in the spinal cord, PGi and DRN (Alba-Delgado et al. 2011). However, in this study, the spontaneous tonic activity of LC neurons, which was measured by electrophysiology procedures, was not significantly changed in seven days CCI rats when compared with control rats, in agreement with what had already been described (Viisanen and Pertovaara 2007). Additionally, microdialysis studies pointed to similar extracellular NA levels in the LC and PFC of control and CCI7D rats (Alba-Delgado et al. 2011). Remarkably, at this time-point, CCI7D rats did not display any affective/emotional impairment (i.e., anxiety and depression) as a consequence of the exposure to the painful situation (Alba-Delgado et al. 2013). Overall, the data obtained at this specific time-point of neuropathic disease suggested that ERK1/2 activation was playing a role in the processing of specific pain conditions although the electrophysiological activity of the LC neurons was conserved (Alba-Delgado et al. 2011). Thus, we expanded our studies to evaluate the pattern of pERK1/2 immunoreactivity in further supraspinal structures, mainly noradrenergic or serotonergic, and spinal cord, and following the application of a noxious mechanical stimulation to the hind paw of anaesthetized CCI7D rats (**Publication I**). The main rationale of the procedure was based on the fact that LC neurons might be phasically activated by noxious external stimuli (Valentino and Foote 1988). Moreover, painful

stimulation was already shown to imply central noradrenergic regulation in patients (Chapman et al. 2014). Besides, it was still unknown if, in neuropathic pain conditions, pERK1/2 was also expressed/altered in other supraspinal structures implicated in sensory, cognitive and affective functions.

At the spinal cord level, we observed increases in the amount of pERK1/2 detected ipsilaterally in neuropathic rats, in accordance with data from other authors (Ji et al. 1999; Song et al. 2005; Zhuang et al. 2005; Ji et al. 2009; Yu et al. 2013). The spinal cells expressing pERK1/2 in consequence of a painful situation were mostly localized in the superficial dorsal horn, more precisely, in the superficial laminae (lamina I to III), as has already been shown (Cruz et al. 2005). This reflects a certain spinal cytoarchitectonic organization, indicating the zone where the nociceptive inputs enter.

Interestingly, in the LC, the data regarding pERK1/2 expression in control and CCI conditions was quite unexpected as significant decreases in the expression of pERK1/2 in the LC of CCI animals, either stimulated or not, were observed, despite the absence of significant electrophysiological changes in the LC neurons of CCI rats (Alba-Delgado et al. 2011). This suggested there was a higher inhibitory input, probably provided by GABAergic input from the PrH or serotonergic input from the DRN that, somehow, counterbalanced the excitatory glutamatergic input from the PGi. However, the data obtained did not allow any further conclusions and anesthesia-induced decreased ERK1/2 activation was barely likely to be the cause (please, consult supplementary discussion of **Publication I**). Later on, further behavioral,

electrophysiological and immunohistochemical studies on the CCI model by other members of the team indicated that in fact, the LC-NA system is impaired at longer time-points of the disease, and that concomitantly anxiety and depressive-like behaviors start developing in those animals (Alba-Delgado et al. 2012; Alba-Delgado et al. 2013; Bravo et al. 2013; Bravo et al. 2014). This was the motivation for proceeding with the remaining studies in a model of chronic pain with different characteristics and etiology, as is the monoarthritis induced by intra-articular CFA, and look out for the involvement of the LC-NA system and the associated molecular mechanisms.

The next step was then to evaluate prolonged inflammatory conditions which, in the clinical field, are recognized to be accompanied by the emergence of anxio-depressive symptoms (Covic et al. 2012). However, despite the knowledge that the CFA injection produced clear pain symptoms, such as allodynia and hyperalgesia in the injected and adjacent area, at the time these experiments were performed it was not that clear if it could trigger the onset of anxio-depressive symptoms, as observed in clinical settings. Thus, the next goal was to evaluate the time-course for the development of these symptoms using well known behavioral paradigms (see Behavioral considerations, below). Interestingly, we observed that four weeks (28 days) after the inoculation of the CFA solution, rats displayed evident anxiety and depressive behaviors. Additionally, anxiety but not depression was also detected at 2 weeks (14 days) after MA induction using the marble burying test. Although some questions remain regarding the validity of this behavioral paradigm for evaluating anxiety-like behaviors, as discussed below (see Behavioral considerations), the

resulting outcome is nevertheless indicative of an abnormal behavioral state of these animals. At this time-point, the data obtained with the elevated zero maze were similar between experimental groups, indicating no anxiety-behaviors. At earlier time-points of MA disease (4 days) no anxio-depressive symptoms were detected (**Publication II**). Overall, it may be argued that a certain chronological cascade of events occurs in the development of affective disorders associated to chronic pain, where anxiety precedes the emergence of depression. This has already been described by other authors (Yalcin et al. 2011). Moreover, the development of anxiety and depressive disorders in the CFA model has already been described in mice (Narita et al. 2006; Maciel et al. 2013) and rats (Kim et al. 2012; Parent et al. 2012). The presence of these disturbed mood behaviors was also evident in other models of persistent inflammation or neuropathic pain (Alba-Delgado et al. 2013; Amorim et al. 2014; Caspani et al. 2014), suggesting that severe painful conditions, independently of their etiology, trigger the affective component of pain, although some differences are identified regarding the time-course of development of anxiety and depression.

In order to investigate the possible implication of the LC-NA system and some of the associated molecular mechanisms in these MA-induced behavior changes, we then quantified the expression of phosphorylated ERK1/2 (**Publication II**)**.** While at 4 and 14 days after MA induction no significant differences in the pattern of ERK1/2 expression in LC were detected, the 28 days' time-point was accompanied by significant increases in ERK1/2 phosphorylation. Indeed the difference in the results obtained at the earliest time-points, where the anxio-depressive phenotype had still

not been developed neither in the CCI (**Publication I**) nor in the MA (**Publication II**) models of pain, are quite interesting. In fact, they reinforce that there is a different LC modulation in neuropathic or inflammatory conditions, at least at the earliest time points of the diseases. It is still lacking the physiological meaning of these events because pain is clearly present in both models at these time-points.

The increased pERK1/2 levels in the LC at 28 days after the induction of MA suggests an increased activation of this signaling cascade in chronic inflammatory conditions, leading to anxious and depressive behaviors. Further, we explored the ERK1/2 activation in the CNS structures that, together with the LC, form an ascending pathway, namely in the spinal cord and the PGi, that project to LC, and the PFC, the main afferent of LC neurons (Ennis and Aston-Jones 1988; Samuels and Szabadi 2008); see section 2.6.2.). In the PGi and PFC an increased pERK1/2 expression was detected only at 28 days after MA induction. The increased ERK1/2 activation in the PFC is, somehow, expected since it includes the rostral part of the anterior cingulate cortex (ACC, (Krettek and Price 1977; Ray and Price 1992)), a structure already shown to be implicated in the regulation of the affective and emotional responses to pain (Lane et al. 1998; Vogt 2005). Indeed, activation of ERK1/2 in the ACC was already shown to be an important contribution to the expression of affective pain (Cao et al. 2009). Corroborating this, we then observed that microinjection of SL327, an inhibitor of ERK1/2 activation, in the LC was able to reverse the anxiety-like behavior in the monoarthritic rats with 28 days after CFA injection (MA4W), as well as restored the ERK1/2 activation levels in the ACC of these animals (**Publication III**). Thus, we were

able to conclude that ERK1/2 activation in the PGi-LC-PFC pathway is implicated in the signaling events underlying the onset of anxio-depressive behaviors in animals with prolonged conditions of chronic inflammatory joint pain.

In what regards to the pattern of ERK1/2 activation in the spinal cord at distinct time-points of the MA conditions, it was already shown that at 4, 7 and 14 days after CFA injection, the expression of activated ERK1/2 forms was mostly observed when there was application of an ankle bend stimulation (innocuous to control rats) (Cruz et al. 2005). In our western blot studies, we also found that 4 days after CFA injection were not accompanied by significant increases of ERK1/2 activation in the lumbar spinal cord (**Publication II**). However, on the contrary to what we have found (**Publication II**) and previous reports (Ji et al. 2002; Cruz et al. 2005), other authors were able to detect significant increases of pERK1/2 expression 96 hours (4 days) after CFA injection into the hind paw (Xu et al. 2008). At a later time-point of disease, namely at 14 days after MA induction, our data was not in accordance with Cruz et al, (2005) studies. The different procedure used for the quantification of pERK1/2 levels may underlie such differences. Indeed, Cruz et al., (2005) quantified the number of cells immunoreactive to pERK1/2 per section and per sub-region of the grey matter of the spinal cord of animals anaesthetized with sodium pentobarbital. In contrast, we quantified pERK1/2 expression levels by western blot in the ipsilateral or contralateral sides of the lumbar (L3-L6) spinal cord segments (**Publication II**), the most important segments implicated in chronic painful conditions from the periphery, particularly from the hind paws (Rigaud et al. 2008). Thus, although some of the available data regarding

ERK1/2 expression in chronic pain conditions are contradictory, they are undoubtedly indicative of ERK1/2 implication in pain processing at various levels of the pain matrix.

The following step was to evaluate if these behavioral and biochemical changes were, in fact, a consequence of the prolonged nociceptive inputs from the inflamed paw in the monoarthritic condition. Thus, we adopted the strategy of topically blocking these nociceptive inputs from the inflamed paw with a potent anti-inflammatory cream, as is the commercial diclofenac-based cream Voltaren® emulgel (**Publication II**). The topic application was used in order to avoid the well-known side effects of the oral administration and the stress associated with daily intraperitoneal injections, and, at the same time, to produce a local analgesic effect, without affecting other body parts. As expected, the inflammatory signs (edema) and pain sensitivity of MA rats were significantly reduced to control levels. Similar analgesic effects using topic diclofenac cream were also observed in other studies (Dawane et al. 2011; Takayama et al. 2011). Moreover, in diclofenac-induced analgesia conditions, anxiety- and depression-like behaviors were no longer observed in MA4W rats and the increased pERK1/2 levels previously observed in MA4W rats were reversed to almost normal levels in the spinal cord, PGi, LC and PFC. These data suggested that the anxio-depressive behaviors were indeed triggered by the existence of continuous painful input arising from the inflamed paw, and when this nociceptive input has been subsided, the affective-associated behaviors became improved. Similar data was obtained by Amorim et al., (2014) using another experimental model of monoarthritis, the kaolin/carrageenan injected in the knee joint. Indeed, these authors showed that 4 weeks after the induction of arthritic

conditions, rats displayed and anxiety- and depressive phenotype and that amitriptyline, a tricyclic antidepressant used in chronic pain treatment (Jann and Slade 2007), was able to reverse the hyperalgesia and the anhedonia-like effect, a core symptom in depression (Amorim et al. 2014). Additionally, it was also shown that tramadol, a potent analgesic compound, was able to reverse the mechanical allodynia and anxio-depressive phenotype observed in the CCI condition (Caspani et al. 2014). All these studies suggest that drugs that relieve pain have also repercussion on the indirect consequences and comorbidities associated to chronic painful states. In our **Publication II**, also the levels of pERK1/2 expression have been restored indicating a direct functional correlation between the activation of these signaling cascades in the spinal cord-PGi-LC-PFC pathway and the nociceptive-, anxiety- and depressive-like behaviors. Indeed, although a systemic effect of the diclofenac cream is not totally discarded, as argued in **Publication II,** it is the topic effect that is producing the central changes observed in the pERK1/2 levels, by directly blocking the nociceptive inputs from the inflamed paw. Overall, the data obtained in the **Publication II** lead to the conclusion that a chronic inflammatory condition is able to induce affective disorders associated with increased pERK1/2 expression in the LC and its afferents and projections, and that the blockade of the nociceptive inflammatory inputs is capable of reversing all the CFA-induced changes.

Although the quantification of pERK1/2 expression is a widely used method to explore the degree of activation of certain encephalic structures in specific experimental conditions, it does not allow going further on in the understanding of the

implicated mechanisms and, also, does not allow the interpretation of the two most important modes of discharge of LC neurons: the tonic and the phasic modes. In order to overcome this barrier, we used electrophysiological procedures to infer about the activity of the LC neurons in chronic inflammatory conditions. Thus, in **Publication III**, we evaluated the LC discharge characteristics at baseline and following noxious stimulation of the inflamed paw in an early (non anxio-depressive phenotype) and late (anxio-depressive phenotype) phase of the disease development. Using rats with 1 week of MA (MA1W) as a control of a pain condition without emotional changes and rats with 4 weeks of MA (MA4W) because of their anxio-depressive phenotype, we showed that prolonged time-points of MA were susceptible to deep electrophysiological changes in both the tonic and phasic activity of LC neurons. Indeed, while tonic activity was significantly decreased at 4 weeks after MA induction, the sensory-evoked activity (phasic activity) was significantly increased (**Publication III**). Additionally, the incidence of burst activity was also decreased in MA1W and MA4W conditions, although it did not reach the significance level. These results suggest a decrease in the spontaneous tonic activity of the LC neurons in prolonged monoarthritis. Corroborating this, significant increases in TH expression were found in MA4W rats suggesting a noradrenergic dysfunction in terms of demand of this neurotransmitter. This is consistent with our finding of an altered climbing behavior in the forced swimming test in MA4W rats (**Publication II**), which can be interpreted as altered availability of noradrenaline (Detke et al. 1995; Cryan et al. 2005). Altogether, these results indicate an impairment in noradrenergic transmission to the spinal cord

(descending pathways) and to the cortical areas (ascending pathways), in chronic inflammatory conditions.

To evaluate the sensory-evoked activity of the LC neurons several noxious stimulations were performed in the injured paw while the discharge was recorded in the contralateral LC (**Publication III**). Contralateral LC neurons were activated by noxious stimuli in the hind paw in control animals, as expected (Simson and Weiss 1988; Valentino and Foote 1988), but this activation was exacerbated in MA4W rats. It has already been shown that this type of response of the LC neurons has mostly an "all-or-none" nature and the magnitude of the response is not altered when the pressure of the paw compression slightly varies (Simson and Weiss 1988). Thus, overall, the results are greatly suggesting that an increased ratio of the tonic/evoked activity, as observed in prolonged monoarthritic conditions (MA4W), enhances the pain perception to noxious events, with repercussions on the signaling to the forebrain areas. Similar electrophysiological data and TH expression were observed in rats with chronic neuropathic pain and under social stress (Bravo et al. 2013) and in a rat model of post-traumatic stress (George et al. 2013), suggesting that prolonged monoarthritis may produce similar LC changes as those observed in stressful conditions.

At this point we were speculating if the increased pERK1/2 levels observed in the LC of rats with 4 weeks of MA (**Publication II** and **Publication III**) were playing a role in this sensory-evoked response in MA4W rats. Thus, we explored the effect of the pharmacological blockade of ERK1/2 phosphorylation, achieved by intra-LC administration of the SL327 compound, on the neuronal activity of the contralateral

LC. Although the microinjection of SL327 did not significantly affect the LC tonic activity in MA4W rats, it caused a reversion to control levels, or even below, of the sensory-evoked parameters that had been found altered in response to the prolonged inflammatory condition (**Publication III**). This suggests that increased ERK1/2 activation in the LC is implicated in the sensory-evoked response of LC neurons in rats with prolonged monoarthritis (MA4W). Moreover, the microinjection of SL327 reversed the anxiety-like behavior to control levels but the mechanical hyperalgesia remained unchanged. Thus, it is possible that the changes in the LC neuronal electrophysiological activity observed in MA4W animals reflect mechanisms related with the anxiety-like behaviors which involve ERK1/2 activation in the LC. In accordance, the absence of effect of the SL327 in the electrophysiological activity of control rats is corroborated by the lack of behavioral changes in control rats in the elevated zero-maze (**Publication III**). Therefore, altogether these data suggest that increased levels of ERK1/2 phosphorylation in the LC at the prolonged times of monoarthritis disease are more related with the onset of "affective-behaviors" associated with pain. Indeed, the present data demonstrate that pain-related anxiety is dissociated from the sensorial pain component in an animal model of arthritic pain.

Finally, we were questioning what extracellular input in the LC would be producing increased ERK1/2 activation upon prolonged painful conditions and, consequently, would be responsible for the development of anxiety-like behaviors. Taking into account that, as mentioned above, the electrophysiological data together with the increased TH expression in the LC of MA4W rats were quite similar with what

had been observed in stressful conditions (Bravo et al. 2013; George et al. 2013; Bravo et al. 2014), we hypothesized that CRF would play a role in these mechanisms (**Publication IV**). In support of this, pain, and particularly the inflammatory pain, was recognized as a potent stressor activating the Hypothalamic–pituitary–adrenal axis (HPA; Hummel et al. 2010). Furthermore, chronic stress produces persistent ERK1/2 activation in dendrites of the layers I and II in the PFC (Trentani et al. 2002), precisely where we found an increased number of neurons expressing pERK1/2 in MA4W rats. Thus, the hypothesis that prolonged painful conditions were producing stress-like effects mediated by CRF neurotransmission was highly attractive. Moreover, we observed a significant increase in ERK1/2 activation in the PVN of the hypothalamus, the main producer of CRF, at prolonged time-points of monoarthritis (MA4W), indirectly indicating an increased activity in the CRF-based system. Thus, we microinjected a CRF antagonist in the LC and assessed the nociceptive and anxiety-like behavior in control and MA4W rats. Similarly to what occurred after microinjection of SL327 in the LC, here we also observed a reversion in the anxiety-like behaviors that had been observed in chronic inflammatory conditions, without affecting the nociceptive threshold. Additionally, the microinjection of the CRF antagonist in the LC was also able to reverse the increases in ERK1/2 activation in LC neurons, highly pointing to an effect of CRF through the ERK1/2 cascade in the LC (**Publication IV**). This increased CRF neurotransmission in the LC would explain the increased expression of TH in prolonged inflammatory conditions, as it was already observed in previous studies (Melia and Duman 1991). However, it is well known that CRF activates the LC

neurons, as shown by the increase in the tonic discharge of the LC neurons upon local CRF microinfusion (Curtis et al. 1997), which could be against our findings of decreased tonic discharge in MA4W rats (**Publication III**). Nevertheless, a dual role for the CRF in acute or chronic stressful conditions is, nowadays, documented. Acute stress promotes sensitization of the LC neurons to CRF neurotransmission, producing high tonic but low phasic activation (Curtis et al. 1995). Indeed, in **Publication III**, a weak decrease in the sensory-evoked response of the LC neurons of MA1W rats was observed, although the tonic discharge rate was maintained at normal levels. In contrast, chronic stressors that more closely relate to psychopathology, such as repeated auditory stress, desensitize LC neurons to CRF (Conti and Foote 1996) and induce intracellular trafficking of CRF receptors (Reyes et al. 2014) producing opposite effects to those observed in acute stress. Thus, in conclusion, although some studies are still needed to clarify the role of CRF in prolonged monoarthritic conditions, altogether the data obtained seem to implicate CRF as an important mediator in the onset of anxiety in inflammatory painful conditions, through mechanisms that involve the activation of the ERK1/2 signaling cascade in the LC. Future experiments, as immunohistochemistry and electrophysiology, are planned in order to further understand the effect of blocking CRF neurotransmission in anxio-depressive monoarthritic rats. A schematic representation of the data obtained using the monoarthritis model of chronic inflammation can be found in Figure 10.

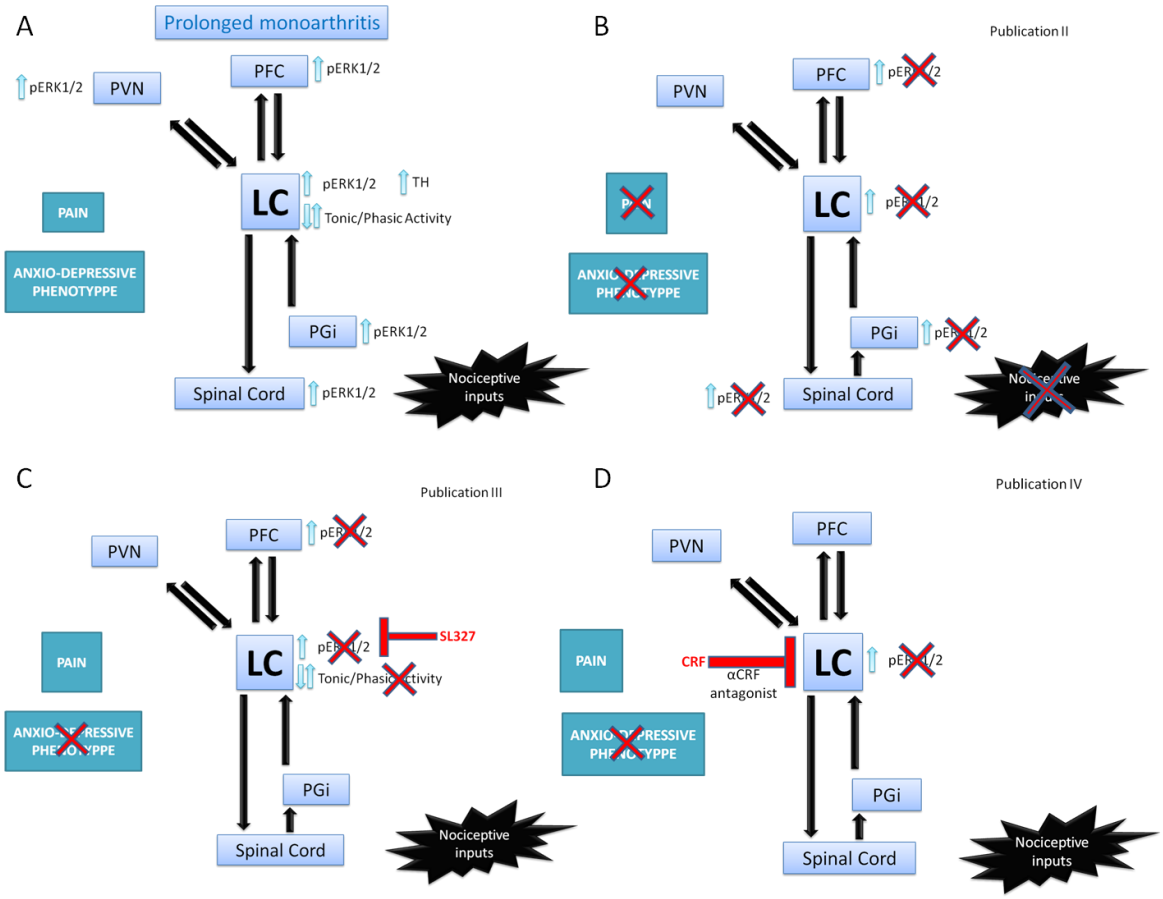

Figure 10: Schematic representation of the main results obtained using the monoarthritis model of chronic inflammation. A) Main features observed in prolonged monoarthritis. B) Results obtained upon blocking the peripheral nociceptive inputs with diclofenac topical application, in Publication II. C) Results obtained upon inhibition of ERK1/2 activation in the LC by using SL327, in Publication III. D) Results obtained upon blockade of the endogenous CRF activity in the LC, in Publication IV.

Behavioral considerations

The study of pain neurobiology, and of its associated comorbidities, relies in a large amount on behavioral data, as happens in many research areas within neurosciences. Indeed, the assessment of behavioral changes in nociception, and in the case of the present work, the search for anxiety- and depressive-like behaviors,

allows to validate the experimental animal pain models under study and to correlate molecular and cellular data with function.

In the present work, the nociceptive tests aimed to assess the nociceptive threshold in CFA-injected and CCI rats, confirming that thermal and/or mechanical hypersensitivity was increased. The most widely known standard pain assays or nociception tests that are used in the pre-clinical pain research, and were used in the monoarthritic and CCI animals, consist in applying either noxious (hyperalgesia tests) or innocuous (allodynia tests) mechanical or thermal stimuli to an animal's body part (often the hind paw). This procedure leads to a simple behavior that can be scored, such as the nocifensive withdrawal or vocalization, and allows assessing evoked nociceptive behaviors, as opposed to spontaneous pain. In the monoarthritis model, it is also well described another test that evaluates nociception associated to the physiological movement of the joints, mimicking the movement that the animal would perform during locomotion. Described by Butler and Weil-Fugazza (1994), the ankle-bend test (or foot-bend test) consists in scoring the animals behavior in response to five alternate flexions and extensions of the arthritic joint, in this case the ankle, and has been reliably used to test pharmacologically the antinociceptive efficacy of several compounds (Neto and Castro-Lopes 2000; Potes et al. 2006; Potes et al. 2006; Pozza et al. 2010). As it allows obtaining different information regarding the animals' well-being and nociception, this ankle-bend test was also used in the present work in the monoarthritis model.

In order to assess the onset of anxiety- and depressive-like behaviors resulting from the progression of the chronic pain condition, it was necessary to perform other specific behavioral paradigms. In the case of anxiety, the marble-burying and the elevated zero maze tests were used. The marble-burying test is based on the fact that rodents display a burying behavior (known as defensive burying) in response to an aversive or non-aversive but unknown stimulus (De Boer and Koolhaas 2003). This behavior refers to the displacement of bedding material using the snout or the forepaws in an effort to cover the foreign object (Pinel and Treit 1978). In the context of the behavioral test, rodents bury the higher number of glass marbles in response to non-aversive stimuli, according to their anxious state. This assay has been used as a tool for assessing the anxiety-like behavior mainly due to its sensitivity to several anxiolytic drugs (Broekkamp et al. 1986; Njung'e and Handley 1991; Nicolas et al. 2006; de Almeida et al. 2014). Nevertheless, some authors argue that the burying behavior reflects a repetitive and preservative behavior (obsessive-compulsive) rather than novelty-induced anxiety (Thomas et al. 2009).

The elevated zero maze is another paradigm used for the assessment of anxiety-like behavior. It consists of a modification of the elevated plus maze model comprising an elevated annular platform with two opposite and enclosed quadrants and two open quadrants. The “zero” shape removes any ambiguity in the interpretation of the time spent in the central square of the traditional “plus” design, allowing uninterrupted exploration. It is based on the fact that when rodents are exposed to a novel open and elevated alley, they exhibit a conflict between exploring

the new areas or staying in closed and safe areas. Thus, an increase in the amount of time spent in the closed areas is correlated with anxiety-like behavior (Shepherd et al. 1994).

Depression-like behavior was evaluated using a modified version of the forced swimming test (FST), as described previously by Detke et al. (1995). It is based on the fact that when confined to a limited space, rodents engage in a vigorous escape behavior. When it becomes clear that escape is impossible, these animals adopt an immobile posture, performing only the necessary movements required to keep their head above the water. Accordingly, in the FST, rats are placed for 15 minutes (pre-test) in a clear cylindrical acrylic container filled with tepid water to induce a depressive state in these animals. After 24 hours, the rats are once again exposed to the same conditions for 5 minutes (test) and they are videotaped to score their immobility (floating without struggling, using small movements to maintain the head above water), swimming (actively moving limbs more than is required to maintain the head above water) and climbing (active forepaw movements in and out of the water, often directed at the wall of the tank) (Detke et al. 1995). While longer periods of immobility are taken as an indication of depression-like behavior, changes in the time spent climbing or swimming have been correlated with alterations in the availability of noradrenaline and serotonin, respectively (Detke et al. 1995; Cryan et al. 2005). The FST keeps being one of the most used assays for the detection of pro-depressive and anti-depressive effects due to its good predictive validity, and it allows a rapid and

cheaper way for the assessment of antidepressant activity in a wide range of substances and pro-depressive activity in several experimental conditions.

Conclusions

6. Conclusions

The main conclusions of this work are:

1) The ERK1/2 cascade is differentially activated/deactivated in the spinal cord and in the PGi-LC-PFC pathway according to the duration of the exposure to a painful condition (acute vs chronic) and the type of pain (inflammatory vs neuropathic);

2) Chronic and painful inflammatory conditions are accompanied by the development of anxiety and depression which are a direct consequence of the increased and persistent nociceptive input due to inflammation;

3) In chronic inflammatory conditions, the ERK1/2 cascade in the LC is implicated in the altered perception of the noxious stimulus and modulates the development of the observed affective disorders;

4) The normal noradrenergic circuitry is compromised in chronic inflammatory conditions triggering the affective component of pain rather than participating in the nociceptive response;

5) Electrophysiology recordings of LC neurons points to a low tonic activity and high phasic activity in chronic inflammatory conditions leading to an anxio-depressive phenotype;

6) Increased CRF neurotransmission in the LC upon chronic inflammatory conditions seems to be one of the mechanisms triggering the activation of the ERK1/2 cascade and subsequent effects.

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