Neurobiological mechanisms involved in chronic pain

Roger Negrete Buela

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Thesis Director

Prof. Rafael Maldonado

Departament de Ciències Experimentals i de la Salut

Abstract

Chronic pain is currently a major clinical issue that represents huge economic and social burdens. Chronic pain treatment currently available presents limited efficacy and significant side effects. One of the reasons for this lack of effective therapeutic approaches is the insufficient knowledge of the mechanisms involved in the development and maintenance of chronic pain and pain-related comorbidities, such as emotional and cognitive manifestations. These associated symptoms negatively affect the life quality of the patients. In our study, we have first validated the different outcomes to measure the nociceptive, emotional and cognitive components of chronic neuropathic pain in mice, and the effects of repeated treatment with pregabalin on these manifestations. We have then identified the influence of different personality traits in the individual variability to chronic neuropathic pain perception in mice, and the emotional and cognitive manifestations associated to this chronic pain using different behavioural, electrophysiological and genetic techniques. The opioid system is a crucial therapeutic target for the treatment of chronic pain, despite the multiple side

effects associated to opioid compounds. Thus, we have evaluated the involvement of specific components of the endogenous opioid system in the behavioural, emotional, cognitive, neurochemical and epigenetic manifestations of chronic osteoarthritis pain in mice. We have identified the endogenous dynorphin / κ opioid receptor system as an interesting pharmacological target for the treatment of the different manifestations of chronic pain.

Resum

El dolor crònic és actualment una barrera clínica greu que representa una enorme càrrega econòmica i social. Els tractaments disponibles actualment per al dolor crònic presenten una eficàcia limitada amb considerables efectes adversos. Un dels motius d'aquesta manca d'eficàcia terapèutica recau en l'insuficient coneixement dels mecanismes involucrats en el desenvolupament i el manteniment del dolor crònic i el seus components emocionals i cognitius. Aquests símptomes associats tenen un impacte negatiu en la qualitat de vida dels pacients. En el nostre estudi, primer hem validat les diferents manifestacions nociceptives, emocionals i

cognitives de dolor neuropàtic crònic en ratolins, i els efectes del tractament crònic amb pregabalina en aquestes manifestacions. A continuació, hem identificat la influència dels diferents trets de personalitat en les diferències sobre la percepció del dolor en ratolins, així com les manifestacions emocionals i cognitives del dolor crònic en ratolins mitjançant diferents tècniques comportamentals, electrofisiològiques i genètiques. El sistema opioide representa una diana terapèutica crucial per al tractament del dolor crònic, tot i els seus múltiples efectes adversos associats als compostos opioides. Per això hem avaluat la implicació de components específics del sistema opioide endogen en les manifestacions conductuals, emocionals, cognitives, neuroquímiques i epigenètiques en el dolor osteoartrític crònic en ratolins. Hem identificat el sistema endogen dinorfina/receptor opioide κ com a una interessant diana farmacològica per al tractament de les diferents manifestacions clíniques del dolor crònic.

Abbreviations

AC: adenylyl cyclase

ATP: adenosine triphosphate

ACC: anterior cingulate

AMY: amygdala

BDNF: brain-derived neurotrophic factor

cAMP: cyclic adenosine monophosphate

CeA: central nucleus of the amygdala

CNS: central nervous system

COX: cyclooxygenase

CRF: corticotropin-releasing factor

DOR: δ (delta) opioid receptor

DRG: dorsal root ganglion

DSM: Diagnostic and Statistical Manual of Mental Disorders

fMRI: functional magnetic resonance imaging

FR: fixed ratio

GABA: gamma aminobutyric acid

Gadd45: growth arrest and DNA damage

GPCR: G protein-coupled receptor

H3K9: lysine 9 of histone 3

HIP: hippocampus

IASP: International Association for the Study of Pain

IC: insular cortex

IL: interleukin

KO: knockout

KOR: κ (kappa) opioid receptor

MAP: mitogen-activated protein

MIA: monosodium iodoacetate

MOR: μ (mu) opioid receptor

MRI: magnetic resonance imaging

NAc: nucleus accumbens

NGF: nerve growth factor

NK1: Neurokinin 1

NMDA: N-methyl-D-aspartate

NOP: nociceptin / orphanin

NR3C1: nuclear receptor subfamily 3, group C, member 1

NSAID: nonsteroidal anti-inflammatory drugs

ORL-1: orphanin-receptor like 1

PAG: periaqueductal grey

PDYN: prodynorphin

PENK: proenkephalin

PFC: prefrontal cortex

POMC: proopiomelanocortin

PR: progressive ratio

PSNL: partial sciatic nerve ligation

PTX: pertussis toxin

RVM: rostral ventromedial medulla

TLR: toll-like receptor

TNFα: tumor necrosis factor α

TrkB: tropomyosin receptor kinase B

TRP: transient receptor potential

TRPV1: transient receptor potential vanilloid type-1

VGCC: voltage gated calcium channel

WT: wild type

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INTRODUCTION

1. Pain: General considerations

1.1 A brief history of pain

In the Greek Mythology, Algos was the personification of pain, both physical and emotional. Hesiod, in his 8th-7th century B.C. poem Theogony, used the plural Algea as the female spirits who brought sorrow and cries to the men. Pain, grief or its relief were part of the open-handedness that the gods of Mount Olympus bestowed on mortals.

The Ancient Greeks moved forward from the Myth to the Logos between the age of Hesiod and the fifth century around the age of Plato. Plato, among others, advocated the brain as the centre of sensations and thoughts, and explained pain as a sensation. His pupil Aristotle hypothesized that a noxious stimulus travel from the skin to the heart through the blood, and the pain would be an emotion more than a sensation.

More than twenty centuries beyond, influenced by earlier Greek thinkers as Plato and Aristotle, Galen elaborated a complex theory suggesting the brain as a receptor and a modulator of sensations,

including pain. However, the Aristotelian conjecture of pain prevailed until the XVI century, when Descartes retook the Galen's hypothesis and confronted it with the Aristotle principles. Descartes conceived that the sensations travel through the nerves to the brain, were they were made conscious in the pineal gland (Baños et al., 2006; Bosch and Baños, 2009) (Figure 1).

Fig. 1. Descartes' concept of the pain pathway. He wrote: "If for example fire (A) comes near the foot (B), the minute particles of this fire, which as you know move with great velocity, have the power to set in motion the spot of the skin of the foot which they touch, and by this means pulling upon the delicate thread *c-c*, which is attached to the spot of the skin, they open up at the same instant the pore, *d-e*., against which the delicate thread ends (F), just as by pulling at one end of a rope one makes to strike at the same instant a bell which hangs at the other end."

Almost 2000 years after the Greek period, between 1803 and 1806, a pharmacist's apprentice in Paderborn called Friedrich Sertürner, was the first to isolate the alkaloid morphine from opium. Here, in the ancient relation between Myth and Logos, Sertürner named that substance as morphium by the Greek god of dreams, Morpheus.

Concurrently, Johannes Muller introduced “The Doctrine of Specific Nerve Energies” in 1840, postulating that nerves transmitted both external and internal inputs to the brain, and every sensory nerve corresponded to a concrete sensation when stimulated. In the 1890s, Maximilian von Frey, and Austrian-German physiologist, finally demonstrated the theory of nerve specificity, where cold, heat and touch were associated with the stimulation of high threshold free nerve endings. In 1894, Alfred Goldscheider developed the summation theory of pain maintaining that pain was based in the intensity of the stimuli and the summation of those impulses from other sensory pathways in the central nervous system (CNS).

At the beginning of the XX century, three different theories about the physiology of pain coexisted: the theory of nerve specificity of von Frey, the summation theory from Goldscheider and the ancient Aristotelian theory where pain would be an emotion more than a sensation. In 1965, Ronald Melzack and Patrick David Wall revolutionized research field by establishing the gate control theory of pain, in an article published in Science (Melzack and Wall, 1965). They postulated that the information coming in over C-fibres was modulated through presynaptic inhibition from incoming beta fibres. Then, the 'gating' mechanism depends upon the relative quantity of information coming in over the larger fibres versus the smaller fibres. Thus, the larger fibre activity relative to thin fibre activity at the inhibitory cell, the less pain is felt. This theory introduced the spinal and supraspinal mechanism of pain. In 1971, Melzack himself proposed the neuromatrix theory, where he defended the presence of a neuronal network located in the limbic areas of the brain, where sensorial information was received and processed. Those neural circuitries produce the emotional component of pain. Nowadays, all these studies converged in the

actual neuroanatomy and physiology theories of pain, accepting that pain is a sensation influenced by the emotional and cognitive components as well. An important occurrence during XX century was the creation of specific centres with the exclusive purpose of treating pain.

1.2 Definition

The International Association for the Study of Pain (IASP) describes pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Baños et al., 2006). However, pain is always subjective, and cannot be only defined as a nociceptive experience since it incorporates different behaviours that finally construct the symptom of pain. The definition of pain proposed by the IASP could be better comprehended if we inquire into two considerations about the components of pain (Baños et al., 2006):

- Nociception: the consequence of the painful stimuli transmission from peripheral nerves to brain cortex.

- Affection: the modulation of the grief associated to pain. Many psychological issues can modulate the perception of the painful experience.

1.3 Classification of pain

Pain can be classified in many different manners depending on the duration, intensity, localization or even its association with diseases. Considering the aim of this Thesis, we prefer to discern pain taking into account the duration of the pain (acute, chronic) and the pathophysiological aspects (nociceptive, inflammatory and neuropathic) (Cervero, 1991).

a) From acute to chronic pain

The distinction between acute and chronic pain is due to a temporally issue, but also because of the essential differences in the physiological and pathophysiological mechanisms of these two pain modalities (Aliaga et al., 2002). The transition from acute to chronic pain also appears to occur in separate pathophysiological and histopathological stages (Voscopoulos and Lema, 2010).

Generally, acute pain is defined as an immediate sensorial event in the nociceptive system. Somatic or visceral tissue damage leads into acute pain that is extended during the process of reparation and cicatrisation of the lesion. However, chronic pain is described as a pain that lengthen beyond the injury and remains once the lesion disappears (Aliaga et al., 2002). Indeed, different theories propose that a prolonged experience of acute pain may create chronic pain (Voscopoulos and Lema, 2010).

A large growth in the knowledge of the trip from acute to chronic pain has emerged in the last decades, although further investigations are needed for a better understanding of the pathophysiology of chronic pain. In this Thesis, we will focus our attention in the study of the different components involved only in chronic pain.

b) Pathophysiological mechanisms of pain

Temporal criteria may not be the best method to classify pain. Indeed, three different distinctions are currently used to categorise pain considering its pathophysiological mechanisms: nociceptive,

inflammatory and neuropathic pain. Nociceptive pain serves as a warning system activated to evade physiological damage to the organism. Nociceptive pain occurs with a normally functioning somatosensory nervous system, and arises from damage to non-neural tissue due to the presence of noxious stimuli acting on specialized high-threshold sensory machinery of the nociceptive pathway.

Inflammatory pain results of an activation of the nociceptive pathway by a variety of mediators released at a site of tissue damage. This inflammatory soup is rich in mediators that directly activate nociceptors and produce a sensitization of the somatosensory nervous system. As a consequence of this perturbation of the nociceptive system with peripheral and/or central sensitization, hyperalgesia and allodynia emerge. The IASP defines hyperalgesia as increased pain from a stimulus that normally provokes pain. However, allodynia is defined as an unexpectedly painful response due to a stimulus that does not normally provoke pain.

Neuropathic pain is described as a pain caused by a lesion or disease of the somatosensory nervous system. Neuropathic pain appears at both peripheral and central levels. It is characterised by the existence of spontaneous (i.e. non stimulus-evoked) pain and abnormal stimulus-evoked pain. Hyperalgesia and a severe allodynia emerge as a clinical manifestations (Maldonado et al., 2016; Merskey, H. & Bogduk, 1994; Scholz and Woolf, 2002; Woolf, 2010) (Figure 2). For the purpose of this Thesis, we mainly focus our attention in chronic neuropathic and osteoarthritis pain that has an important inflammatory component.

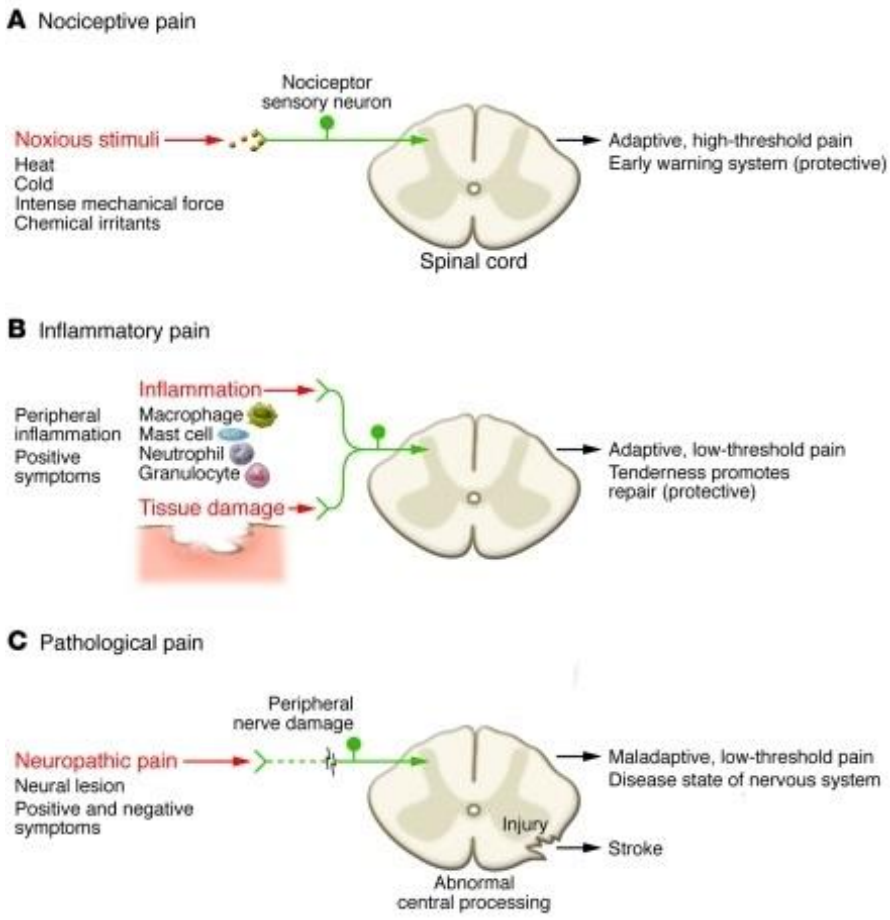

Figure 2. Pathophysiological classification of pain. Pain can be divided into three classes depending on its pathophysiological mechanisms. (**A**) Nociceptive pain represents the sensation associated with the detection of potentially tissue-damaging noxious stimuli and is protective. (**B**) Inflammatory pain is associated with tissue damage and the infiltration of immune cells and can promote repair by causing pain hypersensitivity until healing occurs. (**C**) Pathological pain is a disease state caused by damage to the nervous system (neuropathic pain) (adapted from Woolf, 2010).

1.4 Physiology of pain: The transmission of the painful stimuli

Nociception has been defined as the process of encoding noxious stimuli, which is detected by a subpopulation of peripheral nerve fibres called nociceptors. The cell bodies of nociceptors are located in the dorsal root ganglion (DRG), presenting a peripheral and a central axonal prolongation that innervates their target tissue and the dorsal horn of the spinal cord, respectively (Basbaum et al., 2009).

There are four major groups of sensitive fibres. First, fast and large diameter myelinated fibres (Aα), that are mainly responsible for proprioceptive transmission. The second group of myelinated large diameter afferent fibres are the Aβ fibres, that mainly respond to innocuous mechanical stimulation (i.e. light touch). Third, a medium diameter myelinated (Aδ) afferents that considerably differ from the larger and rapidly conducting fibres, mediate a first and fast well-localized pain transmission as well as mechanical and cold temperature perception. Finally, a last group of fibres includes small diameter and unmyelinated (C) fibres that mediate slow pain and

hot temperature perception (Basbaum et al., 2009; Woolf and Doubell, 1994) (Figure 3).

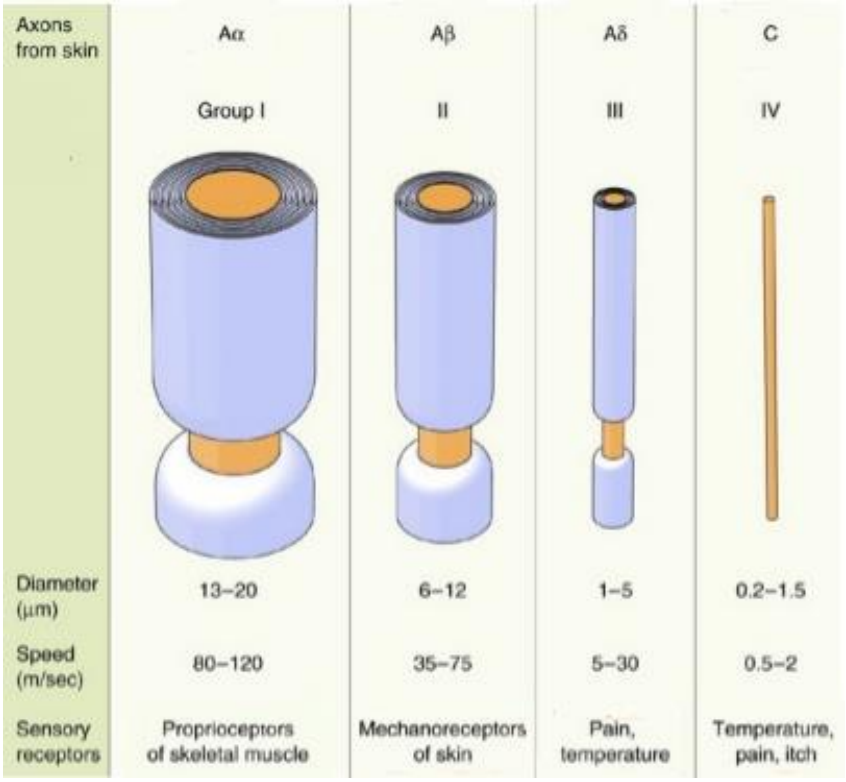

Figure 3. Classification of nociceptors. Group I (Aα) large-diameter and fast myelinated fibres, conduct proprioception. Group II (Aβ), large-diameter and myelinated fibres mainly responsible for the mechanosensitivity of the skin. Group III (Aδ), myelinated medium-diameter afferents that mediate well-localized acute pain. Group IV (C), small-diameter non-myelinated fibres that mediate slow pain (Bear et al., 2015).

In a physiological environment, Aβ fibres are associated to low-threshold mechanoreceptors in the periphery, and are extremely sensitive to skin stimulation, although they do not elicit a pain sensation. Only Aδ and C fibres transmit nociceptive information. However, in pathophysiological conditions, ectopic firing occurs in Aδ, C and also in Aβ fibres. Thus, both exaggerated responses evoked by a low threshold of Aβ fibres, and high threshold of Aδ and C fibres may be involved in the generation of peripheral sensitization (Schaible, 2007). Several specific changes at the peripheral level underlie this nerve sensitization. Thus, tissue injury produces an altered chemical environment on the peripheral terminals of nociceptors, common referred as 'inflammatory soup', including neurotransmitters, peptides (substance P, CGRP, bradykinin), eicosinoids and related lipids (prostaglandins, thromboxanes, leukotrienes, endocannabinoids), neurotrophins, cytokines, and chemokines, as well as extracellular proteases or protons, among others. Some of these inflammatory mediators, such as bradykinins, activate neurons directly and sensitize them for mechanical, thermal and chemical stimuli. As a result, this

inflammatory environment produces dramatic changes in the excitability of nociceptors and amplify the signal transduction transmitted to the spinal cord (Basbaum et al., 2009; Schaible, 2007; Scholz and Woolf, 2002) (Figure 4).

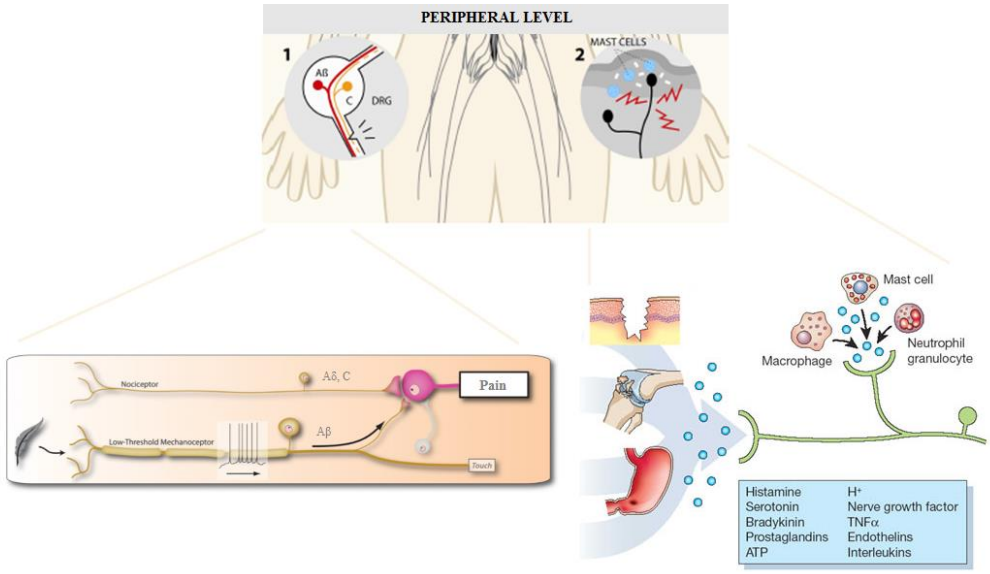

Figure 4. Pathophysiology on the periphery. 1, Painful stimuli are caused by the activation of nociceptive receptors (Aδ and C). A low threshold of Aβ fibres produces exaggerated peripheral sensitization during transmission of nociceptive information. 2, An inflammatory environment releases proinflammatory mediators that produce changes in the excitability and sensitivity of nociceptive afferents and amplify the nociceptive transduction to central neurons (adapted from Scholz and Woolf, 2002; Woolf, 2011).

The ascent Aδ and C nociceptive fibres project mainly to lamina I and II in the spinal cord. By contrast, the non-painful Aβ fibres project to the lamina III, IV and V of the dorsal horn of the spinal cord (Willis and Coggeshall, 2004). A proportion of lamina I neurons project contralaterally via the lateral spinothalamic tract, which plays a crucial role in pain, itch and temperature pathways. In addition, the spinoparabrachial tract is one of the major projection targets to laminae I and II of the dorsal horn, and it is involved in numerous autonomic and emotional responses to painful stimuli (Bester et al., 2000). Therefore, the spinothalamic tract is important for the sensory discriminatory aspects of pain, whereas the spinoparabrachial pathway plays a crucial role in the emotional component of pain and in regulating descending control pathway (Scholz and Woolf, 2002; Suzuki and Dickenson, 2005).

A dramatic activation of microglia in the dorsal horn of the spinal cord has been reported in different models of chronic pain, such as neuropathic and osteoarthritis pain (Sagar et al., 2011; Tsuda, 2016). Activated microglial cells induce the release of inflammatory mediators that in turn contribute to the abnormal growth of the Aβ

fibres, leading to the sensitization phenomena with allodynia and hyperalgesia. In addition, the primary afferent nerve terminal are enclosed by activated microglial cells that maintain the neuroinflammatory environment in the spinal cord and the hiperexcitability in the CNS leading to an enhanced processing of nociceptive information (Basbaum et al., 2009; Marchand et al., 2005). This sensitization of the central mechanisms is characterized by an intensification in the activity of neurons and circuits in the nociceptive pathway triggered by an increase in membrane excitability and a diminished inhibitory transmission (Latremoliere and Woolf, 2009). Inflammatory mediators such as cytokines mediate DRG and spinal cord central sensitization. Glial cells (microglia and astrocytes) also play a crucial role in this process. The activation of Toll-like receptors (TLR), among others, in glial cells mediate pain processing (Milligan and Watkins, 2009). Thus, the glial TLR-4 recognizes opioids, and this opioid-induced activation of glia leads to the release of neuro-excitatory and inflammatory mediators.

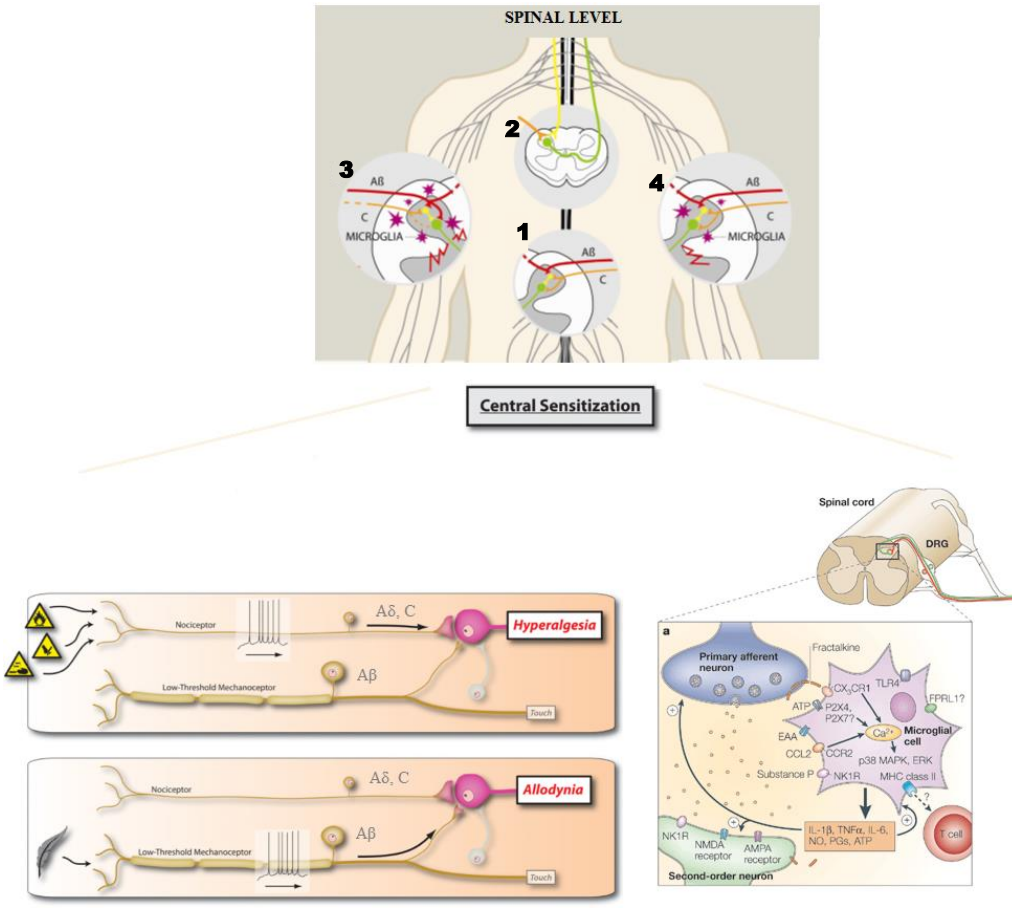

Figure 5. Pathophysiology on the spinal cord. 1 and 2, The painful stimuli are transmitted through the spinal cord to brain areas. 3 and 4, In addition, microglia become activated after peripheral events, and release several inflammatory mediators that leads to central sensitization. This neuroinflammatory environment produces an atypical development of Aβ fibres that finally contribute to allodynia and hyperalgesia (adapted from Marchand et al., 2005; Woolf, 2011).

Finally, astrocytes and microglia express various functional neurotransmitter receptors activated by classic neurotransmitters and neuromodulators (Milligan and Watkins, 2009). Therefore, central sensitization is the result of an enormous plasticity of the CNS finally manifested as hyperalgesia and allodynia even in areas outside the initial trigger zone (Thakur et al., 2014). Once central sensitization begins, pain signalling is no longer the adaptive, and protective mechanism (Milligan and Watkins, 2009b) (Figure 5).

At supraspinal level, several brain regions are activated by nociceptive input and participate in pain perception. Thus, ascending painful information through the spinothalamic tract spreads through the thalamus to the somatosensory cortex and associated brain areas. In parallel, the parabrachial nucleus connects to the hippocampus (HIP) and the central nucleus of the amygdala (AMY), crucial brain areas involved in the cognitive and emotional components of chronic pain (La Porta et al., 2016; Scholz and Woolf, 2002). The nociceptive stimuli projects from thalamus to limbic cortical areas such as anterior cingulate (ACC) and insular cortex (IC). These brain areas have been reported to be related to

the social-emotional components of pain, such as empathy, that are specific to primates (Lieberman and Eisenberger, 2009; Price, 2000). Moreover, neuroimaging studies in humans reported that different subregions within the hypothalamus and sensory cortex are also activated after chronic pain, suggesting that the nociceptive input into these regions underlies the perception of sensory, affective and neurovegetative dimensions of pain (Apkarian et al., 2005).

Beyond the ascending mechanisms, a well-characterised descending pathway originates within the midbrain periaqueductal grey (PAG), which is heavily interconnected with the hypothalamus and limbic structures, such as the AMY, and projects downstream to the rostral ventromedial medulla (RVM), which in turn sends outputs to the dorsal horn of the spinal cord. Inhibitory control from the PAG-RVM system preferentially suppresses nociceptive inputs mediated by C-fibres, preserving sensory-discriminative information conveyed by more rapidly conducting Aδ fibres (Heinricher et al., 2009) (Figure 6).

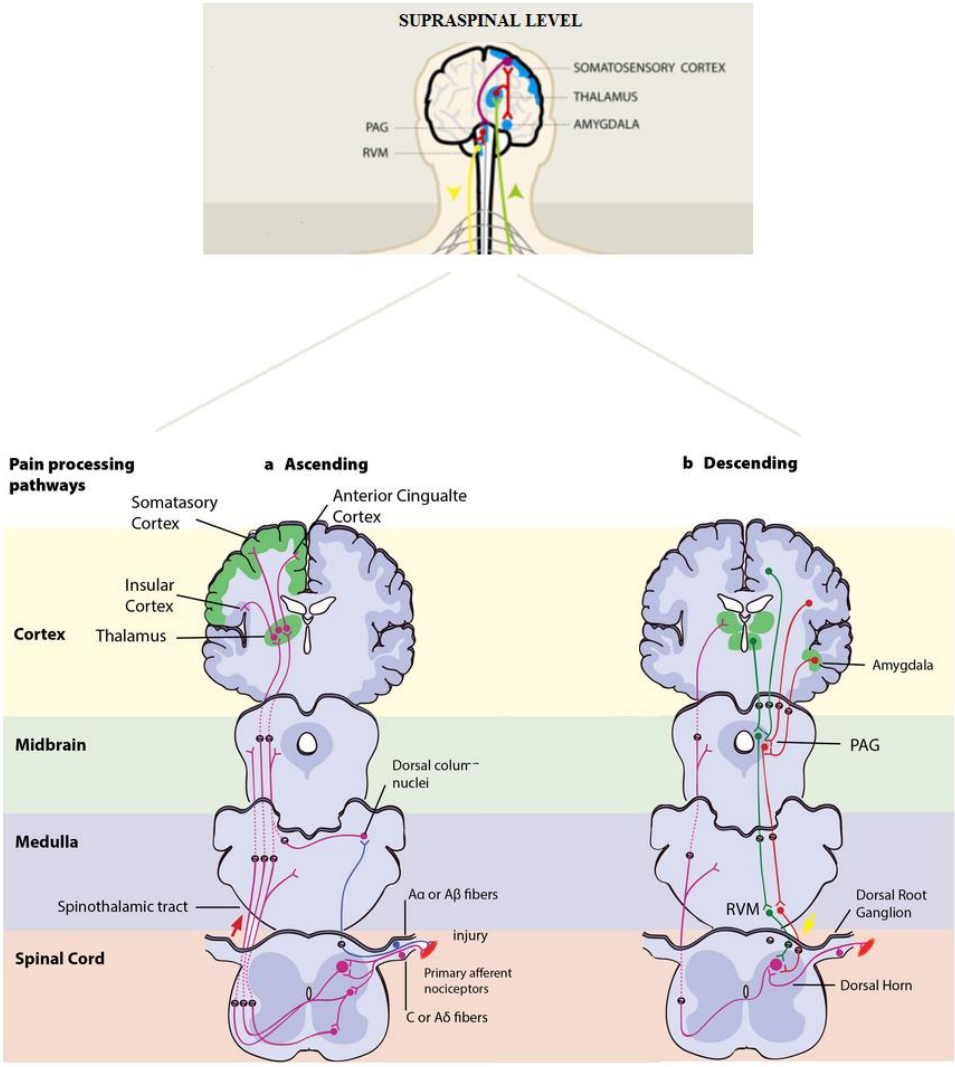

Figure 6. Pain transmission at supraspinal level. Ascending and descending pathways modulate pain in the CNS. A) Ascending pain pathway. After synapsing with the primary afferents, second order neurons in the dorsal horn of the spinal cord decussate contralaterally to ascend through the spinothalamic and parabrachial tracts to the thalamus, that conveys the nociceptive information to the somatosensory brain areas, such as the AMY, HIP, ACC and IC, where the

emotional and cognitive components of pain are mediated. B) Descending pain pathway. Cortical areas connect to the midbrain PAG, which is interconnected downstream to the RVM. The PAG-RVM system inhibits pain through the release of endogenous analgesic compounds, such as opioids. In addition, this inhibitory system blockades the nociceptive inputs mediated by C-fibres in the dorsal horn of the spinal cord.

This descending pathway is of particular interest due to its analgesic responses through the inhibition of the ascending nociceptive transmission. Indeed, PAG and RVM are major sites of analgesic action of opioid. Several studies have shown that opioids produce disinhibition in the PAG-RVM system through both pre- and postsynaptic mechanisms. Thus, opioid agonists directly inhibit a subpopulation of gamma aminobutyric acid (GABA) interneurons through the postsynaptic activation of opioid receptors. This postsynaptic inhibition leads to an increase in K^+ conductance, reducing conduction across the axon, or limiting neurotransmitter release at central terminals, producing analgesia. In turn, opioids agonists inhibit GABAergic inputs onto PAG and RVM neurons through the activation of presynaptic opioid receptors, which is also

mediated by a voltage-dependent K^+ channel mechanism (Lau and Vaughan, 2014). Indeed, different studies have reported that the opening of K^+ channels plays an important role in the antinociception induced by morphine at supraspinal, spinal and peripheral levels (Ocaña et al., 2004).

Nociceptive inputs can also trigger central sensitization at the supraspinal level. Neuroimaging studies revealed an increase in the excitability of neurons in the somatosensory cortex evoked by a low threshold of Aβ fibres (Woolf, 2011). Accordingly, electrophysiological studies demonstrated that neurons of the CeA showed sensitization with noxious stimulation mediated through metabotropic glutamate receptors after chronic pain (Ossipov et al., 2010). This glutamatergic sensitization induces neuroplastic changes that appear to promote chronic pain (Li and Neugebauer, 2004).

1.5 Osteoarthritis pain

a) Epidemiology

Osteoarthritis is the most common form of arthritis presenting the highest occurrence of all rheumatic diseases and one of the most predominant chronic diseases (Puig-Junoy and Ruiz Zamora, 2015). Historically, osteoarthritis has been described as a disease associated with cartilage degradation, although novel studies point osteoarthritis as a disease of the whole joint with the involvement of different genetic, biochemical and biomechanical components. Osteoarthritis is characterized by cartilage degradation, structural and functional deterioration of the synovium, bones and joint tissue, and the presence of local inflammatory mediators plays a crucial role in this complex chronic pain state (Sofat et al., 2011). The prevalence of this chronic disease increases because of an ageing population. According to the Global Burden of Disease study (Murray and Lopez, 1997), an elderly population could place osteoarthritis as the 9th cause of disability-adjusted life years in developed countries by the year 2020. In agreement, between 1990

and 2010 the total number of years lived with disability caused by osteoarthritis in worldwide population growth by 60.2%, positioning this disease to the 11th in the list of the most common disabilities. Osteoarthritis also affects elder and mid-age people, even though young people may also be affected due to an injury or over use. Osteoarthritis affects both men and women, although it arises most commonly in women. Other risk factors such as obesity, reduced physical activity, genetic, diet, hormonal status and different systemic factors may contribute to the onset and progression of the disease (Puig-Junoy and Ruiz Zamora, 2015; La Porta et al., 2014).

This disease represents a high cost for the health system burdens, not only for the osteoarthritis healthcare in itself, but also for the indirect costs such as productivity losses and the cost of associated care with patients with osteoarthritis (Puig-Junoy and Ruiz Zamora, 2015).

b) Pathophysiology of osteoarthritis

Osteoarthritis was previously considered as a disease of the articular cartilage. However, the disease compiles chronic and

progressive structural damage that affects the whole joint. Thus, joints are complex organs where different tissues cooperate to facilitate or limit movement between bones due to the presence of articular cartilage that covers the bones providing smooth and deformable environment to sustenance movements. This tissue is conformed of articular chondrocytes surrounded in a specific extracellular matrix that contains type II collagen and sulphated proteoglycans that absorb and translate to the bones the compressive loading forces applied to the joint during movements. In addition, joint cavity is lined by the synovium thin connective tissue that provides lubricating synovial fluid. Finally, ligaments and capsules contribute to the strength and limiting the degree and the axes of movement of the whole joint (Thysen et al., 2015) (Figure 7).

Osteoarthritis results when the equilibrium between the breakdown and the repair of the whole joint tissue becomes unbalanced leading to the disruption of the normal homeostasis of the joint (Lories and Luyten, 2012). The mechanisms implicated in the onset and progression of the disease are not well understood.

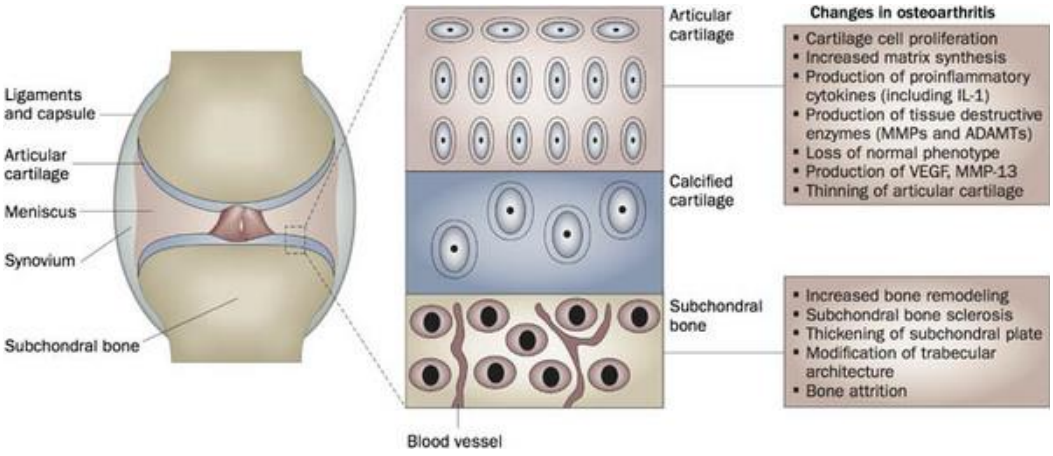

Figure 7. Pathophysiology of osteoarthritis. Joints are complex organs where different tissues cooperate to maintain the structure and functionality of the knee. Progressive development of osteoarthritis results in activation of different processes and pathways in the distinct tissues and cells of the joint. ADAMTs, a disintegrin and metalloproteinase with thrombospondin motifs; IL-1, interleukin-1; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor (Lories and Luyten, 2012).

Regardless of many different risk factors, such as obesity, diet, physical activity, genetic and hormonal status among others, several causes may contribute to the initiation of the disease. Inflammation or trauma triggers the activation of the articular chondrocytes that begin to produce additional molecules to the extracellular matrix and pro-inflammatory molecules, such as interleukins (ILs) or matrix

metalloproteinases (MMPs). These molecules collaborate to a long-term and progressive loss of cartilage with cell death and depletion of the extracellular matrix leading all together to the destruction and disintegration of joint tissue (Lories and Luyten, 2012), which play a crucial role on the disturbed processes implicated in OA pathophysiology (Kapoor et al., 2011). Several studies using magnetic resonance imaging (MRI) and ultrasonography have demonstrated that the presence of synovial inflammation is also characterized by the production and release of these secreted inflammatory molecules onto the synovial fluid where they activate the production of catabolic factors leading to cartilage degradation (Thysen et al., 2015). Numerous cytokine pathways involved in the pathophysiology of osteoarthritis have been investigated as potential therapeutic targets to treat osteoarthritis, although some small clinical trials have questioned the feasibility of those approaches (Jotanovic et al., 2012; Thysen et al., 2015).

Concurrently, a complex set of biomechanical and biochemical dysregulation occurs between the different implicated structures. Thus, progressive loss of the cartilage, remodelling of the

subchondral bone, formation of bone outgrowths (osteophytes), synovial inflammation or damage of tendons, menisci and capsules are some of the processes associated to the pathophysiology of osteoarthritis that finally contribute to joint pain (Thysen et al., 2015) (Figure 8).

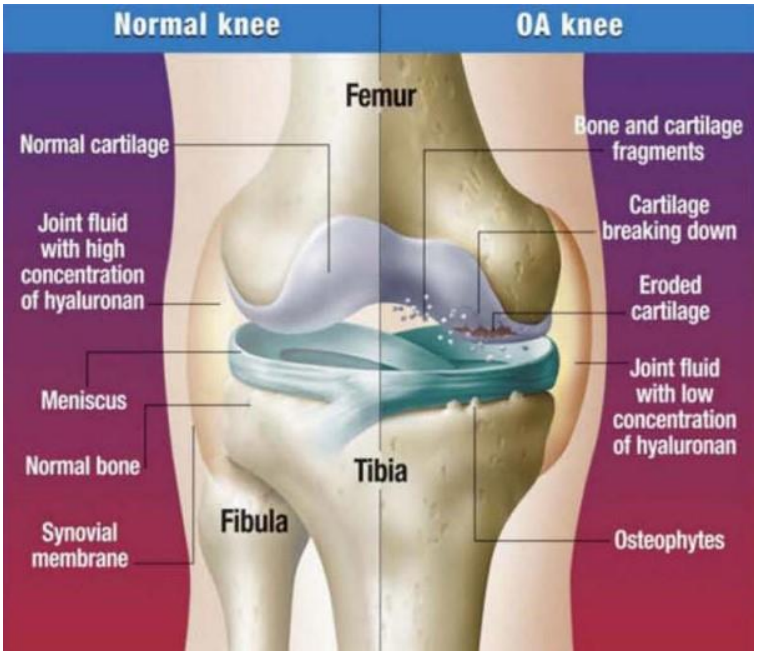

Figure 8. Structural affections during osteoarthritis. Healthy tissue is shown (left): Normal cartilage and bone structure, high levels of joint fluid and synovial membrane. Osteoarthritis knee (right) presents remodelling bone and cartilage, outgrowth of osteophytes and altered joint fluid levels (Bhopal, 2015).

Sensitive diagnostic techniques beyond classical radiography are not available, and physicians cannot predict the progress of the disease, although structural molecules and mediators derived from bone, cartilage and the synovium have been reported to be potential biomarker candidates for osteoarthritis. However, a better knowledge of the disease biomarker field remains a major research challenge (Ishijima et al., 2014).

i) Peripheral mechanisms

The peripheral mechanisms involved in the mammalian bipedalism requires an important mechanical component that eventually may contribute to the development of osteoarthritis pain. Knee joint is a highly innervated tissue with large diameter myelinated nerve fibres encoding and transmitting proprioceptive signals, and short diameter unmyelinated innervations responding to noxious mechanical stimuli, showing the crucial role of mechano-sensitivity in the precise joint function (Malfait, 2016; Malfait and Schnitzer, 2013). Recent studies correlate the mechanical component of osteoarthritis pain with the presence of mechano-gated ion

channels on Aδ and C joint afferents suggesting that these channels might have a crucial role in pain sensing (Heppelmann and McDougall, 2005; Krustev et al., 2015; Malfait, 2016).

Nociceptors express a wide variety of receptors for a broad number of inflammatory mediators in the joint. These mediators, including cytokines, chemokines, prostaglandins, neuropeptides, H^+ ions, IL-1β and tumour necrosis factor α (TNFα), among others, are the major mediators implicated in the synovial inflammation and cartilage degradation during osteoarthritis (Sohn et al., 2012). In vivo studies support a catabolic role for interleukins in the joint. Elevated levels of IL-6 has been shown to activate B cells, T cells and mediate the recruitment of inflammatory cells to sites of inflammation (Sokolove and Lepus, 2013). Moreover, synovitis produced by the elevated plasma protein levels in the synovial fluid and the presence of activated macrophages contribute to inflammation during osteoarthritis (Sohn et al., 2012; Sokolove and Lepus, 2013). As a result, joint movement within the normal range produces mechanical allodynia (Malfait, 2016; Malfait and Schnitzer, 2013) (Figure 9).

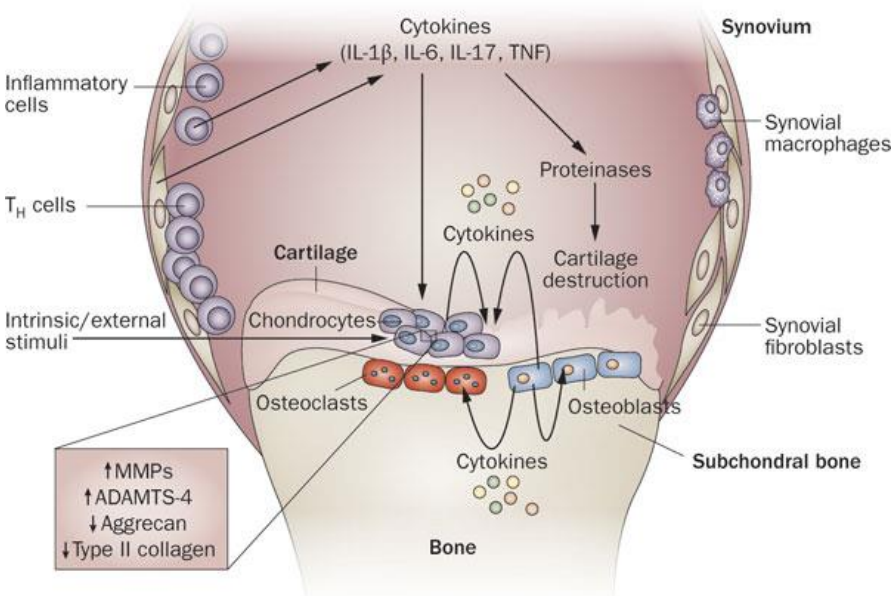

Figure 9. Proinflammatory mediators during osteoarthritis in the periphery. Increased levels of inflammatory mediators, such as cytokines, prostaglandins, chemokines, among others, contribute to cartilage degradation and inflammation, resulting in structural impairments of the joint (Kapoor et al., 2011).

The literature assessing the relation between painful knees and structural changes in the joint have demonstrated strong associations with radiographic and MRI correlations. Patients with osteoarthritis experience fluctuations in the presence and intensity of knee pain, and changes in MRI detectable features such as bone

marrow lesions, meniscal damage and synovitis are associated with these fluctuations (Niu et al., 2015; Zhang et al., 2011).

ii) Central mechanisms

Continued nociceptor input from the DRG may lead to prolonged hyperexcitability of pain circuits in the CNS, the previously explained phenomenon known as central sensitization. Neuroplasticity occurring during central sensitization involves both spinal and supraspinal structures (Lluch et al., 2014). Several neuroimaging studies demonstrate altered activation of different brain regions after different chronic osteoarthritis pain (Malfait, 2016; Malfait and Schnitzer, 2013). These brain anatomical changes after osteoarthritis pain, including volume loss of grey matter of the thalamus, have been shown to be reversible (Gwilym et al., 2010).

In addition to these ascending modulation, descending pathways also play an important role in the central physiopathological mechanisms involved in osteoarthritis.

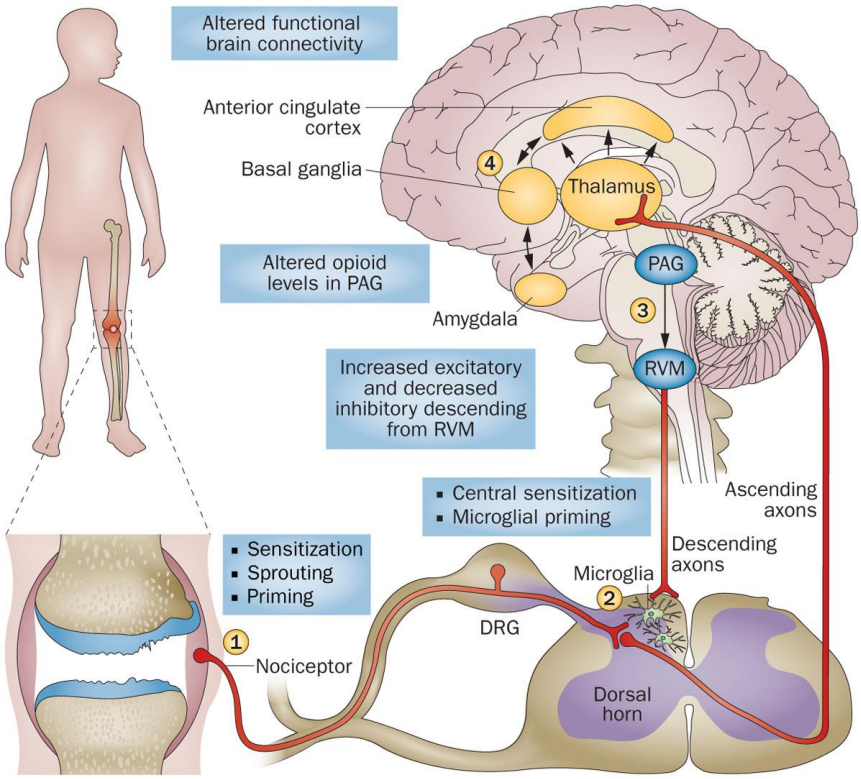

Figure 10. Representation of the central mechanisms during osteoarthritis. (1) Hypersensitive afferent terminals in the DRG synapse to second neuron in the dorsal horn of the spinal cord. (2) Activated glial cells release inflammatory mediators that contribute to central sensitization and pain processing. (3) PAG-RVM system modulates descending pathways through decreased inhibitory and increased excitatory control, and regulates the actions of pain-relieving drugs. (4) Altered activation of different supraspinal regions involved in the emotional and cognitive components of osteoarthritis. Central sensitization at spinal and supraspinal levels also occurs during osteoarthritis pain (La Hausse de Lalouvière et al., 2014).

Central sensitization during osteoarthritis comprise an impairment of descending inhibitory signals and increase descending pain facilitation, as well as altered spatial and temporal summation and abnormal spinal reflexes. These descending signals are relevant to the actions of pain-relieving drugs, including an opioid-sensitive circuit, cannabinoids, nonsteroidal anti-inflammatory drugs (NSAIDs), and serotonin/norepinephrine reuptake blockers (Malfait, 2016; Ossipov et al., 2010) (Figure 10).

c) Therapeutic approaches for osteoarthritis pain

Clinical management of osteoarthritis is mainly symptomatic and usually entails a limited combination of pharmacological and non-pharmacological approaches to reduce pain. These therapies have a modest efficacy and frequently present significant side effects leaving patients with considerable functional disabilities and persistent pain (Yu and Hunter, 2015). According to Osteoarthritis Research Society International, those treatments are intended to reducing joint pain and improving mobility, reducing physical disability, limiting the progression of the disease and educating

patients about the nature of the disorder (Zhang et al., 2013). Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are the most common treatment for rheumatologically conditions including osteoarthritis (Mobasheri and Henrotin, 2015). NSAIDs have some effects in relieving pain and increasing mobility for approximately 60 % of patients with osteoarthritis, through an inhibition of the activity of the cyclooxygenase (COX) enzymes. Aceclofenac reduces pain, disease severity and improves functional capacity in patients with knee osteoarthritis to a similar extent to diclofenac, piroxicam, and naproxen (Dooley et al., 2001). However, NSAIDs present a short-time efficacy and its analgesic effects are highly reduced beyond time, unable to stop the progression of the disease. Moreover, NSAIDs have also been associated with severe gastrointestinal renal and cardiovascular side effects (Tonge et al., 2014).

Considering these pronounced limitations of NSAIDs, other drugs have also been considered as candidates for treating osteoarthritis pain, including opioids and non-standard analgesics. Among these, gabapentinoids, such as pregabalin, have demonstrated efficacy in the monosodium iodoacetate (MIA) model of osteoarthritis

(Rahman et al., 2009; Thakur et al., 2014), and in combination with NSAIDs in preclinical studies (Ohtori et al., 2013). Recent clinical studies indicate that those patients who respond inaccurately or cannot tolerate NSAIDs with the persistent of chronic pain may considered for novel strategies, including opioids (da Costa et al., 2014; Smith et al., 2016), tramadol or tapentadol, acting as μ-opioid receptor agonist and noradrenalin reuptake inhibitors (Steigerwald et al., 2013). These treatments may be of particular relevance in the management of severe osteoarthritis pain. It is also crucial to consider the affective and cognitive components associated with chronic pain, and it is essential to further investigate in those aspects. These non-conventional therapies suggest a dual benefit on both pain and affective disorders during osteoarthritis pain. However, the side effects related to some of these treatments would limit their clinical use.

A new strategy for the treatment of osteoarthritis pain now under investigation consists in disease-modifying osteoarthritis drugs. These drugs are promising adjuncts to symptomatic relief and structure reconstruction during the disease progress (Yu and

Hunter, 2015). Several studies have shown remarkable efficacy in clinical trials and could represent new pharmacological approaches to treat osteoarthritis. One of the first important targets to investigate these drugs was cartilage matrix degeneration. Matrix metalloproteases, a diverse family of zinc-dependent proteolytic enzymes, are involved in tissue remodelling (Li et al., 2014; Tonge et al., 2014). The unbalance between the inhibition and activation of matrix metalloproteases results in excessive degradation of the extracellular matrix. However, the development of matrix metalloproteases inhibitors has been limited by their tendency to elicit various undesirable pathologies such as fibrodysplasia and musculoskeletal syndrome in preclinical animal models and also in clinical trials in osteoarthritis patients (Tonge et al., 2014). These concerns regarding matrix metalloproteases inhibitors safety profile have led to investigate additional disease-modifying osteoarthritis drugs approaches. Aggrecanases are members of the disintegrin and metalloproteinase with thrombospondin motifs family, which are the major enzymes responsible for aggrecan cleavage during early cartilage remodelling. Inhibitors targeting these enzymes may

be promising alternative drugs to treat osteoarthritis symptomatology (Larkin et al., 2015).

Other disease-modifying osteoarthritis drugs are related with the beneficial effects of the nutritional supplements such as chondroitin sulphate, glucosamine or vitamin D, among others. It is shown that patients deficient in vitamin D have an increased risk of knee osteoarthritis progression (Zhang et al., 2014). Chondroitin sulphate and glucosamine have also been observed as targets to exert valuable effects on the metabolism during osteoarthritis in both animal models and humans (Mantovani et al., 2016). However, current studies with chondroitin sulphate and glucosamine suggest controversial results and high-quality clinical trials are needed to determine its possible effectiveness (Akhtar and Haqqi, 2012; Davies et al., 2013). Therefore, pharmacological treatment for osteoarthritis remains unclear, and there is an urgent need to further research to identify more effective drugs to better understand the aetiology and pathophysiology of this disease.

1.6 Neuropathic pain

a) Epidemiology

Neuropathic pain is defined by the IASP as a 'pain caused by a lesion or disease of the somatosensory system'. Given the large and varied population with many neuropathic pain diagnoses, a large amount of literature on the field has been published in the last years. Aged people, female gender and the prevalence of mental disorders may be susceptible factors to promote chronic neuropathic pain (Butler et al., 2013). The multifactorial reasons involving this disease highlight the importance to establish standardised epidemiological studies to better understand neuropathic pain (van Hecke et al., 2014). An accurate estimation of population incidence is found to be between 3% and 17% in chronic pain patients with neuropathic characteristics, although it is revealed a high prevalence variability (0.8–72.0 per 100,000 population/year) in neuropathic pain associated with a specific condition (i.e. phantom limb pain, post-herpetic and trigeminal neuralgia or diabetic neuropathy). Neuropathic pain is often associated with emotional and cognitive

dysfunctions such as depression, sleep disturbances and physical impairments. These patients present a lower labour productivity, having an impact in the health system resources (Gilron and Dickenson, 2014; Maldonado et al., 2016). Recent reports indicate that the costs related to neuropathic pain may represent approximately $160 billion per year in the United States alone (Gilron and Dickenson, 2014).

b) Pathophysiology of neuropathic pain

Neuropathic pain is characterized by the presence of exaggerated response to painful stimuli (hyperalgesia), pain response to innocuous stimuli that normally does not evoke pain (allodynia) and the presence of ectopic and spontaneous pain. However, different mechanisms might be involved in this symptomatology, indicating the complexity of neuropathic pain, but also the significance of understanding these mechanisms in individual patients (Baron et al., 2010). The pathophysiology underlying chronic neuropathic pain comprise complex peripheral and central mechanisms. Peripheral neuropathic pain results from lesions mainly caused by mechanical

trauma on the periphery, metabolic diseases, infections or tumour invasions, whereas central mechanisms are mainly a consequence of spinal cord injury, stroke or multiple sclerosis (Bouhassira and Attal, 2016). These abnormal pain sensations are associated with complex physiological changes in the peripheral and/or the central nervous system.

i. Peripheral mechanisms

Peripheral nerve injury produces damage to primary afferents and nerve fibres that develop ectopic activity and become hyperexcitable. Several specific changes occur at peripheral level. Increased potassium and voltage-gated sodium channels RNA expression in primary afferent terminals lead to ectopic spontaneous activity, which might also contribute to decrease the action potential threshold and in turn generate a membrane hyperexcitability of Aδ and C fibres (Baron et al., 2010).

A recruitment of immune cells releasing proinflammatory cytokines, nerve growth factor (NGF), among other signalling mediators has also been reported. An increase of the purinergic receptors

functionality and calcium channel subunit α2δ1, and a reduction in the opioid receptor expression contributes to the pharmacological dysfunction during neuropathic pain (Baron et al., 2010; Maldonado et al., 2016). In addition, nerve injury induces a downregulation of transient receptor potential V1 (TRPV1) on injured fibres and an upregulation on injured C-fibres. TRPV1 are located predominantly on nociceptive afferent fibres and transmit noxious heat. These changes may contribute to the development of peripheral sensitization and the associated heat hyperalgesia (Basbaum et al., 2009). Moreover, peripheral nerve injury also induces an upregulation of a cold and menthol-sensitive transient receptor potential (TRP) channel related to the temperature-sensitive excitation that is expressed in DRG neurons (Wasner et al., 2004). This mechanism seems to participate in the sensory phenomenon of cold and mechanical hyperalgesia during neuropathic pain (Figure 11).

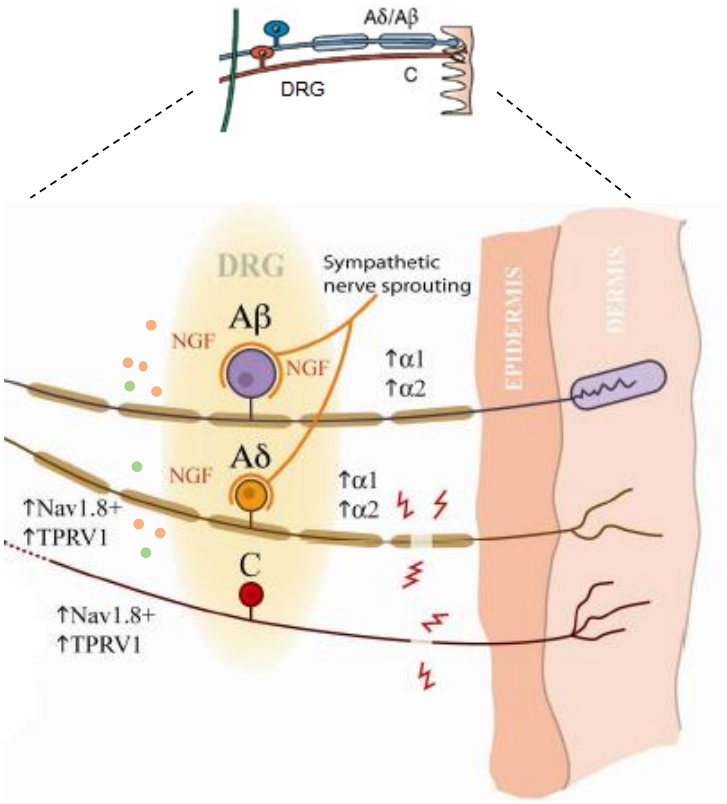

Figure 11. Peripheral mechanisms during neuropathic pain. After peripheral neuropathic pain some nerves are damaged, whereas others are still intact connecting to the peripheral tissue. Different ion channels (sodium, α2δ subunit calcium, potassium, TRPV1, among others) increase their expression, contributing to ectopic activity and a reduction of the action potential threshold on damaged fibres. In addition, the recruitment of immune cells induces the release of inflammatory mediators, such as cytokines and NGF. All together contributes to the development of peripheral sensitization associated to hyperalgesia (adapted from Baron et al., 2010; Nadal, 2011).

ii. Central mechanisms

In the spinal cord, the increased neuronal activity initiated and maintained by sensitized Aδ and C fibres results in central sensitization. In addition, innocuous mechanical stimuli become capable to activate neurons through Aδ and Aβ low-threshold fibres, developing mechanical allodynia (Baron et al., 2010). In turn, damaged fibres contribute to the increase of excitability by releasing glutamate and substance P, which acts on N-methyl-D-aspartate (NMDA) and neurokinin 1 (NK1) receptors increasing calcium levels on the postsynaptic spinal cord neurons. This mechanism is involved in the hyperalgesia (Baron, 2006).

In addition, glial cells also contribute to central sensitization. After nerve injury, microglia surround the cell bodies of the dorsal horn of the spinal cord. Activated microglia induces critical alterations in the expression of different genes involved in the cell-to-cell communication under pathophysiological conditions, such as adenosine triphosphate (ATP), which targets microglial purinergic receptors (Basbaum et al., 2009; Tsuda, 2016). Moreover, ATP-evoked microglia induces the release of brain-derived neurotrophic

factor (BDNF). In turn, these BDNF downregulates the potassium-chloride transporter (KCC2), through tropomyosin receptor kinase B (TrkB) receptor and generates a depolarization of transmembrane anion gradient by increasing intracellular levels of chloride, and the shift of function of GABA and glycinergic interneurons on the dorsal horn neurons, which results in a disinhibition mechanism that increases pain (Coull et al., 2005; Maldonado et al., 2016; Tsuda, 2016) (Figure 12).

In addition, important adaptive changes also occur at different supraspinal levels. Plastic changes are increasingly reported in brain areas involved in emotional and cognitive aspects of neuropathic pain, including AMY, HIP, prefrontal cortex, or nucleus accumbens (NAc), among others (Maldonado et al., 2016; La Porta et al., 2016). Further, potent inhibitory mechanisms, such as opioid, cannabinoid, serotonergic and noradrenergic neurons constitute a descending inhibitory pathway that contribute to modulation of pain processing that also results impaired during neuropathic pain (Maldonado et al., 2016).

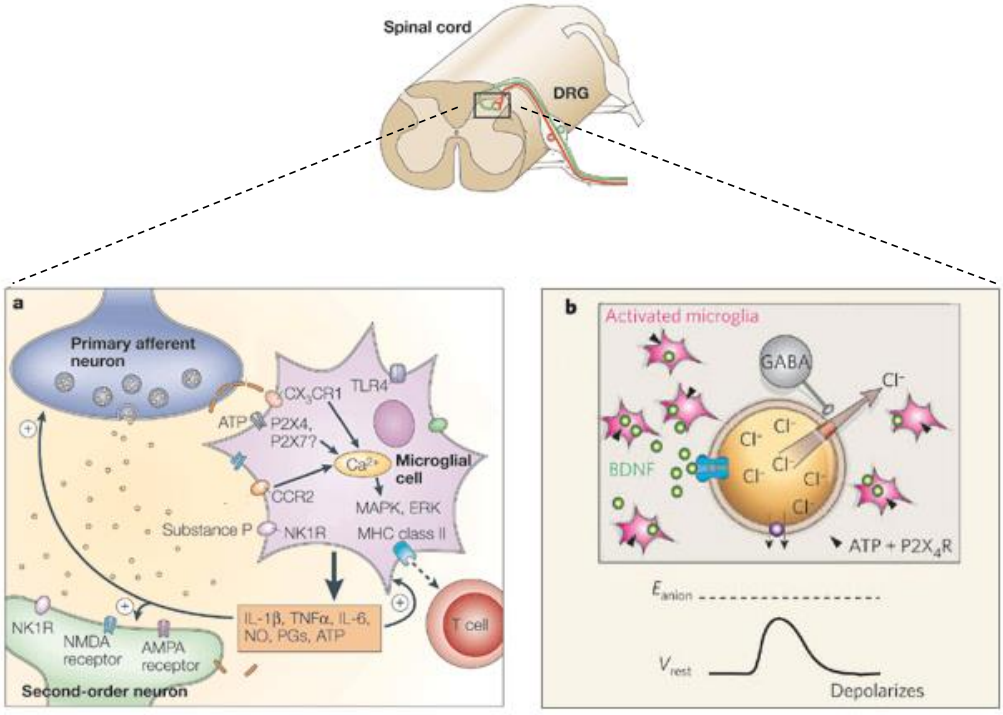

Figure 12. Central mechanisms during neuropathic pain. A) Increased neuronal activity and microglial activation by inflammatory mediators promote central sensitization. Damaged fibres contribute to increase excitability by the presynaptic release of neurotransmitters (glutamate and substance P), which modulate NMDA and NK1 receptors activity in second order neurons. B) Microglial activation induced by ATP produce the release of BDNF, which acts on TrkB receptor increasing intracellular levels of chloride. E_{anion} results now positive compared to V_{rest} producing a shift of GABA function that causes a disinhibition mechanism increasing pain (adapted from Marchand et al., 2005; Torsney and MacDermott, 2005).

c) Therapeutic approaches for neuropathic pain

The current management of neuropathic pain comprises pharmacological and nonpharmacological therapies that also include alternative medicine, behavioural therapy or invasive interventions, such as surgical treatments or spinal cord stimulation (Gilron and Dickenson, 2014; Maldonado et al., 2016). However, there is an important absent of effective treatment for palliating neuropathic pain. This difficulty might be the result of the inter-individual variability of neuropathic pain mechanisms and the coexisting emotional and cognitive components of chronic neuropathic pain (La Porta et al., 2016). Pharmacological treatment for neuropathic pain is reduced to tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors, anticonvulsants, cannabinoids and opioids. However, they present a modest efficacy and significant side effects (Maldonado et al., 2016; La Porta et al., 2014).

Opioids are effective and widely used in clinical management of chronic pain. However, opioid compounds are often discouraged in neuropathic pain due to its uncertain efficacy and its potential for

the development of tolerance and other important side effects. Different opioid receptor ligands have been demonstrated to exert high antinociceptive efficacy after nerve injury (Przewlocki and Przewlocka, 2005). Strong opioid analgesics such as levorphanol, morphine, fentanyl, oxycodone and tramadol are effective in different animal models of neuropathic pain (Desmeules, 2000; Przewlocki and Przewlocka, 2005; Rowbotham et al., 2003). In agreement, tramadol, among other opioid analgesics, are recommended as second-line treatment that can be considered for first-line use in certain clinical circumstances (Dworkin et al., 2010). However, further investigations and randomized controlled trials are necessary to support the use of opioids during neuropathic pain.

The anticonvulsant gabapentin was first successfully reported for neuropathic pain treatment twenty years ago (Mellick, 1995). Since then, gabapentinoids such as gabapentin and pregabalin has been also included as a crucial therapy for different neuropathies including neuropathic pain. Originally designed as derivatives of GABA, neither gabapentin nor pregabalin acts as a direct agonist on

$GABA_A$ or $GABA_B$ receptors. Both compounds prevent transmitter release through a direct inhibition of the voltage gated calcium channels (VGCCs). As pain stimuli ascend from DRG neurones to central terminals, gabapentinoid drugs have been demonstrated to inhibit the α2δ-1 calcium subunit of the VGCCs, producing an increase in descending serotonergic and noradrenergic facilitations and restoring the spontaneous activity in the AMY after neuropathic pain (Patel and Dickenson, 2016). Tricyclic antidepressants have also been used to reduce neuropathic pain, mainly amitriptyline (Kremer et al., 2016; Moore et al., 2015). Although their analgesic effects are independent of their antidepressant effect, these properties may be beneficial due to the emotional comorbidities associated to chronic neuropathic pain. The selective norepinephrine and serotonin reuptake inhibitor duloxetine is also efficacious in painful polyneuropathies and has been approved for neuropathic pain treatment (Baron et al., 2010). Indeed, calcium channel α2δ-1 ligands (i.e. gabapentin and pregabalin), tricyclic antidepressants, and dual reuptake inhibitors of serotonin and norepinephrine are recommended as first-line treatment options

on the basis of the results of randomized clinical trials (Dworkin et al., 2010).

The emergence of animal models in the lasts three decades provides an excellent tool to understand the pathophysiological mechanisms underlying neuropathic pain. Recent preclinical findings reveal the crucial role of the endocannabinoid system in the development and maintenance of neuropathic pain. These studies have reported a potential analgesic effect of cannabinoid agonists in different neuropathic pain models, and they have also identified specific targets in the endocannabinoid system to develop more effective and safe drugs (Maldonado et al., 2016). Orally administered cannabinoids such as cannabidiol, dronabinol, nabilone are the main cannabinoid compounds used with medical purposes. However, moderate evidence supports the use of these drugs in neuropathic pain. Clinical trials with oromucosal spray Sativex have been shown to generate beneficial effects in different chronic neuropathic pain models (Barnes, 2006; Hoggart et al., 2015; Nurmikko et al., 2007; Serpell et al., 2014).

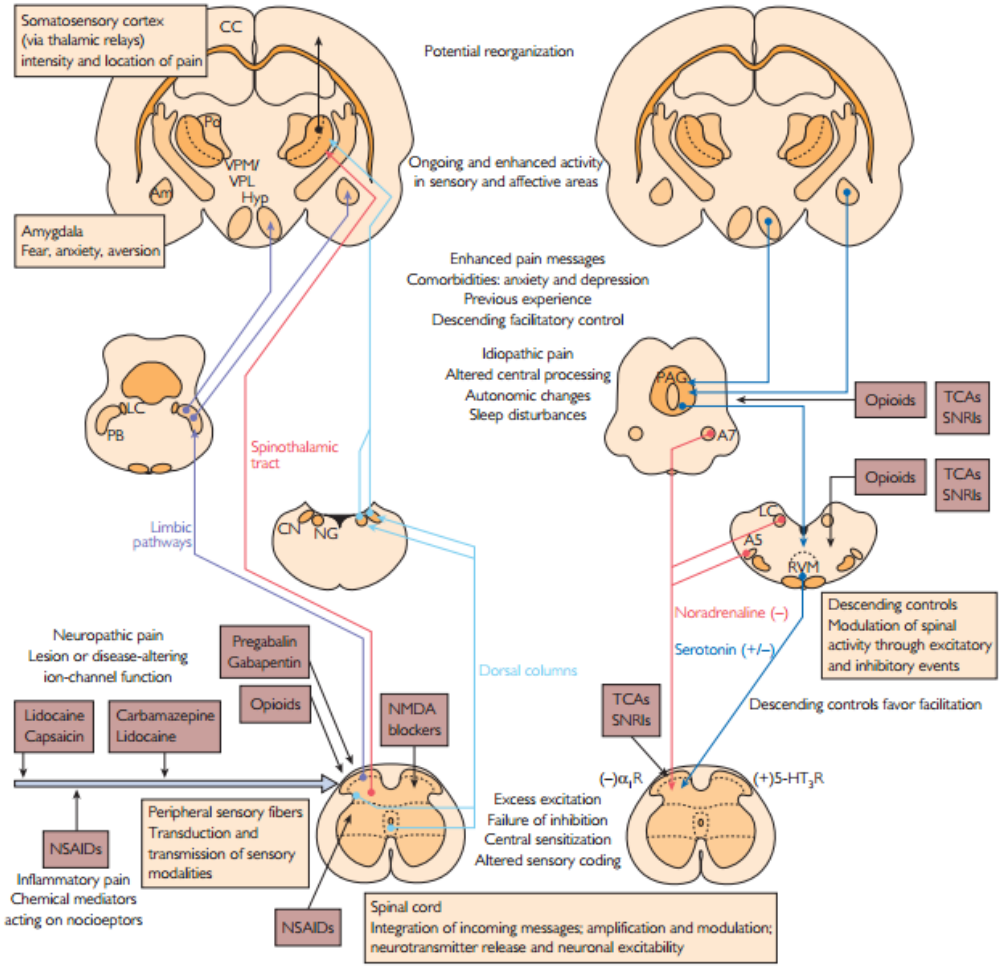

Figure 13. Principal sites for pharmacologic modulation of neuropathic pain. Ascending pathways are shown in the left. Descending mechanisms are shown in the right. Am, amygdala; CC, cerebral cortex; CN, cuneate nucleus; Hyp, hypothalamus; LC, locus coeruleus; NG, nucleus gracilis; NMDA, N-methyl-D-aspartate; NSAID, nonsteroidal anti-inflammatory drug; PAG, periaqueductal gray matter; PB, parabrachial nucleus; Po, posterior nuclei of the thalamus; RVM, rostroventral medial medulla; SNRI, serotonin-norepinephrine reuptake inhibitor; TCA, tricyclic antidepressant; VPM and VPL, ventrobasal thalamus, medial and lateral components (Gilron et al., 2015).

Further high-quality studies are needed to assess the impact of the duration of the treatment as well as the best form of drug delivery. (Boychuk et al., 2015). The large diversity of pharmacological targets investigated reveals the complexity of neuropathic pain. The multifactorial pathophysiological mechanisms are perhaps the greatest barrier to identify efficient targets for neuropathic pain (Figure 13).

1.7 Inter-individual variability in chronic pain

The different peripheral and central manifestations of chronic pain show a very high inter-individual variability that depends on multiple factors, including the different personality traits of the patients (Tang and Gibson, 2005). An advance in the knowledge of the mechanisms involved in the emotional and cognitive manifestations of chronic pain would allow to clarifying the personality traits responsible for this inter-individual variability and would help to develop efficient personalized treatments for chronic pain (Coghill et al., 2003; Elman et al., 2011). In the last years, a growing number of studies have used powerful human genetics approaches to better understand the biological basis of chronic pain (Ritter and Bingel, 2009). Genetic factors influence pain processing, and may cause remarkable personality differences in thresholds, tolerance to treatments and psychophysical ratings (Mogil, 2012; Nielsen et al., 2005). In animal models, different pain models (i.e. thermal, mechanical, chemical) are differentially affected by genetic variability (Mogil, 1999). In humans, studies performed with twin patients also showed a crucial role of genetic and environmental

factors, indicating that the genetic contribution to pain processing is up to 70 % in specific chronic pain conditions such as neuropathic and osteoarthritis pain (Hestbaek et al., 2004; Page et al., 2003).

Recent neuroimaging studies reveal the complex interaction between inter-individual genetics and pain processing in the brain. Indeed, functional magnetic resonance imaging (fMRI) studies have investigated the crosslink between genetic individual variability and opioid drugs effects, elucidating the neuropharmacological mechanisms that underlie the effects of these drugs on sensory affective context during pain experience (Oertel et al., 2008). Moreover, specific brain cortical regions such as the ACC, prefrontal cortex (PFC) and large portions of the somatosensory cortex have been demonstrated to reflect inter-individual differences in pain sensitivity (Coghill et al., 2003) (Figure 14). Other neuroimaging studies suggest that gender may also contribute to the inter-individual variability in central processing of chronic pain (Henderson et al., 2008). However, further investigations are needed to better understand the influence of personality traits

responsible for the inter-individual variability of chronic pain manifestations.

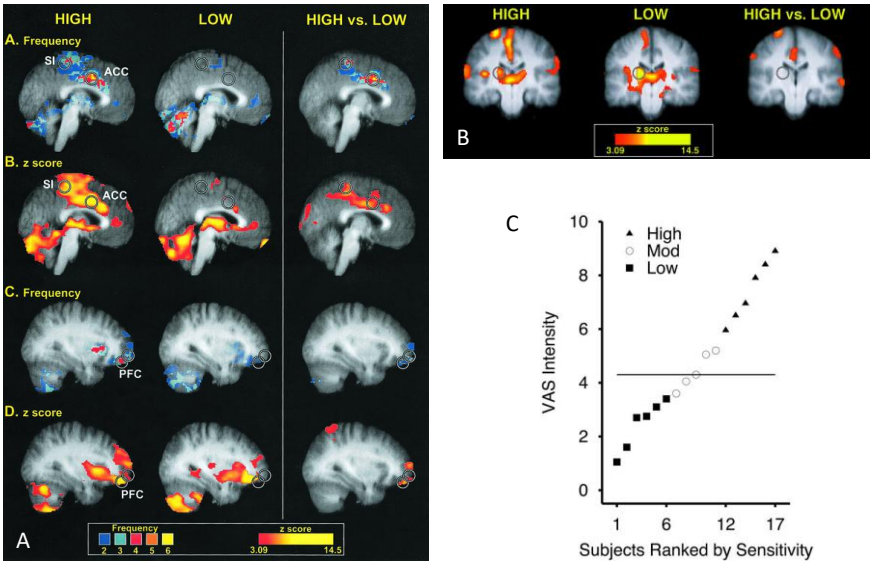

Figure 14. Inter-individual differences in the subjective experience of pain. A) Brain areas displaying different frequencies of activation between high- and low-subjective experience to pain. B) Thalamic activation produced by pain displayed no significant differences between high- and lo- sensitivity groups. C) Distribution of pain intensity ratings during fMRI analysis indicates inter-individual differences in during pain experience (Coghill et al., 2003).

2. Chronic pain manifestations

Pain is a complex experience that can widely diverge between people depending on the personality traits. Nociceptive, affective and cognitive components are common comorbidities of chronic pain that have a crucial influence on pain perception and difficult on pain therapy (Bushnell et al., 2013; Liu and Chen, 2014). Thus, chronic pain is frequently associated with emotional manifestations such anxiety and depression, impaired cognitive functions, including memory and reward sensitivity and motivation that could aggravate each other lessening the quality of life of patients (La Porta et al., 2016) (Figure 15). Indeed, a negative treatment expectancy reverse opioid agonist remifentanil analgesic effects (Bingel et al., 2011), whereas a positive expectation of pain relief plays a critical role on placebo efficacy (Benedetti et al., 2005).

2.1 Emotional manifestations

As pain becomes persistent and severe, patients may experience a difficulty to perform daily simple activities. This may lead to the loss of functional and autonomous capabilities, exerting a major

negative effect on the quality of life and increasing the risk of developing other medical comorbidities.

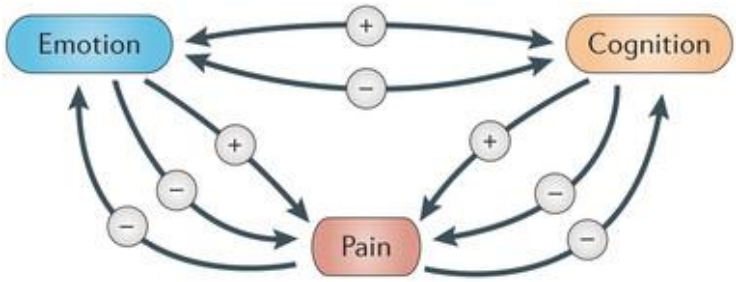

Figure 15. Interaction between pain, emotions and cognition. Pain may have a negative effect on emotions and on cognitive function. Conversely, a negative emotional state can lead to increased pain, whereas a positive state can reduce pain. Similarly, cognitive states such as attention and memory can either increase or decrease pain. Emotions and cognition can also reciprocally interact.

Chronic pain is normally accompanied by emotional disorders (Liu and Chen, 2014). Indeed, a recent study indicates that over 40% of a cohort of patients with lower limb osteoarthritis suffered from clinically significant anxiety or depression (Axford et al., 2010). Different clinical studies demonstrate that occurrence of depression is significantly higher than other mental disorders during chronic pain. Thus, patients with depression report long lasting and severe

pain experiences than non-depressed patients, and patients with chronic pain conditions show complex depressive symptoms (Liu and Chen, 2014). In agreement, neuroimaging studies demonstrate that patients with chronic pain have showed increased activity in brain regions involved in emotional processing pain associated to depressive manifestations (Peters, 2015).

Anxiety represents another emotional comorbid symptom and has also been reported to participate in chronification of pain. Different studies suggest that anxiety can directly or indirectly influence different brain regions involved in sensory and emotional pain manifestations. Indeed, anxiety plays a critical role in hippocampal formation (Ploghaus et al., 2001), and anxious patients show impaired prefrontal activity and connectivity patterns between the anterior insular cortex and the PAG, suggesting a less efficient pain modulation (Peters, 2015). In addition, corticotropin-releasing factor (CRF) has been implicated in the neurobiological basis of both anxiety and depression, and the AMY, closely related to the emotional dimension of pain, is a major site of expression of CRF and its receptors. Endogenous CRF receptor activation in the AMY

contributes to pain manifestations in different models of chronic pain. Indeed, electrophysiological studies showed an important role of endogenous CRF receptors to neuroplasticity in the AMY in a model of arthritic pain, although non-selective CRF receptor antagonist did not modify the emotional pain manifestations in a neuropathic pain model (Neugebauer, 2015).

Therefore, the growing number of studies in this field should encourage preclinical and clinical research to better characterize these pain-related comorbidities, and further investigate novel therapeutic approaches to treat these chronic pain manifestations.

2.2 Cognitive manifestations

Cognitive processing has been widely investigated in different chronic pain conditions. Dysfunctions in an extensive range of cognitive outputs such as attention, concentration, psychomotor activity, executive function, decision-making or memory have been largely reported during chronic pain experiences (Liu and Chen, 2014). Animal models of neuropathic and arthritic pain have been used to report cognitive alterations, although contradictory data

have been reported due to the large experimental variability including the use of different strains, pain models, time courses and lesion sites (Leite-Almeida et al., 2015). Studies in neuropathic pain patients show that nerve injury induces attention and social-recognition memory deficits. Similarly, these cognitive deficits have also been found in osteoarthritis patients (Moriarty and Finn, 2014; La Porta et al., 2015). Moreover, memory impairments have also been reported in a variety of diseases having chronic pain as a symptom, such as fibromyalgia (Glass et al., 2011).

Interestingly, neural substrates involved in cognitive and pain processing seem to be linked, and the two systems modulate each other reciprocally (Moriarty and Finn, 2014). Neuroimaging techniques have allowed to better understand the interaction between these two responses at neuroanatomical level. Indeed, an integrated neuromatrix participates in the processing of cognitive and nociceptive information. The ACC receives the signal from the limbic areas such as the AMY and the HIP, which control different cognitive functions, including attention or working memory, among others (Moriarty and Finn, 2014).

Moreover, electrophysiological and morphological studies confirm significant changes in the PFC firing activity and an overall decrease in the information transmission of the hippocampal pathway, as well as altered fronto-hippocampal circuit morphology after chronic pain (Moriarty and Finn, 2014; Oertel et al., 2008). In addition, patients suffering from chronic pain (i.e. lower back pain and fibromyalgia) have a significantly reduced grey matter volume than healthy controls, which is essentially associated with cognitive dysfunction (Moriarty et al., 2011; Moriarty and Finn, 2014).

Further investigations are required to better understand the neuroanatomical mechanisms involved in cognitive-pain related conditions. Cognitive modulation of pain may be an important approach to develop novel analgesic compounds to reduce these chronic pain manifestations.

3. The opioid system

3.1 A brief history of opioids

It is out of question to determine when opium poppy was first used by humans already. On the ninth century B.C., Homer wrote on The Odyssey a reference presumably related to opium use: "Presently she cast a drug into the wine of which they drank to lull all pain and anger and bring forgetfulness of every sorrow". The Ancient Greeks used the word opium (from "*opos*") as a word for vegetable juice. However, before that age, the Sumerians cultivated opium poppies and isolated opium from their seed at the end of the third millennium B.C. Their ideograph for poppies was "*hul gil*", the word for "plant of joy", and the Ebers Papyrus, one of the oldest treatise in medicine dating to 1500 B.C., includes the use of opium as a "remedy to prevent excessive crying of children". Beyond years, ancient traders spread opium to other places. From Asia Minor (modern-day Turkey) to India, China and finally to all parts of Europe (Figure 16).

A

B

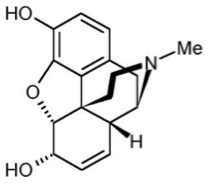

Figure 16. The History of opioids.

A) Method of extracting the juice from the poppy (adapted from Sala, 1618). B) The first isolated alkaloid, Morphine (Blakemore et al., 2002).

It was at the beginning of 1800 when Friedrich Wilhelm Adam Sertürner, a German pharmacologist, isolated the first alkaloid from the opium extract, and named it Morphine, by the God of dreams. The discovery and isolation of morphine brought Sertürner several honorary doctor's degrees from outstanding universities and even a prize of 2,000 francs as a "Benefactor of Humanity". In his later life, he suffered from gout and alleviated his pain with the morphine he had isolated.

After the invention of the hypodermic syringe and needles in the 1850s, Claude Bernard, a French physiologist who first performed blind experiments, used morphine in experimental animals. He found that morphine reduced the amount of chloroform to induce

anaesthesia. However, the abuse potential of morphine was extremely higher compared to opium and was not safe to use either. Consequently, in 1898, heroin was synthesized with the initial purpose to obtain a drug more potent than morphine and safer in terms of abuse liability. From then, the opiate bandwagon claims for novel opiates (Aggrawal, 1995; Brownstein, 1993).

In the decade of 1960s, it became apparent that those opiate compounds exert their actions at specific receptors. In 1971, a new radiobinding methodology was described to analyse the association between the morphine derivative levorphanol and the brain tissue, suggesting that those membrane fractions in brain homogenates were the opiate receptors (Goldstein et al., 1971). Few years later, three laboratories simultaneously succeeded in demonstrating the existence of opioid binding sites in the CNS (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). With those discoveries, people fortunately reasoned that the opioid receptors might be the binding sites of endogenous neurotransmitters. Two years later, endogenous opioid compounds that bind to opioid receptors were discovered. Enkephalins were isolated from guinea pigs brain

extracts and identified based on the determination of the amino acid sequence (Hughes et al., 1975; Kosterlitz and Waterfield, 1975). One year later, endorphin, a second endogenous opioid peptide derivative from enkephalin sequence was isolated (Bradbury et al., 1976). Finally, the full primary structure of the potent opioid peptide dynorphin was determined (Goldstein et al., 1981). The demonstration of the existence of both, the opioid receptors and the endogenous opioid ligands, was the first step to identify the endogenous opioid system.

3.2 Endogenous opioid system

Opioid receptors, their endogenous peptide ligands and the enzymes involved in their metabolism comprise the endogenous opioid system. This system is extensively distributed through the CNS and peripheral tissues. The wide distribution of the endogenous opioid system is determinant to its involvement in multiple physiological responses including control of pain, emotional behaviour, learning and memory functions or regulation of reward circuitry, among others (Bodnar, 2015). During the mid-

1990s, the molecular characterization and cloning of the different opioid receptors widely improved the knowledge and advances in this system (Kieffer, 1995).

a. Opioid receptors

Three opioid receptors have been identified and cloned in experimental animals and humans (Kieffer, 1999): μ (mu opioid receptor, MOR), δ (delta opioid receptor, DOR) and κ (kappa opioid receptor, KOR). Recently, a non-classical opioid receptor, the nociceptin / orphanin (NOP) or orphanin-receptor like 1 (ORL-1) is also accepted to be part of the opioid receptors family (Bodnar, 2015).

Opioid receptors belong to the superfamily G protein-coupled receptor (GPCR). They present a structure of seven transmembrane domains with an extracellular N-terminal domain and an intracellular C-terminal domain. Opioid receptors present a high homology in the sequence identity in both, transmembrane (73%-76%) and intracellular domains (63%-66%), while it is reported a

large divergence in the extracellular N- and C- domains (34%-40%) identity (Pogozheva et al., 2005) (Figure 17).

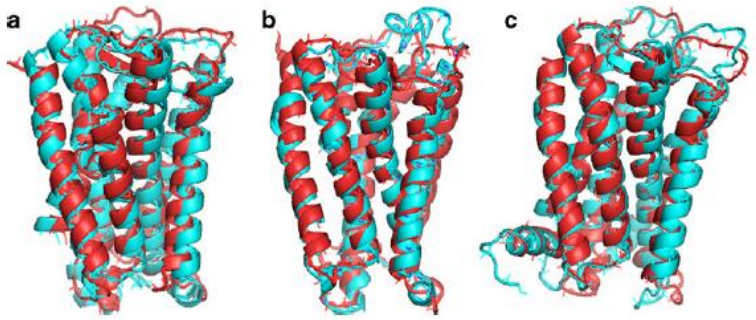

Figure 17. Superimposition of homology models based on single templates from transmembrane 1 to transmembrane 7. a) KOR, b) DOR, c) MOR. (Bera et al., 2011).

Opioid receptors mainly interact with the pertussis toxin (PTX) sensitive G α-subunit of the G_i and G_o proteins. Activation of opioid receptors leads to the split of the G protein into two different subunits, G_α and $G_{\beta\gamma}$ that activates an intracellular second-messenger cascade. The $G_{\beta\gamma}$ subunit activates neuronal K^+ channels and inhibits high threshold voltage activated Ca^{2+} channels, leading to inhibition of neurotransmitter release and reduction excitability. In turn, the G_α subunit inhibits the adenylyl cyclase (AC) and reduces

the adenosine monophosphate (cAMP), leading to a reduction of the neuronal excitability (Law et al., 2000). Moreover, opioid receptors also regulate the expression of second messengers, such as the mitogen-activated protein (MAP) kinase, which is activated by the $G_{\beta\gamma}$ subunit activity (Williams et al., 2001). As a result, the activation of opioid receptors produces a hyperpolarization of the membrane and a reduction in neuronal activity, mediating inhibitory actions (Henriksen and Willoch, 2008; Law et al., 2000) (Figure 18).

Autoradiography anatomical studies indicates that opioid receptors are widely distributed throughout central and peripheral system (Mansour et al., 1988; Stein, 1993). Although there is overlap in the localization of the different opioid receptors, the precise anatomical distributions of MOR, DOR and KOR is clear.

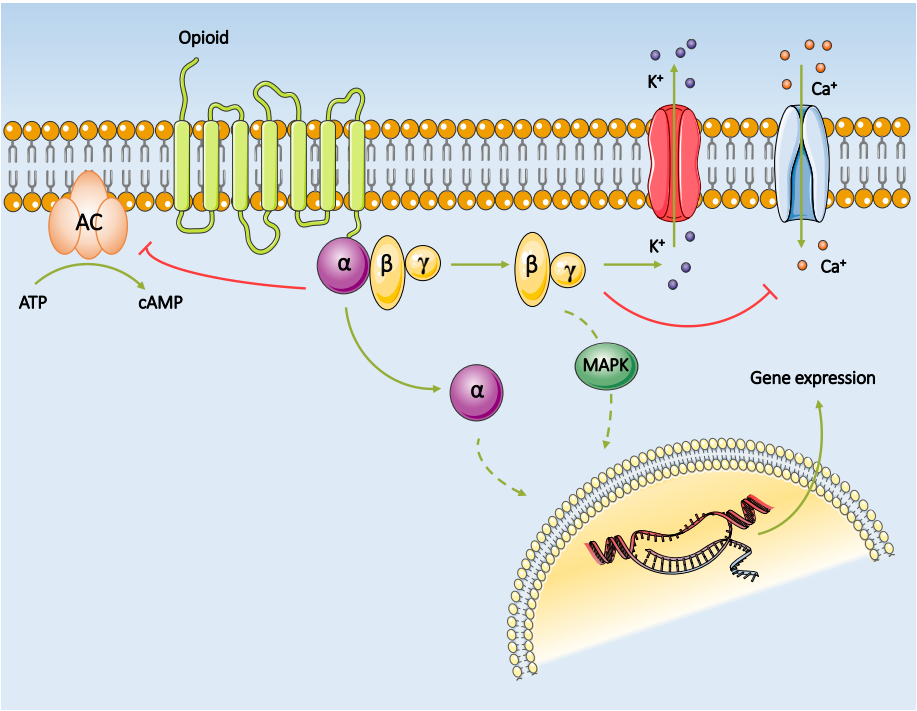

Figure 18. Schematic representation of opioid receptor signalling. Activation of the receptor separate G_{α} and $G_{\beta\gamma}$ subunits. $G_{\beta\gamma}$ activates and inhibits the K^{+} and Ca^{2+}channels, respectively, reducing neuron excitability. Moreover, $G_{\beta\gamma}$ is involved in the activation of second messengers such as MAP kinases. Concurrently, G_{α} subunit inhibits AC and reduces cAMP. Together, activation of opioid receptor leads to the reduction of neuronal activity.

In the CNS, the MOR present an extensive distribution in the nociceptive, motor and motivation related structures in the CNS. It is expressed at high levels in the neocortex, caudate-putamen, PAG, septum, thalamus, HIP, substantia nigra ventral tegmental area, raphe nuclei, striatum, locus coeruleus and the primary sensory afferents of the spinal cord. The distribution of DOR is more restricted than MOR, and it is mainly distributed throughout the forebrain structures, such as the olfactory bulbs, striatum, caudate-putamen and AMY as well as in the lamina I of the spinal dorsal horn. KOR is widely localized in the preoptic area, hypothalamus, striatum, tractus solitarius, ventral tegmental area, raphe nuclei substantia nigra, PAG, AMY, HIP and in the substantia gelatinosa of the spinal cord (Mansour et al., 1988) (Figure 19).

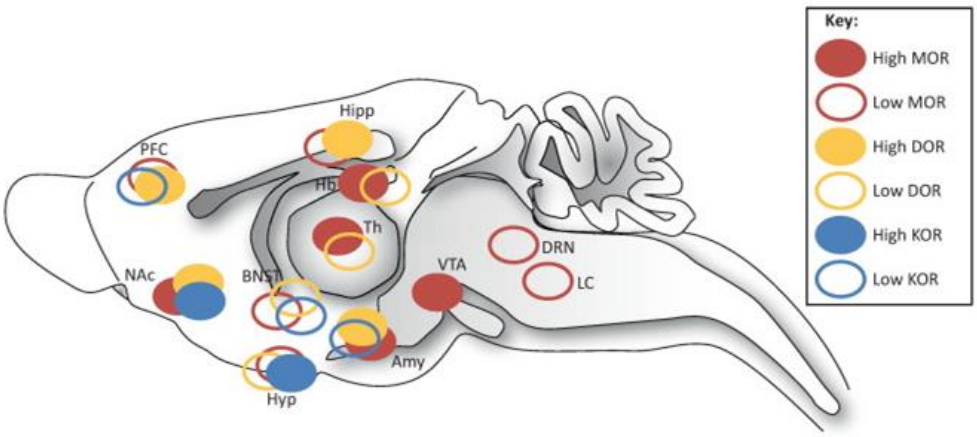

Figure 19. Anatomical distribution of opioid receptors. Opioid receptors overlap their localization throughout rodent brain. PFC, prefrontal cortex; NAc, nucleus accumbens; BNST, bed nucleus of the stria terminalis; Hyp, hypothalamus; Hipp, hippocampus; Hb, habenula; Th, thalamus; Amy, amygdala; VTA, ventral tegmental area; DRN, dorsal raphe nucleus; LC, locus coeruleus (Lutz and Kieffer, 2013).

In the peripheral nervous system, opioid receptors have also been found in various tissues including immune cells, gastrointestinal system, dermis and epidermis, around hair follicles, bone, joint tissue and in dental pulp (Bigliardi and Bigliardi-Qi, 2014). They are located in sensory and sympathetic fibres in those tissues where they modulate different physiological effects (Mansour et al., 1988; Przewłocki and Przewłocka, 2001).

b. Endogenous opioid ligands

Three families of endogenous opioid peptides derived from their precursor proteins proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) have been identified and cloned. These three proteins generate several active peptides, including β-endorphin, met- and leu-enkephalins, dynorphins and neo-endorphins, respectively (Kieffer and Gavériaux-Ruff, 2002). These endogenous ligands exhibit different affinities for each opioid receptor (Table 1), whereas all the opioid peptide present an N-terminal sequence (Tyr-Gly-Gly-PheMet-Leu) indispensable to activate opioid receptors (Akil et al., 1997).

Endogenous ligands	MOR	DOR	KOR
β-endorphin	+++	+++	-
Leu-enkephalin	+	+++	-
Met-enkephalin	++	+++	-
Dynorphins	++	+	+++

Table 1. Selectivity of endogenous opioid ligands for opioid receptors. Increased selectivity is represented from -, (no selectivity) to +++, (high selectivity) (adapted from (Kieffer, 1995).

3.3 Enzymes in the catabolism of opioids

Opioid peptides catabolism results in the production of metabolites. Once released into the synaptic cleft, opioid ligands are metabolized by two zinc metallopeptidases, the endopeptidase neprilysin and the aminopeptidase N, that catalyses the cleavage of peptide bonds on the N-terminal side of Tyr-Gly-Gly and Tyr residues, respectively (Roques et al., 2012) (Figure 20). The distribution of both enzymes coincide with the same brain areas of the opioid receptors expression. Aminopeptidase N is distributed throughout the cerebral cortex, the caudate, and moderately expressed in the hippocampus, and neprylisin distribution coincides with that of the MOR and DOR (de Gortari et al., 2007). In situ hybridization studies reported that neprylisin is mainly expressed in the hippocampus, cerebral cortex, caudate nucleus, substantia nigra and the NAc, among others (Gaudoux et al., 1993)

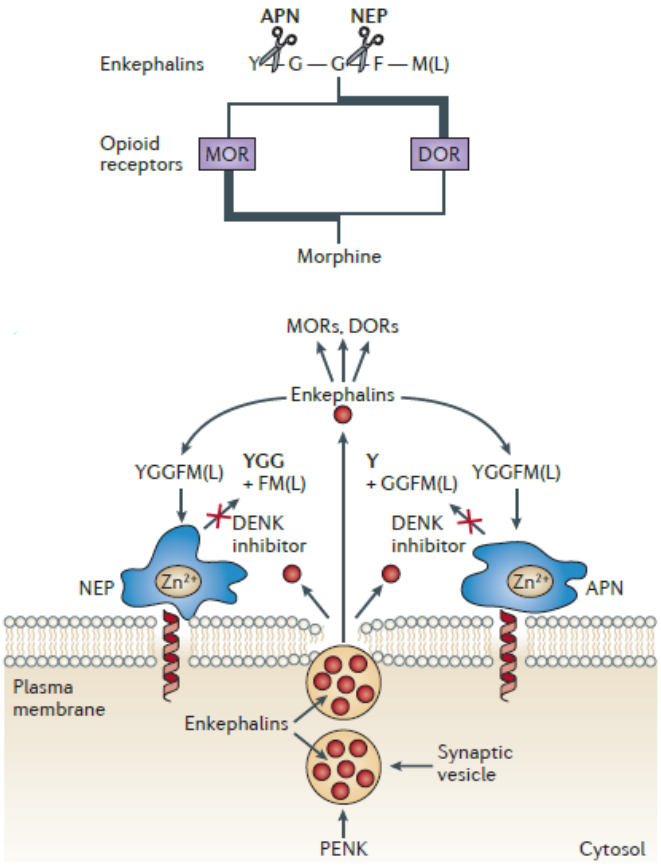

Figure 20. Schematic representation of the endogenous opioid catabolism. The arrows indicate the sites of the opioid peptide cleavage by aminopeptidase N (APN) and neprylisin (NEP) of the peptide bonds on the N-terminal side Tyr-Gly-Gly and Tyr, respectively. In this case, enkephalins are synthesized intracellularly from enzymatic processing of the gene-derived precursor preproenkephalin (PENK). Stored in large synaptic vesicles, they are released (under basal or phasic conditions) by a Ca2+-dependent exocytosis mechanism. Outside the cells, enkephalins interact with opioid receptors, and their signal is interrupted by the concomitant action of NEP and APN that generate inactive metabolites (Roques et al., 2012).

3.4 Endogenous opioid system functions

The study of the broad role of the endogenous opioid system in the control of multiple physiological responses has been the subject of a vast number of investigations for decades. The control of pain is probably the most well studied physiological function of the endogenous opioid system. However, this system is involved in a wide range of functions related to behaviour, such as reward and addiction, stress and social status, learning and memory, mental and mood disorders, food intake, gastrointestinal, respiratory, cardiovascular and immunological responses, among others (Bodnar, 2015). For the aim of this Thesis, we will focus our attention on the role of the endogenous opioid system in pain and mood disorders and memory.

a. Endogenous opioid system and pain

The endogenous opioid system controls nociceptive responses at peripheral, spinal and supraspinal levels.

At the peripheral level, endogenous opioid peptides are produced and released by leucocytes upon inflammatory stimulation (Lesniak

and Lipkowski, 2011). Opioid ligands are synthesized in the DRG, where chemokines released from leucocytes and endothelial cells increase the avidity of adhesion molecules and migration through the neuronal axons to peripheral nerve terminals producing analgesic actions. This chemokine signalling contribute to the peripherally mediated endogenous opioid antinociception (Rittner et al., 2008) (Figure 21).

Neuropathic pain resulting from nerve injury is another condition that involves the endogenous opioid system at the peripheral level. Opioid receptor expression was upregulated in peripheral sensory neurons in different models of neuropathic pain (Lesniak and Lipkowski, 2011). In addition, endogenous opioid ligands suppress TRPV1 and other nonselective cation currents, which may account for the efficacy of peripheral opioids in both inflammatory and neuropathic pain conditions (Stein and Baerwald, 2014).

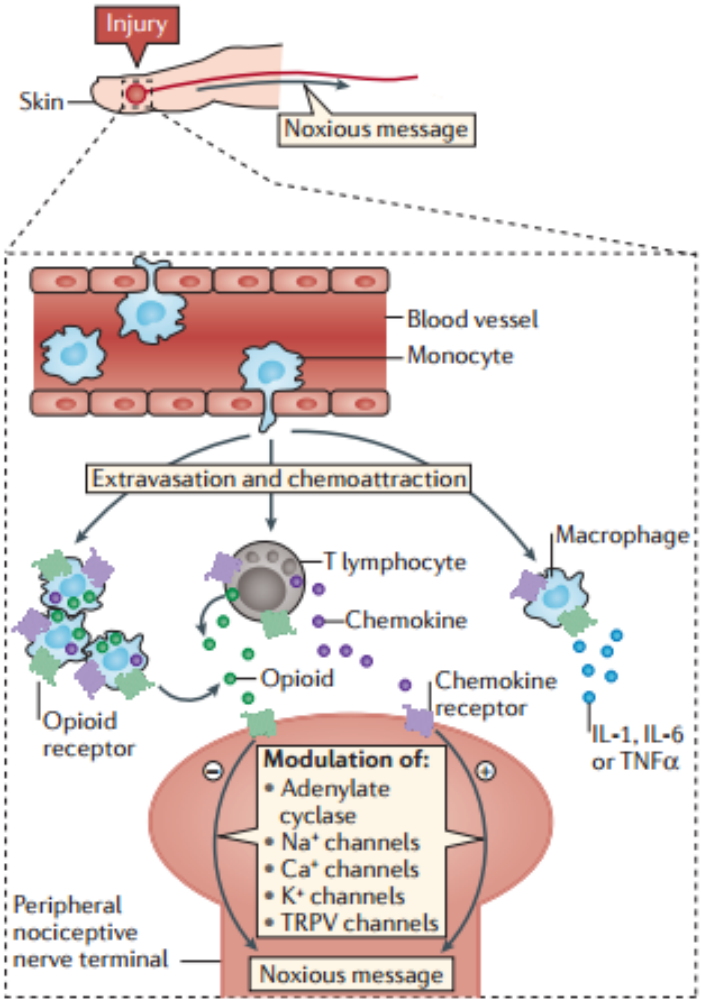

Figure 21. Endogenous opioid peptides in peripheral nociception. After peripheral injury, immune cells migrate from blood vessels to inflamed tissues by extravasation and chemotaxis processes controlled by chemokines. Immune cells release pro-inflammatory cytokines chemokines that initiate and maintain the noxious message. Opioid peptides secreted by immune cells promote an antinociceptive effect through the activation of their peripheral receptors (adapted from Mélik Parsadaniantz et al., 2015).

At the spinal level, the endogenous opioid system inhibits the nociceptive transmission conveyed of Aδ and C fibres at the level of the dorsal horn. The opioid receptors are expressed presynaptically in the terminals of these fibres and modulate the release of excitatory molecules, such as substance P and glutamate (Przewłocki and Przewłocka, 2001) (Figure 22). The endogenous peptides levels are dramatically increased in the dorsal horn of the spinal cord in both acute and chronic pain conditions (Iadarola et al., 1986; Noguchi et al., 1989; Przewłocki and Przewłocka, 2001).

The endogenous opioid system has also been reported to play an important role in the spinal cord in the control of both inflammatory and neuropathic pain. Chronic inflammatory pain increases the spinal levels of different endogenous opioid ligands. Indeed, dynorphin peptides and the PDYN mRNA levels were increased in the lumbar part of the spinal cord after chronic inflammation in rats (Iadarola et al., 1986).

The endogenous opioid system also plays a role in the spinal cord in the control of neuropathic pain. Elevated levels of dynorphins in the superficial laminae I and II, as well as in deeper laminae V-VII have

been shown after nerve injury in rats (Przewlocki and Przewlocka, 2005). An increased Met enkephalin immunoreactivity was also observed in the spinal cord during the late phase of chronic constriction injury of sciatic nerve in rats (Sommer and Myers, 1995).

At the supraspinal level, the opioid receptors and peptides are expressed in different brain areas involved on the control of pain, such as the thalamus, hypothalamus, limbic system, cerebral cortex, PAG, locus coeruleus and nucles raphe magnus (Kanjhan, 1995). The endogenous opioid system inhibits the nociceptive transmission of the ascendant pathway that innervates the thalamus that produces and inhibits the vegetative component of pain in the hypothalamus (Flórez, 2007). In addition, the endogenous opioid system is also abundant in the limbic system, where it mediates the emotional response to pain (Przewłocki and Przewłocka, 2001).

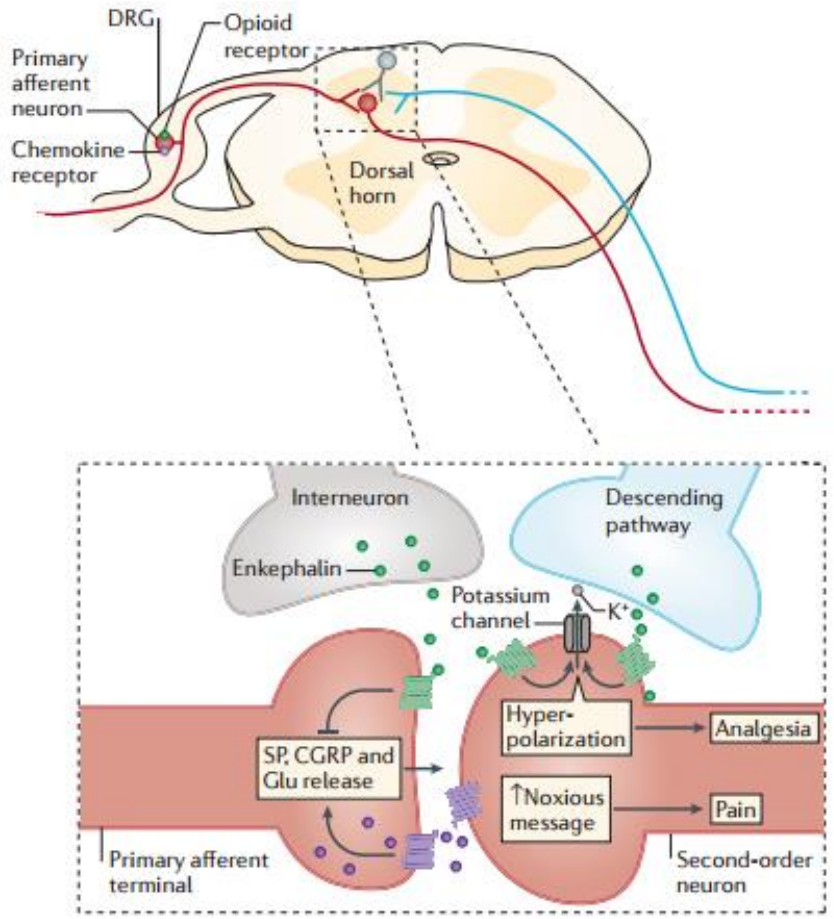

Figure 22. Potential sites of action of endogenous opioid system at the spinal level.

The endogenous opioid system mediates analgesia by the inhibition of substance P, glutamate and by the hyperpolarization of spinal neurons (adapted from Mélik Parsadaniantz et al., 2015).

Furthermore, the endogenous opioid system controls the thalamic projections to the cerebral cortex, where the painful information is integrated and defined in terms of intensity, localization and sensation (Flórez, 2007).

It has been reported an increase of endogenous opioid ligands in the thalamus and hypothalamus during chronic inflammatory pain. Indeed, β endorphin levels were increased at early time points after induction of inflammation in the ventro-medial hypothalamus, ventro-basal thalamus and the PAG in rats (Porro et al., 1991). The endogenous opioid system also plays an important role at supraspinal level in neuropathic pain. PDYN-positive neurons are present through the limbic system after nerve injury, and neurons expressing POMC are also present in the hypothalamus. Thus, the activation of these circuits may evoke analgesia in rats (Przewlocki and Przewlocka, 2005) (Figure 23).

In addition to the ascending pain pathway, the well-characterized descending PAG-RVM analgesic mechanism is also closely related to the physiological role of the endogenous opioid system in the control of pain.

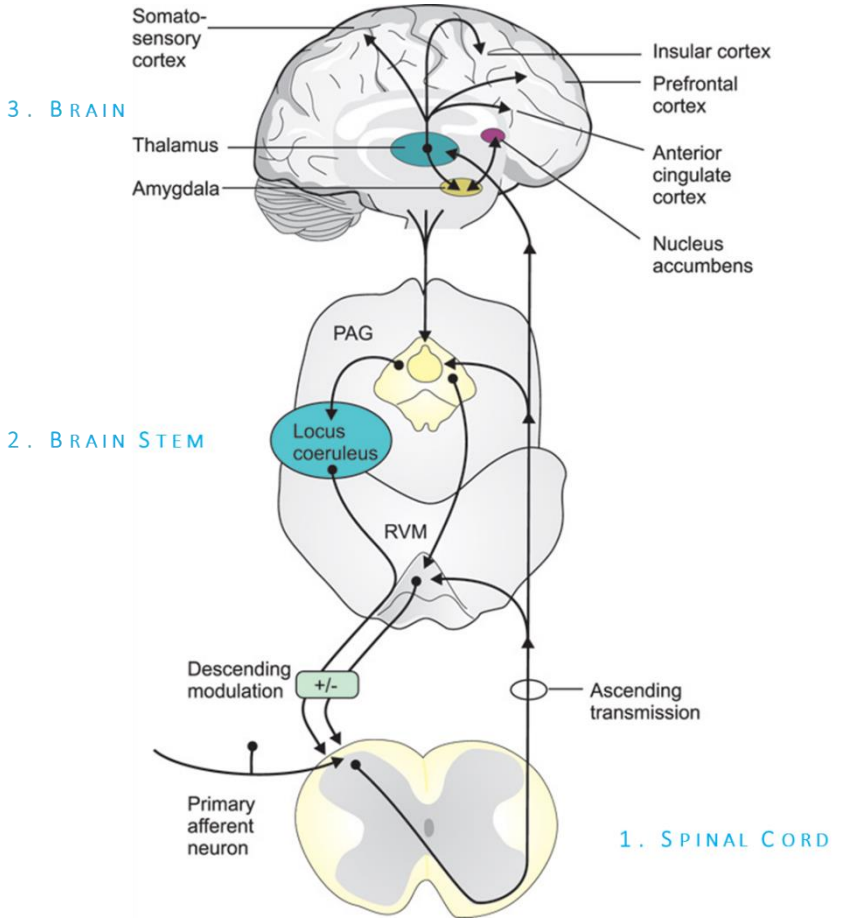

Figure 23. Potential sites of action of endogenous opioid system at the supraspinal level. 1) The endogenous opioids system produces the inhibition of pain transmission in the ascending pathway through the spinal cord to the brain stem. 2) In the brain stem, the activation of the PAG-RVM descending pathway and the projection of the locus coeruleus to the dorsal horn is also modulated by the endogenous opioid system. 3) In the brain, the endogenous opioid system mediates the perception and significance of pain through the projections from the thalamus to the high centres in the limbic system and the somatosensory cortex (adapted from Brodin et al., 2016).

This descending pathway is involved in the analgesic actions of opioids, and it is the major site of the stress-induced analgesia, mediated by the endogenous opioid system.

As stated in section 1.4, the GABA disinhibition promoted by the endogenous opioid system is the main mechanism involved in the opioid analgesia mediated by the descending PAG-RVM pathway. Accordingly, GABAergic interneurons are present in PAG and RVM, which release GABA and in turn produce the inhibition of the output neurons. Opioid peptides activate the PAG-RVM mechanisms inhibiting those GABAergic interneurons and thereby produce a shift in the GABA effects from inhibition to disinhibition of the antinociceptive descending pathway to the spinal cord during acute pain (Lau and Vaughan, 2014). In addition, noradrenergic terminals that depart from the locus coeruleus and project into the dorsal horn are also regulated by the endogenous opioid system (Flórez, 2007) (Figure 24).

During chronic pain, it has been shown that an upregulation of the endogenous opioid peptides expression during neuropathic pain might be a consequence of the descending activation of RVM

neurons (Gardell et al., 2003; Lau and Vaughan, 2014). Thus, enkephalin-positive neurons in the PAG synapse with raphe magnus nucleus that project to the dorsal horn of the spinal cord.

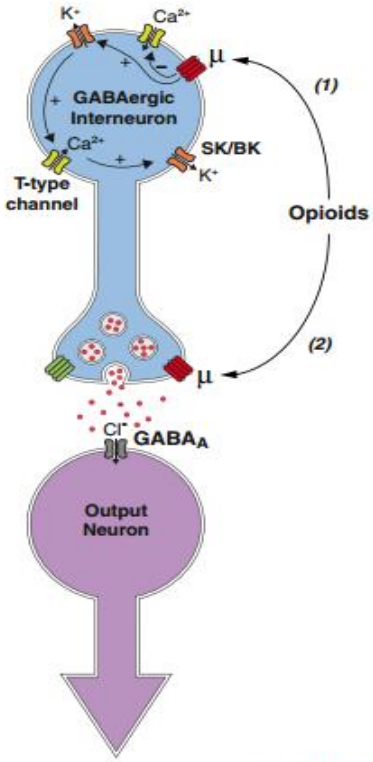

Figure 24. Cellular descending mechanisms underlying opioid disinhibition. (1) Opioids act through postsynaptic opioid receptors to directly inhibit GABAergic interneurons. (2) Opioids act via presynaptic opioid receptors to inhibit neurotransmitter release from GABAergic neurons. Raphe magnus nucleus and locus coeruleus also project to the dorsal horn thorugh endogenous opioid system control. Together, presynaptic and postsynaptic inhibition leads to the excitability of descending output neurons (adapted from Lau and Vaughan, 2014).

Besides the antinociceptive effects of endogenous opioid ligands, they have also been related to pronociceptive actions in specific situations involving the activation of NMDA receptor by dynorphins that produces excitatory nociceptive responses and toxic effects

(Laughlin et al., 2001). Indeed, some of the increased spinal dynorphins actions during pain are blocked by the NMDA antagonist dizocilpine, but not by the general opioid antagonist naloxone, indicating an interaction with these receptors (Wang et al., 2001).

b. Endogenous opioid system and mood disorders

Affective disorders, such as depression and anxiety are a worldwide leading cause of disability recognized in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Association Psychiatric, 2013). The high density of endogenous opioid peptides in different mood-regulating brain areas defines this system as a crucial contributor in mood control.

Observational studies show that treatment with MOR agonist has been associated with significant mood elevation during depression (Lutz and Kieffer, 2013). Pharmacological activation of MOR produces euphoria in humans and antidepressive-like effects and reinforcement in animal models (Lalanne et al., 2014). However, clinical evidence point to the implication of MOR in prodepressive states (Berrocoso et al., 2009). These results remain difficult to

evaluate as a result of the potent abusive liability of MOR agonist (Lutz and Kieffer, 2013).

The anxiolytic- and antidepressive-like activity of DOR activation is also well documented. Thus, exogenous administration of enkephalins and β endorphin produces antidepressant effects in animal models of depression (Peppin and Raffa, 2015). In addition, inhibitors of enkephalins catabolism (Tejedor-Real et al., 1993). Moreover, DOR selective agonists, such as SNC-80, BU-48 and AZD2327 among others, have been shown to produce antidepressant-like responses in animal models (Hudzik et al., 2011; Peppin and Raffa, 2015; Shimazoe et al., 1987). Moreover, a recent pilot study showed promising anxiolytic effects of a selective DOR agonist AZD2327 in patients with anxious major depressive disorder (Richards et al., 2016).

Interestingly, it has been suggested that the effects of tricyclic antidepressants on neuropathic pain require DOR stimulation in mice (Peppin and Raffa, 2015).

Pharmacological activation of KOR produces dysphoria, anxiety and psychotomimetic effects in humans. Increasing evidences indicate

that both KOR agonists and intra-amygdala injection of dynorphin A produce anxiogenic-like responses in rodents (Narita et al., 2006). However, salvinorin A, a preferential KOR agonist, and the selective KOR agonist U50488 increased the time in the open arms in the elevated plus maze test in mice (Kudryavtseva et al., 2004). Moreover, the KOR antagonist norbinaltorphimine showed anxiolytic-like effects in different animal models of anxiety (Hang et al., 2015). Discrepancies among these studies may be due to the different experimental paradigms, test conditions, animal strains, and laboratory specific basal stress levels.

In addition to pharmacological studies, constitutive knockout (KO) mice for MOR, DOR and KOR were generated and characterized several years ago (Kieffer, 1995), and have been excellent tools to better understand the physiological role of the endogenous opioid system.

The deletion of MOR in mice produced an increase in the time spent and the percentage of visits to the open arms of the elevated plus maze, and longer times and higher number of entries in the illuminated compartment of the light-dark box, indicating anxiolytic-

like responses in both cases (Filliol et al., 2000). Accordingly, the forced swim test also indicated that MOR-KO mice decreased the time of immobility, suggesting that the blocking of MOR contributes to the establishment of antidepressive-like effects in mice (Filliol et al., 2000).

Mice deficient in DOR exhibit opposing affective responses than MOR-KO mice. DOR-KO mice spent less time in the open arms and a decreased percentage of entries to the open arms in the elevated plus maze model of anxiety (Filliol et al., 2000). In addition, mice lacking DOR showed aversive behavioural in the light-dark box, suggesting that the activity of DOR may contribute to diminish anxiety-like responses. This is also in accordance with the anxiety-like behaviour revealed in mice lacking preproenkephalin gene (Peppin and Raffa, 2015). Moreover, DOR-KO animals also showed increased immobility time in the forced swim test, suggesting that the blocked DOR may contribute to the development of depressive-like behaviour (Filliol et al., 2000; Lutz and Kieffer, 2013).

Finally the deletion of KOR and PDYN gene produces anxiolytic and antidepressive-like effects in rodents (Hang et al., 2015). However,

deletion of the components of the dynorphin/KOR system has also been reported to increase anxiety-like responses in mice (Bilkei-Gorzo et al., 2008; Femenía et al., 2011). These differences between studies may be due to the different genetic constructs for generating mutant mice, test conditions and experimental paradigms.

Taken together, the three opioid receptors widely differ in the control of emotional processes (Table 2).

Opioid Receptor	Behavioural Phenotypes
MOR	• Euphoria / Reinforcement • Contradictory emotional responses
DOR	• Anxiolytic-like effects • Antidepressive-like effects
KOR	• Dysphoria • Anxiogenic-like effects • Prodepressive-like effects

Table 2. Opioid receptors phenotypes. Different behavioural effects are produced on the modulation of mood by the activation of opioid receptors.

c. Endogenous opioid system and cognition

The role of the endogenous opioid system in learning and memory is well documented. Systemic pharmacological activation of MOR has been reported to produce learning and memory impairments in rodents (Sala et al., 1994). Since clinical studies have also shown that opioid addicts may have significant cognitive impairments with the duration of the addiction (Curran et al., 2001), several lines of evidences suggest that molecular and synaptic plasticity changes in the hippocampus are also modulated by endogenous opioids (Dacher and Nugent, 2011).

DOR have also been shown to play an essential role in learning and memory processes. They are highly expressed in brain regions involved in cognitive functions, such as hippocampus and amygdala (Klenowski et al., 2015). Activation of DOR inhibited the presynaptic neurotransmitter release and increased excitation of pyramidal cells in the CA1, CA3 and dentate, leading to the facilitation of the long term potentiation in the hippocampus (Klenowski et al., 2015). Accordingly, mice lacking DOR display impaired performance in learning tasks (Le Merrer et al., 2013).

Finally, the involvement of KOR in memory function is also reported. Both KOR and its endogenous ligand dynorphin are present in the hippocampus and amygdala (Schwarzer, 2009). When released, dynorphins modulate the information between the dentate gyrus and the CA3 region of the hippocampus decreasing excitatory glutamatergic signalling and therefore diminishing hippocampal activity (Bilkei-Gorzo et al., 2014). In addition, pharmacological KOR activation produces aversive emotional behaviours that contribute to the stress-induced learning and memory dysfunctions in mice (Carey et al., 2009). Interestingly, a human genetic study also revealed that subjects with a rare gene polymorphism associated with reduced PDYN expression is associated with a better episodic memory (Kölsch et al., 2009). Accordingly, genetic ablation of PDYN gene enhanced social memory in mice, and this recognition ability was reduced after pharmacological activation of KOR (Bilkei-Gorzo et al., 2014).

3.5 The dynorphin/KOR system in chronic pain

Although the role of MOR and DOR in the control of pain has been widely investigated in the past, recent investigations have focussed a particular attention in the dynorphin/KOR system. For the aim of this Thesis, we will closely analyse the involvement of the dynorphin/KOR system on the nociceptive, affective and cognitive manifestations of chronic pain (Figure 25).

a) Dynorphin/KOR system in the nociceptive manifestations of chronic pain

KOR activation produces analgesia (Cahill et al., 2014) and this receptor is involved in chronic pain states, including neuropathic pain and osteoarthritis chronic pain. Functional KORs are expressed in human fibroblasts-like synoviocytes, which may contribute to opioid-induced antinociceptive effects during osteoarthritis (Shen et al., 2005). Indeed, a reduction of KOR expression at both mRNA and protein levels has been shown in patients with osteoarthritis and rheumatoid arthritis compared to healthy volunteers (Shen et al., 2005).

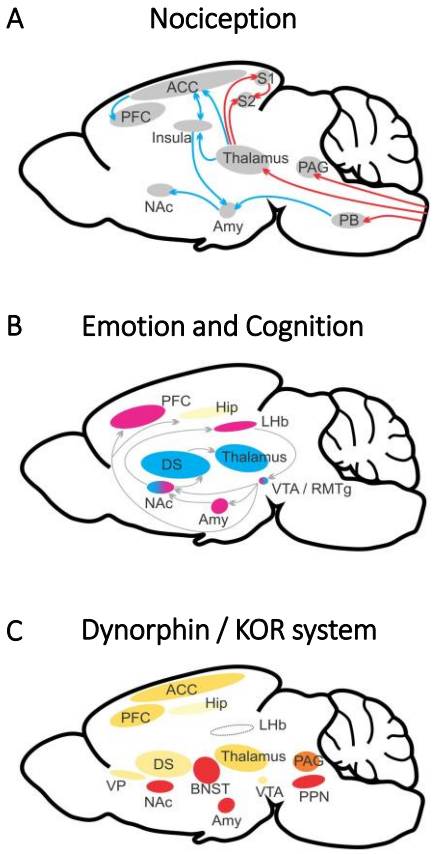

Figure 25. Schematic illustration of the localization of the dynorphin/KOR system and the major brain structures involved in the nociceptive, emotional and cognitive components of chronic pain. A) Nociceptive information conveys to multiple brain areas related to pain processing, including these regions related to sensory (red arrows) and affective (blue arrows) dimensions of chronic pain. B) Emotional and cognitive components involve multiple central structures that overlap and interact with different brain areas related to nociceptive processing. C) Dynorphin/KOR system expression in these relevant structures related to the

nociceptive, emotional and cognitive components of chronic pain. ACC, anterior cingulate cortex; Amy, amygdala; BST, bed nucleus of the stria terminalis; DS, dorsal striatum; LHb, lateral habenula; NAc, nucleus accumbens; OFC, orbitofrontal cortex; PAG, periaqueductal gray; PB, parabrachial nucleus; PFC, prefrontal cortex; PPN, pedunculopontine nucleus/pedunculopontine tegmental nucleus; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; VP, ventral pallidum; VTA, ventral tegmental area (adapted from Cahill et al., 2014).

Moreover, the increased levels of different proinflammatory mediators in arthritis, such as TNFα and IL-1β contribute to the suppression of the KOR mRNA expression (Shen et al., 2005). These data suggest that the reduction of KOR might be critical in the progression and maintenance of the osteoarthritis disease. In addition, pharmacological evidences demonstrate that selective KOR agonists attenuate mechanical allodynia and inflammation during osteoarthritis, and peripheral KOR agonists present the ability to restore the KOR expression in the fibroblasts-like synoviocytes of patients with osteoarthritis (Shen et al., 2005).

Several studies have also revealed the role of KOR-induced analgesia in neuropathic pain. Activation of KOR produces

antinociceptive activity after both peripheral and central administration, although these effects appears to be weaker than that evoked by MOR or DOR agonists (Przewlocki and Przewlocka, 2005). It has been reported that the intraplantar injection of peripherally-selective KOR agonists induced antinociceptive effects in a rat model of neuropathic pain, indicating a peripheral component of this KOR agonism during neuropathic pain (Catheline et al., 1998; Walker et al., 1999). Accordingly, mechanical and thermal allodynia was significantly enhanced after treatment with KOR antagonists norbinaltorphimine and GNTI in rodent models of neuropathic pain (Obara et al., 2003). In addition, neuropathic pain is associated with high levels of spinal dynorphin in superficial laminae I and II as well as in deeper laminae V-VII on the ipsilateral side to the sciatic nerve injury in rats (Obara et al., 2003), and spinal cord injury also increased levels of prodynorphin mRNA and dynorphins in the spinal cord (Przewłocki et al., 1988). Interestingly, genetically modified mice lacking PDYN did not exhibit neuropathic pain after nerve injury (Gardell et al., 2004), suggesting that

increased levels of dynorphin may be a requisite in the development of neuropathic pain.

Besides the antinociceptive effects, KOR agonists also produce undesirable effects. Thus, central KOR agonists produce sedative, dysphoric, diuresis, immunompdulation, and psychomimetic effects (Cahill et al., 2014; Lalanne et al., 2014). Therefore, although central KOR agonists exerts analgesic effects in many chronic pain models, including neuropathic and osteoarthritis (Cahill et al., 2014; Mika et al., 2011; Przewłocki and Przewłocka, 2001), they have not been proved to be viable in humans due to these negative effects (Negus et al., 2012). A possible strategy to avoid these dysphoric and psychomimetic effects might be the use of peripheral dynorphin/KOR compounds that do not cross the blood brain barrier and, in consequence, dissociate the analgesic properties of those central undesirable effects (Negus et al., 2012). However, further investigations are needed in this issue.

b) Dynorphin/KOR system in emotional manifestations of chronic pain

Chronic pain and mood are processed within overlapping brain regions and can modulate each other. The dynorphin/KOR system seems to plays an important critical role in the modulation of both the nociceptive and the affective component of pain. In general, the activation of this system causes dysphoria and psychotomimetic properties in humans and aversive response in animals, whereas the decrease of its activity produces antidepressive- and anxiolytic-like effects (Cahill et al., 2014). Moderate to high expression levels of dynorphins and KOR have been shown in different brain areas implicated in mood control, including AMY, HIP, NAc, PFC and the ventral tegmental area (VTA), among others, in both animals and humans (Peckys and Landwehrmeyer, 1999).

One of the main mechanisms through which KOR activation exerts dysphoric effects is the ability to decrease mesolimbic dopaminergic transmission. The mesolimbic system, which includes the NAc and the VTA, is responsible for the reinforcement produced by natural and drug rewarding stimuli. A decrease in dopamine transmission in

the VTA seems to be a common mechanism underlying mood disorders, such as stress, depression and anxiety (Shelton et al., 2012). The PAG projects directly to the VTA neurons supplying information for nociceptive and mood responses to opioids, and it participate in the rewarding effects of opiate drugs (Cahill et al., 2014). Thus, the PAG projections to the VTA may contribute to this specific activation in response to opioids by form excitatory synaptic inputs to the dopaminergic cells and inhibitory inputs directed to the GABAergic neurons (Omelchenko and Sesack, 2010). In agreement, electron microscopy revealed axons from the PAG synapse onto dopamine and GABA neurons in the VTA (Omelchenko and Sesack, 2010).

The AMY is one of the main limbic structures involved in the emotional component of chronic pain, and exerts a link between brain areas participating in processing sensory information and those regions involved in emotional responses. Here, the anxiety modulator CRF and dynorphins are co-expressed (approx. 30-40%) in the basolateral and CeA (Tejeda et al., 2012). Thus, CeA neurons co-release CRF and dynorphins in monoaminergic nuclei, such as

the VTA, playing a critical role in the modulation on dopaminergic activity (Tejeda et al., 2012). As a result, the anxiogenic effects encoded in the basolateral and CeA are partially mediated by the activation of the dynorphin/KOR system (Hang et al., 2015).

The dynorphin/KOR system also modulates depressive-like behaviours. Accordingly, a positive correlation has been shown between dynorphin expression and anhedonia in depressive disorders (Cahill et al., 2014). KOR activation showed prodepressive-like effects in both humans and rodents, and these effects tend to be blocked by the disruption of the dynorphin/KOR system (Knoll and Carlezon, 2010). In agreement, systemic KOR agonist administration and microinfusion of these compounds into the mesocorticolimbic dopamine structures produce aversive effects in rodents (Knoll and Carlezon, 2010). These KOR agonists do not produce such effects in animals lacking KOR (Simonin et al., 1998), demonstrating that these dysphoric effects require intact KOR signalling. However, it is interesting to note that different studies in some strains of PDYN KO and KOR KO mice have reported increases in dysphoric responses such as anxiety, depression and social

disorders (Bilkei-Gorzo et al., 2014; Knoll and Carlezon, 2010), or no modification of these emotional responses (McLaughlin et al., 2006), these discrepancies highlight the complexity of these interactions that depend of basal stress levels, behavioural paradigms as well as strains or procedure to generate the mutant mice.

The pharmacological potential of KOR antagonism in the treatment of depressive and anxiety-related disorders is well reported. KOR antagonists may increase monoaminergic signalling pathways, producing similar responses to those obtained by typical antidepressants (Berrocoso et al., 2009). Central administration of the KOR antagonist norbinaltorphimine produced antidepressive-like responses in rodent models of depression (Mague et al., 2003) and infusion of norbinaltorphimine into the hippocampus produced antidepressive responses in a learned helplessness paradigm (Shirayama et al., 2004). Moreover, KOR antagonists also produces anxiolytic-like effects. Indeed, systemic administration of KOR antagonists increased the exploration time in the open arms in the elevated plus maze in rats (Knoll and Carlezon, 2010). However, it

has been reported significant differences between the duration and the effectiveness of these antidepressive- and anxiolytic-like responses that may be due to the time point of administration or the pharmacokinetics of the current available KOR antagonists (Knoll and Carlezon, 2010). Regarding the therapeutic strategies for emotional manifestations, the interpretation of animal behavioural is difficult to directly translate to humans. Indeed, significant differences in the distribution of PDYN mRNA in the AMY have been reported in humans and rodents (Lin et al., 2006), and it is also shown the involvement of KOR in the anxiolytic effects of diazepam (Tsuda et al., 1996). Interestingly, patients with major depression showed decreased mRNA levels of PDYN in different brain areas of the limbic system, including the AMY and the hippocampus (Hurd, 1996).

Taking together, all these data show the role of the dynorphin/KOR system in the modulation of mood disorders. A better understanding of the mechanisms of action of this system may provide an important tool to improve the development of psychiatric medications (Figure 26).

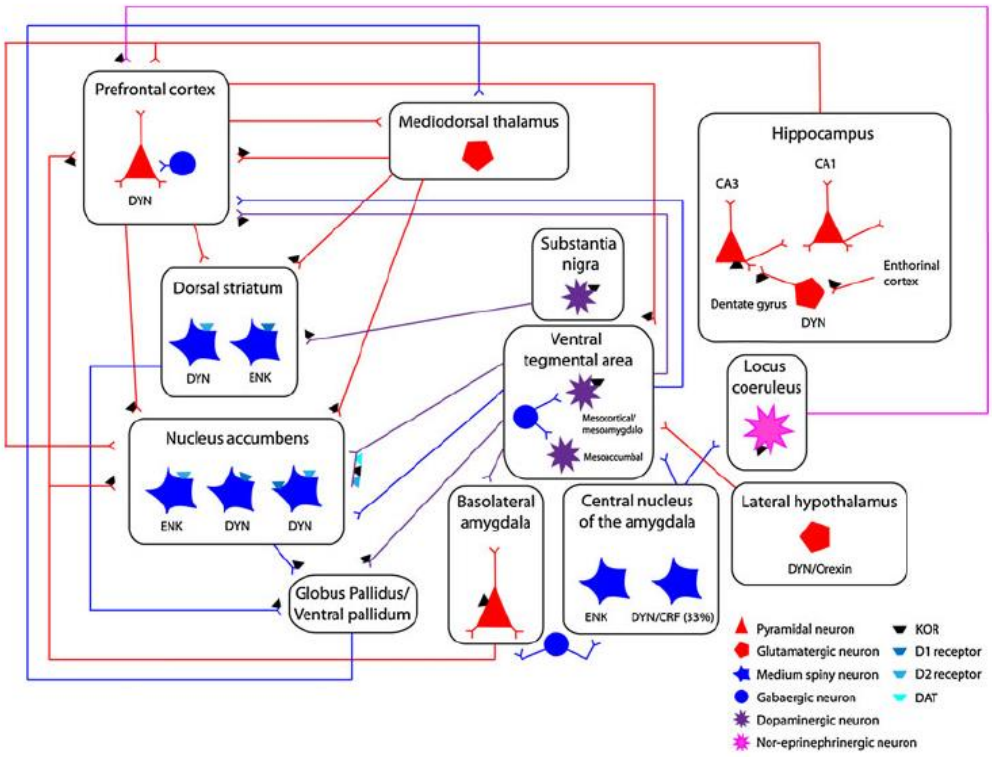

Figure 26. Scheme of the brain circuitry involved in emotional disorders modulated by dynorphin/KOR system. Dynorphin-positive neurons are located in the hypothalamus, central nucleus of the amygdala, striatum and nucleus accumbens and innervate neural substrates rich in KORs. Dynorphin-positive neurons innervate by glutamatergic and monoaminergic fibres, providing a neurobiological substrate for dynorphin/KOR system responses (Tejeda et al., 2012).

c) Dynorphin/KOR system in cognitive manifestations of chronic pain

Learning and memory processes are strongly influenced by emotional and affective responses. The dynorphin/KOR system plays a crucial role in the affective component and participate in the cognitive modulation associated to chronic pain. The HIP is the major structure of the limbic system implicated in the neurobiology of learning and memory processes (Klenowski et al., 2015). The HIP and the AMY are closely related and play an important role in the formation and consolidation of emotional memories (Bilkei-Gorzo et al., 2014).

The dynorphin/KOR system plays an important role in the control of hippocampal functions. As stated in section 2.2, dysfunctions in cognitive mechanisms can be dramatically altered during chronic pain (Moriarty and Finn, 2014) and stress-induced conditions (Carey et al., 2009), and the dynorphin/KOR system contributes to these cognitive responses. Dynorphins are expressed and localised in the granular cells of the dentate gyrus of the HIP and they modulate signal transmission to the CA3 region (Bilkei-Gorzo et al., 2014). Several studies suggest that the activation of KOR produces

memory impairments. Indeed, intra-hippocampal microinjection of dynorphin B or the exogenous agonist U50,488, decreases spatial working memory, and this effect is reversed by the KOR antagonist norbinaltorphimine, suggesting that activation of CA3 hippocampal KOR modulates spatial memory (Tejeda et al., 2012). Moreover, mice exposed to stressed conditions impaired memory functions by a decrease in the novel object recognition performance (Carey et al., 2009). Interestingly, the administration of the KOR agonist U50,488 was sufficient to induce these cognitive deficits and dynorphins administration in the hippocampus also produced cognitive impairments in a rat model of stress (Carey et al., 2009). In turn, the blockade of the dynorphin/KOR system with norbinaltorphimine or PDYN gene disruption prevented the cognitive impairments in the novel object recognition induced by stress, suggesting that the activity of this system also contributes to the maladaptive behaviours related to learning and memory (Carey et al., 2009).

3.6 Therapeutic implications of the dynorphin/KOR system

The use of KOR ligands as therapeutic targets must be carefully considered since the dynorphin/KOR system is implicated in many different neurobiological mechanisms. Initially, the endogenous dynorphin/KOR system is suggested to regulate acute exposure to drugs of abuse by counteracting the rewarding responses. However, the effects of KOR ligands on drug-seeking behaviours are limited (Tejeda et al., 2012).

The dynorphin/KOR system has also been implicated in another severe mental disorder, schizophrenia. Schizophrenia presents some negative symptoms that are also present in chronic pain patients, such as social withdrawal, anxiety, depression, decreased motivation, anhedonia and cognitive deficits, among others (Tamminga and Holcomb, 2005). Unfortunately, schizophrenia has a polygenetic component and complex mechanisms that difficult the effectiveness of these therapeutic approaches (Gonzalez-Burgos et al., 2010).

In spite of these limitations, the dynorphin/KOR system may represent a therapeutic target to search for new therapeutic

approaches for schizophrenia. KOR agonists produce several psychotic effects similar to those present in schizophrenia, such as hallucinations, perceptual distortions or depersonalization. Indeed, Salvinorin A, the psychoactive compound in the hallucinogenic plant *S. divinorum*, is a potent KOR agonist that have raised interest in this mental disorder, although its effects has not been examined in patients with schizophrenia (Tejeda et al., 2012).

Different neurodegenerative or genetic disorders, such as Alzheimer's disease, Parkinson's disease or Down syndrome, also present different comorbidities similar to those observed during chronic pain states. Depression occurs in up to 50% of patients with Parkinson's disease (Ravina et al., 2007), and depressive symptoms dramatically exaggerate the functional decline levels associated with dementia in people with Down Syndrome (Wark et al., 2014). Cognitive impairments are clearly related to Alzheimer's disease, and depressive symptoms were also associated to the neurodegenerative processes of the clinical expression of the disease (Monsell et al., 2013). The dynorphin/KOR system could also be implicated in those disorders. Indeed, enhanced KOR binding in

the AMY is shown in Alzheimer's patients (Barg et al., 1993), and increased subtypes of dynorphin A immunoreactivity are observed in the PFC of patients with Down syndrome Parkinson's and Alzheimer's disease (Risser et al., 1996; Tejeda et al., 2012). The activation of KOR by the enhanced dynorphin tone in the PFC may raise the possibility of an inhibition of the dopamine function in those mental disorders, since dopamine in the PFC plays a critical role for memory functions and behavioural flexibility (Tejeda et al., 2012).

KOR antagonists has been well defined as antidepressant drugs in several animal models. However, the translation of these findings from animal models to humans has yet to be established.

Future research is needed to better understand the different mechanisms underlying the dynorphin/KOR system and to provide insight to the contribution of this system in the neurobiological substrates of mental disorders.

OBJECTIVES

Treatment of chronic pain is at present a major difficulty due to the complex outcomes of human pain experience. Several studies reported the association of chronic pain with affective disorders including anxiety and depression, and memory impairments. In the present Thesis, we validated a model to evaluate the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice by using pregabalin, a first-line drug. Pregabalin has been reported to successfully alleviate both allodynia and hyperalgesia in animal models of neuropathic pain (Verma et al., 2014), although no information is available on affective and cognitive manifestations associated to neuropathic pain.

These behavioural manifestations associated to chronic pain present a high inter-individual variability that depends on the personality traits of the patient. This individual variability represents a critical concern for the development of highly efficient and personalized treatments for chronic pain. Thus, we aimed to identify the influence of different behavioural traits in the inter-individual vulnerability to the nociceptive, emotional and cognitive manifestations of neuropathic pain.

To further understand the neurobiological mechanisms involved in these emotional and cognitive manifestations of chronic pain, we have evaluated the specific involvement of the dynorphin/KOR system in these manifestations associated to osteoarthritis chronic pain by using KO mice deficient in specific components of this endogenous system. Osteoarthritis is a chronic joint disease related to the degradation of articular cartilage that also includes similar affective and cognitive manifestations associated to other chronic pain states (La Porta et al., 2015). Current therapies for osteoarthritis are limited, and several findings support the dynorphin/KOR system as a promising target to develop therapeutic approaches to treat osteoarthritis pain (Cahill et al., 2014), and this system is also implicated in affective and cognitive control (Bilkei-Gorzo et al., 2014). Thus, we aimed to evaluate the involvement of the dynorphin/KOR system in the nociceptive, affective, cognitive, neurochemical and epigenetic manifestations induced by the MIA model for osteoarthritis (Guingamp et al., 1997).

Objective 1

To validate different behavioural outcomes to measure the affective and cognitive manifestations of neuropathic pain in mice and the effects of repeated treatment of pregabalin on these alterations.

Article 1.

Effects of pregabalin on the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice

La Porta C, Lara-Mayorga IM, Negrete R, Maldonado R

European Journal of Pain, 2016

Objective 2

To identify the effect of different personality traits in the inter-individual vulnerability to the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice.

Article 2.

Influence of behavioural traits in the inter-individual variability of nociceptive, emotional and cognitive manifestations of neuropathic pain

Lara-Mayorga IM, Martínez-Navarro M, Negrete R, Gonçalves L, Dickenson AH, Przewlocki R, Baños JE, Maldonado R

(In preparation)

Objective 3

To evaluate the involvement of the dynorphin/KOR system in the behavioural, emotional, cognitive, neurochemical and epigenetic manifestations of chronic osteoarthritis pain in mice.

Article 3.

Involvement of the dynorphin/KOR system on the nociceptive, emotional and cognitive manifestations of osteoarthritis pain in mice

Negrete R, García-Gutiérrez MS, Manzanares J, Maldonado R

Neuropharmacology, 2016

RESULTS

Objective 1

To validate different behavioural outcomes to measure the affective and cognitive manifestations of neuropathic pain in mice and the effects of repeated treatment of pregabalin on these alterations.

Article 1.

Effects of pregabalin on the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice

La Porta C, Lara-Mayorga IM, Negrete R, Maldonado R

Eur J Pain, 2016

La Porta C, Lara-Mayorga IM, Negrete R, Maldonado R. Effects of pregabalin on the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice. Eur J Pain. 2016 Oct;20(9):1454–66. DOI: 10.1002/ejp.868

Objective 2

To identify the effect of different personality traits in the inter-individual vulnerability to the nociceptive, emotional and cognitive manifestations of neuropathic pain in mice.

Article 2.

Influence of behavioural traits in the inter-individual variability of nociceptive, emotional and cognitive manifestations of neuropathic pain

Lara-Mayorga IM, Martínez-Navarro M, Negrete R, Gonçalves L, Dickenson AH, Przewlocki R, Baños JE, Maldonado R

(In preparation)

Declaration of contribution

Our results demonstrated the influence of personality traits in the inter-individual vulnerability to neuropathic pain manifestations. Behavioural, electrophysiological and genetic approaches provide new insights to better understand the mechanisms underlying the inter-individual variability of the nociceptive, emotional and cognitive manifestations of neuropathic pain. This study contributes to optimize future personalized treatments for chronic pain.

My specific contribution to this study has been:

1. To support in the selection of the extreme phenotypes. Responses to 250 mice to affective and social behaviours were evaluated and classified in high, low and intermediate percentiles for each behavioural trait. After verify the selection criteria for these mice, 15 animals per group were used to assess the nociceptive, emotional and cognitive manifestations to neuropathic pain.
2. The induction of the neuropathic pain by using the PSNL model previously described in mice (Malmberg and Basbaum, 1998) and similar to the approach described in

rats (Seltzer et al., 1990). As stated before, mice were anaesthetized with isoflurane, and the common sciatic nerve was exposed at the level of the mid-thigh of the right hind paw., a tight ligature was created around 33–50% of the sciatic nerve at 1 cm proximally to the nerve trifurcation by using a non-absorbable virgin silk suture. Control mice were sham operated, consisting in the same procedure used for PSNL, but in this case the sciatic nerve was not ligated. Thus, nociceptive responses were assessed on basal conditions and on days 3, 6, 11, 16 and 21 after the induction of neuropathic pain.

3. Mechanical and cold allodynia, as well as thermal hyperalgesia were evaluated after PSNL. First, mechanical allodynia was assessed by measuring the hind paw withdrawal response to von Frey filaments stimulation applying the up-down paradigm (Chaplan et al., 1994) in both ipsilateral and contralateral paws. Then, thermal hyperalgesia was measured with the plantar test apparatus by determining the withdrawal latency response to radiant

heat, as previously reported (Hargreaves et al., 1988). The mean paw withdrawal latencies for both ipsilateral and contralateral paws were evaluated from the average of three separated trials in 5-10 min intervals to prevent thermal sensitization. Finally, cold allodynia was also assessed at a temperature of 5 ± 0.5ºC during 5 min by the hot/cold plate test, as previously reported (Bennett and Xie, 1988). A score was calculated as the difference of number of elevations between ipsilateral and contralateral paws.

The manifestations of neuropathic pain show a high inter-individual variability that mainly depends on the personality traits of patients. The selection of mice displaying extreme phenotypes on affective and social responses was crucial to better characterize the inter-individual vulnerability to nociceptive manifestations of neuropathic pain. We provide relevant results to better predict the inter-individual variability to neuropathic pain manifestations, and therefore to propose a possible mechanism involved in chronic pain manifestations.

Influence of behavioural traits in the inter-individual variability of nociceptive, emotional and cognitive manifestations of neuropathic pain

Lara-Mayorga IM[1*], Martínez-Navarro M[1*], Negrete R[1], Bilecki W[3], Bargiela A[3], Gonçalves L[2], Dickenson AH [2], Przewlocki R[3] Baños JE[1] and Maldonado R[1]

[1] Laboratory of Neuropharmacology. Department of Experimental and Health Sciences. Universitat Pompeu Fabra. Barcelona, Spain.

[2] Neuroscience, Physiology and Pharmacology, University College, London WC1E6BT, United Kingdom

[3] Department of Molecular Neuropharmacology, Institut of Pharmacology, Polish Academy of Sciences. Krakow, Poland.

* These authors contributed equally to this work

Abstract

Chronic neuropathic pain is a complex disorder often associated with emotional and cognitive deficits that can enhance pain manifestations. There is high inter-individual variability in the manifestations of neuropathic pain, which largely depends on personality traits of the patient. We aim to identify the influence of different behavioural traits in the inter-individual vulnerability to the nociceptive, emotional and cognitive manifestations of neuropathic pain using behavioural, electrophysiological and genetic approaches. For this purpose, we selected mice displaying extreme phenotypes on social and emotional responses, and evaluated the possible correlation between the spontaneous and evoked CeA neuronal activity and the behavioural responses. In a second step, neuropathic pain was induced to these extreme phenotype mice to evaluate the possible influence of the behavioural traits on the inter-individual variability of pain manifestations and gene expression profiles in amygdala. Our results show a correlation of the spontaneous CeA neuronal activity with the sociability-like behaviour but not with emotional-like

responses. They also show that the anxiety-like behavioural trait correlates with several manifestations of neuropathic pain. Gene expression analysis identifies *Pdyn* as a potential marker of anxiety and sociability and *NR3C1* genes as potential molecular markers and depressive-dependent susceptibility. These results suggest that anxiety-like behaviour can be of relevance to predict the inter-individual vulnerability to the different manifestations of neuropathic pain.

1. Introduction

Chronic neuropathic pain is a complex disorder that includes nociceptive, emotional and cognitive alterations (Apkarian et al 2004, La Porta 2016). Several reports have established its association with emotional disorders, such as anxiety and depression (Neugbauer et al. 2004, La Porta 2016), as well as with cognitive deficits, including memory, learning and decision making impairment (Apkarian et al 2004, Conrad et al. 2007). Nociceptive, emotional and cognitive alterations could aggravate each other leading to an impairment of the quality of life of patients with neuropathic pain (Apkarian et al 2004, Conrad et al. 2007). Therefore, the consideration of the three factors is relevant in the treatment of these patients.

The manifestations of neuropathic pain show a high inter-individual variability that depends on multiple factors, including the personality traits of patients (Ashgari et al. 2006). It has been well documented that emotional, cognitive and social personality traits can modulate pain perception (Rhudy et al. 2008, D'Amato et al. 2012). The brain areas responsible of such influences are not well

known, although several evidences strongly support an important role of the amygdala in the emotional-affective dimension of pain (Neugbauer et al. 2004 and 2009, Ikeda et al 2007). The amygdala plays a key role in the formation of fear-related memories and emotional processing (Phelps et al. 2005). The amygdala contains several nuclei, including the lateral (LA), basolateral (BLA) and central (CeA) nuclei, that are important for sensory processing (Neugebauer et al. 2009). Strong neuronal responses to peripheral stimuli have been reported in the CeA, which have been defined as the 'nociceptive amygdala' (Neugebauer et al. 2004). Increased excitability of CeA neurons have been reported in models of arthritic, visceral (Neugebauer et al. 2004) and neuropathic pain (Ikeda et al. 2007). High levels of amygdala activation have also been reported in patients with generalized anxiety, social phobia, panic and posttraumatic stress disorder (Etkin et al. 2007).

In this study, we evaluated the influence of specific behavioural traits on the nociceptive, emotional and cognitive manifestations of neuropathic pain. We selected mice displaying extreme phenotypes on social and emotional responses, and evaluated the

possible correlation between the spontaneous and evoked CeA neuronal activity and behavioural responses. In a second step, neuropathic pain was induced to these extreme phenotype mice to evaluate the possible influence of the behavioural traits on the inter-individual variability of pain manifestations. Gene expression profiles in the amygdala were also studied to elucidating the possible mechanisms involved.

2. Materials and methods

2.1. Animals

Swiss albino male mice with initial body weight between 20-22g (Charles River, Lyon, France) were used in these experiments. Mice were housed in groups of 2 to 4 with free access to water and food. The housing conditions were maintained at 22 ± 1˚C and 55 ± 10% relative humidity in a controlled light/dark cycle (light on between 8:00 A.M. and 8:00 P.M.). All experimental procedures and animal husbandry were conducted according to standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and were approved by the local

ethical committee. All the experiments were performed under blinded conditions.

2.2. Experimental protocol

Mice were exposed to locomotion, sociability-like, anxiety-like and depressive-like behavioural tests as shown in Figure 1. Animals showing extreme locomotor responses were excluded following established criteria in order to avoid possible bias with other behavioural tests (Table 1). Those displaying high, low and intermediate social and emotional responses were selected. Spontaneous and evoked CeA neuronal activity were recorded in selected mice of each phenotype. Another group of animals were exposed to a partial sciatic nerve ligation or sham surgery to induce neuropathic pain. Nociceptive responses were assessed under basal conditions and on days 3, 6, 11, 16 and 21 after surgery. Anhedonic state, anxiety-like behaviour and cognitive performance were evaluated on day 10, 15 and 20, respectively, using different paradigms than in the initial screening step to avoid double exposition of mice to the same model (Figure 1).

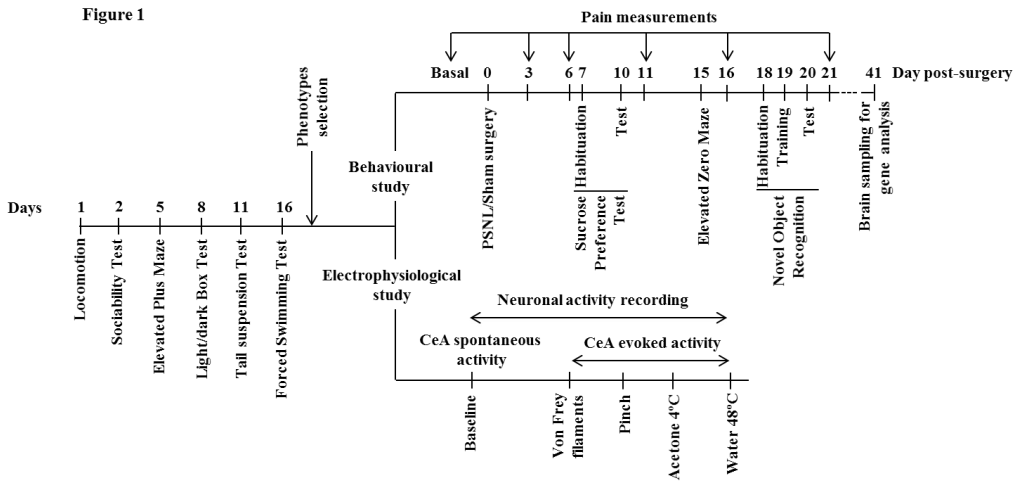

Figure 1. Experimental sequence to select mice displaying extreme phenotypes on different behavioural responses, for the assessment of electrophysiological correlates and for the evaluation of the nociceptive, affective and cognitive behaviours in mice exposed to neuropathic pain.

Finally, brain samples of amygdala were isolated at day 41 after surgery from animals used for the behavioural study. Transcriptional modifications in these areas were examined.

2.3. Behavioural tests

2.3.1. Locomotion activity

Locomotor activity was evaluated as previously described (Martin et al. 2000) by using actimetry boxes (9 × 20 × 11 cm) (Imetronic,

Lyon France) in a low luminosity room (5 lux), and with white noise. Each box contained two lines of photocells located 2 and 6 cm above the floor to measure horizontal and vertical movements, respectively. Mice were individually placed in the boxes and the number of activity counts was recorded for a period of 30 min.

2.3.2. Sociability-like behaviour

Sociability test was performed the day after the locomotor activity evaluation to determine the extreme phenotypes. A black Plexiglas V-maze was used with 15 cm bars of transparent Plexiglas placed at 6.5 cm of the end of each arm that separate both sides but allow exploration (Panlab). The mouse was first habituated to the empty maze during 5 min. In a second step, sociability-like behaviour was evaluated during 5 min by placing one stranger animal in the maze, behind the Plexiglas bars. A sociability index was calculated as the difference between the time spent exploring either the stranger mouse and the empty space divided by the total exploration time.

2.3.3. Anxiety-like behaviour

Three experimental paradigms were used: the elevated plus maze and light/dark box test were used to determine the extreme phenotypes and the elevated zero maze was performed after nerve injury.

The elevated plus maze was performed 3 days after social behaviour evaluation using a black Plexiglas apparatus with 2 open (45 lux) and 2 closed (5 lux) arms (29 cm long x 5 cm wide), set in cross from a neutral central square (5x5 cm) elevated 40 cm above the floor. The percentage of entries and time spent in the open arms was determined during 5 min, as previously reported (Busquets-Garcia et al. 2011).

The light/dark box test was performed 3 days after the elevated plus maze, as previously described (Filliol et al. 2000). A Plexiglas box composed of a small dark compartment (15×20×25 cm, 10 lux) and a large light compartment (30×20×25 cm, 500 lux) separated by a connecting 4 cm long tunnel was used. Floor lines separated the light compartment into three equal zones, from the tunnel to the opposite wall, designated as proximal, median and distal zones.

The percentage of distal entries, the time in the light compartment and total light entries were calculated during 5 min.

The elevated zero maze was performed 15 days after nerve injury, as previously described (Valverde et al. 2004), using a circular black Plexiglas apparatus (5.5 cm wide and with inner diameter of 46 cm) with two open (100 lux) and two wall-enclosed sections (10 lux) elevated above the floor (50 cm). A ratio between the time in open arm and the number of total entries (animal enters with 4 paws) was calculated during 5 min.

2.3.4. Depressive-like behaviour

Three experimental paradigms were used: the tail suspension and forced swimming to determine the extreme phenotypes and the sucrose preference test was performed after sciatic nerve injury.

Tail suspension test was performed 3 days after the elevated plus maze, as previously described (Steru 1985). Mice were suspended by their tails with tape, in such a position that it cannot escape or hold on to nearby surfaces during 6 min. The immobility time was recorded during the last 4 min of the test.

Forced swimming test was performed 5 days after the tail suspension. Mice were placed in a narrow (17.5 x 12.5 cm) Plexiglas cylinder containing water to a depth of 15 cm (22 °C ± 0.2 °C) (Porsolt et al. 1977). Each animal was submitted to a forced swimming during 6 min and the total duration of immobility, including small maintenance movements, was measured during the last 4 min.

Sucrose preference test was performed 10 days after nerve injury, using a new extremely high sensitivity (0.02 g) monitoring system (Phecomp, Panlab, ES), recently validated in our laboratory (Bura et al. 2013). Two different drink solutions were used: plain water and a palatable drink solution (2% sucrose). Three days before the test day, a 24 h session was performed to habituate the mice to the environment and the different drink solutions. The anhedonic-like state was evaluated during a test session of 24 h. The percentage of mean sucrose preference was calculated as the ratio of the sucrose solution intake to total liquid intake x 100.

2.3.5. Cognitive evaluation

Novel object recognition test was performed as previously described (Puighermanal et al. 2009) 20 days after nerve injury in the same maze used for sociability-like behaviour evaluation without the transparent Plexiglas bars, three 9-min phases were performed on consecutive days. Mice were first habituated to the V-maze. On the second day, two identical objects were presented to the mice, and the time that they spent exploring each object was recorded. The third day, one of the familiar objects was replaced with a novel object, and the time spent exploring each object (novel and familiar) was computed. A discrimination index was calculated as the difference between the times spent exploring either the novel or familiar object divided by the total time exploring the two objects.

2.4. Electrophysiological procedures

Extracellular recordings were made from single neurons in the right CeA after the behavioural tests in selected extreme phenotypes mice. Parylene coated tungsten electrodes were applied (A-M

Systems, USA) using the following stereotaxic coordinates (Paxinos et al. 2007): 4.4 mm dorsoventral, 2.4 mm lateral and 1.06 mm caudal to bregma. During the recordings animals were anesthetized and maintained for the duration of the experiment with isofluroane (1.5–1.7%) delivered in a gaseous mix of N_2O (66%) and O_2 (33%). After the animals were fixed in the stereotaxic device, the skull was exposed and the amygdala coordinates found. A small craniotomy was performed and the dura matter taken, allowing access to the brain. All the neurons found in the CeA that fired spontaneously for at least 20 min were recorded. Besides neuronal spontaneous activity (baseline), the activity of CeA neurons after the following stimuli was recorded: von Frey filaments (0.008, 0.16, 0.4, 0.6, 1.4, 6.0 and 10.0 g) applied to both hind paws, and pinch, cold (4° C, acetone) and heat (48° C water) applied to both hind paws, ears and tail. Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design; PSTH and rate functions).

2.5. Neuropathic pain induction and assessment

2.5.1. Pain induction

A partial ligation of the sciatic nerve (PLSN) was used to induce neuropathic pain to the selected mice (Malmberg et al. 1998). Briefly, mice were anaesthetized with isoflurane (induction 5%; surgery 2%) and the common sciatic nerve was exposed at the level of the mid-thigh of the right hind paw. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33-50% of the sciatic nerve using an 18-in non-absorbable virgin silk suture (Alcon® Surgical Inc., USA). The remaining nerve was left undamaged. The muscle was stitched and the incision was closed with wound clips. Sham mice underwent the same procedure without the nerve ligation.

2.5.2. Nociceptive behaviours

Mechanical allodynia, heat hyperalgesia and cold allodynia were used as outcome measures of neuropathic pain, as previously reported (La Porta et al. 2016). Mice were tested in each paradigm at different time points (see experimental protocol), using the

same experimental sequence (mechanical allodynia, thermal hyperalgesia and cold allodynia).

Mechanical allodynia was evaluated by measuring the hind paw withdrawal response to von Frey filaments stimulation. Animals were placed in Plexiglas boxes (20 cm high, 9 cm diameter) placed on a grid surface through which the von Frey calibrated filaments (North Coast Medical, USA) were applied by using the up–down paradigm. The threshold of response was then calculated by the up–down Excel program generously provided by A. Basbaum (University of California, San Francisco, USA). Animals were habituated for 1 h before testing. Clear paw withdrawal, shaking, or licking was considered as nociceptive-like response. Both hind paws were tested.

Heat hyperalgesia was evaluated by measuring paw withdrawal latency in response to radiant heat with plantar test apparatus (Ugo Basile, Italy). Mice were placed in Plexiglas boxes (20 cm high, 9 cm diameter) positioned on a glass surface and habituated to the environment for 30 min before testing. The mean paw withdrawal latencies for the ipsilateral and contralateral hind paws were

determined from the average of 3 separate trials, taken at 5-10 min intervals to prevent thermal sensitization. A cut-off time of 20 s was used to prevent tissue damage.

Cold allodynia was assessed with the hot/cold plate analgesia meter (Columbus, USA). A glass cylinder (25 cm high, 20 cm diameter) was used to keep mice on the cold surface of the plate, which was maintained at 5±0.5º C. The number of elevations of each hind paw was recorded for 5 min. A score was calculated as the difference of number of elevations between ipsilateral and contralateral paws.

2.6. Gene expression analysis

Tissue samples of amygdala were placed in RNAlater reagent (Qiagen Inc., USA) and preserved at -70°C. Samples were homogenized in 1 ml of TRIzol® reagent (Invitrogen, USA). RNA was isolated following the manufacturer's protocol and was further purified using the RNeasy Mini Kit (Qiagen Inc.). Total RNA concentration was measured using a NanoDrop ND-1000

(NanoDrop Technologies Inc., USA). RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent, USA).

2.7. Quantitative PCR

Reverse transcription was performed using Omniscript Reverse Transcriptase (Qiagen Inc.) in tissue samples from amygdala. The qPCR reactions were performed using isoform specific TaqMan® probes (BDNF-Mm01334042_m1, C1qa-Mm00432142_m1, FKBP5-Mm00487401_m1, Gadd45g-Mm00442225_m1, GFAP-Mm01253033_m1, HPRT1-Mm01545399_m1, Il1b-Mm00434228_m1, Il6-Mm00446190_m1, Nr3c1-Mm00433832_m1, PDYN-Mm00457573_m1, Tsc22d3-Mm00726417_s1, Egr1-Mm00656724_m1) designed by the Custom TaqMan® Assay Design Tool (Life Technologies) and were run on the CFX96 Real-Time system (BioRad). Each template was generated from an individual animal, and the amplification efficiency for each assay was determined by running a standard dilution curve. Expression of the hypoxantine-guanine phosphoribosyltransferase 1 (Hprt1) transcript with stable levels

following drug treatment was quantified to control for variation in cDNA amounts. The abundance of RNA was calculated as $2^{-(\text{threshold cycle})}$.

2.8. Statistical analysis

Data obtained in nociceptive behaviour, sucrose preference, elevated zero maze and novel object recognition test were analyzed by one-way ANOVA followed by *Post hoc* analysis (Fisher's LSD) when appropriate. STATISTICA 6.0 software was used. Electrophysiological data were analysed through one-way ANOVA Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistical analysis of gene expression was performed using a two-way ANOVA followed by Tukey's and Bonferroni's multiple-comparisons *post hoc* tests. T-test was used for comparison of gene expression between sham and PSNL groups. Prism 6 (GraphPad) was used for statistical analysis. Differences were considered to be statistically significant when $p < 0.05$. Data are expressed as means ± SEM.

3. Results

3.1. Selection of the extreme phenotypes

Responses of 250 mice to anxiety, depressive and sociability-like behaviours were recorded. Mice with extreme locomotor responses were excluded following the criteria defined in Table 1 in order to avoid a possible bias of this abnormal basal behaviour. After excluding these animals (21 mice), mice were classified in three phenotypes (high, low and intermediate percentiles) for each behavioural trait, using the criteria defined in Table 1. Each behavioural trait was analysed independently without excluding the possibility to incorporate the responses of a particular mouse in the phenotypes corresponding to different behavioural traits. The responses of 151 mice were selected to define the different phenotypes corresponding to each behavioural trait (25 mice per each high, low and intermediate percentile). Ten animals per group were used for electrophysiological studies, and 15 mice per group were used to evaluate the emotional and cognitive manifestations of neuropathic pain, and to provide tissue samples for the biomarkers identification. From each subgroup of 15 animals, 10

mice underwent the surgical procedure and the 5 remaining animals were sham operated. Sham animals of the three phenotypes were considered as a unique sham-balanced group (n=15) per behavioural trait.

Table 1. Summary of selection and exclusion criteria for classify mice within the three phenotypes defined for each behavioural trait

Behavioural trait	Test	Parameters	Exclusion criteria	Inclusion criteria		
				High	Intermediate	Low
Locomotor activity	Actimetry boxes	Horizontal movement	Below10th percentile Above 90th percentile			
		Vertical movement	Below 5th percentile Above 95th percentile			
Sociability-like behaviour	Sociability Test	Sociability index		Above 85th percentile	Between 40th and 60th percentile	Below 5th percentile
Anxiety-like behaviour	Light Dark Box Test	% Light time		3 of the 5 parameters below 25th percentile	3 of the 5 parameters between 35th and 65th percentile	3 of the 5 parameters above 75th percentile
		Total light entries				
		% Distal entries				
	Elevated Plus Maze	% Entries open arms				
		% Time open arms				
Depressive-like behaviour	Tail Suspension Test	Immobility time		2 parameters above 75th percentile	2 parameters between 35th and 65th percentile	2 parameters below 25th percentile
	Forced Swimming Test	Immobility time				

3.2 Spontaneous and evoked neuronal CeA activity in the extreme anxiety, sociability and depressive-like behavioural phenotypes

Electrophysiological data were obtained from selected mice displaying extreme phenotypes. Neurons of mice presenting a phenotype of sociability high percentile had a significantly higher activity than those in the lower percentile, with the intermediate percentile standing between the other two (Figure 2A). Neurons of mice of the anxiety group showed no significant differences between low, intermediate and high percentile (Figure 2B). Neurons from the intermediate percentile in the depression group showed a significantly higher activity than the high percentile, with the low percentile standing between the other two (Figure 2C). There were no significantly different neuronal responses to any stimuli applied peripherally to mice belonging to any of the group of behavior phenotype. Neurons were individually analyzed and separated according to any change observed in the activity after application of stimuli.

Figure 2

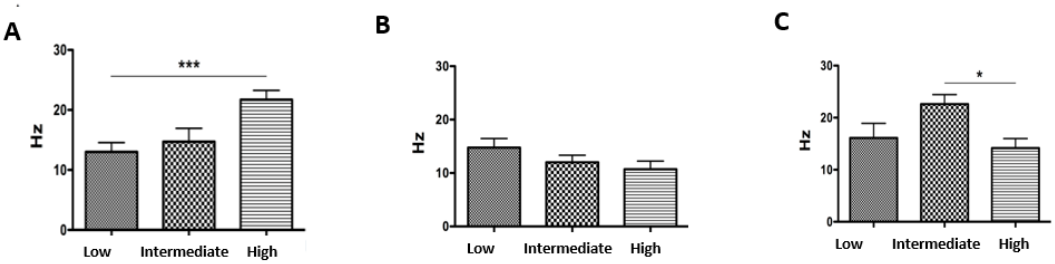

Figure 2. Electrophysiological evaluation of mice displaying extreme phenotypes. Central amygdala (CeA) spontaneous activity of mice in low, intermediate and high phenotypes for (A) sociability-like, (B) anxiety-like and (C) depressive-like behaviour. ★P < 0.05, ★★★P < 0.001 between groups.

After re-grouping in “Increase” and “Decrease” groups, within the respective phenotype group and

percentile, there was still no significant difference between spontaneous and evoked activity.

3.3. Nociceptive behaviours

3.3.1. Nociceptive, emotional and cognitive consequences of neuropathic pain according extreme sociability-like behavioural phenotypes

One-way ANOVA for the ipsilateral responses to mechanical, heat and cold stimulation in sham operated and nerve injury groups revealed a significant effect of group (Table 3). No significant effect was revealed neither for the baseline values nor the contralateral responses (data not shown).

Table 3. Expression profile of selected genes affected in the amygdala of mice displaying extreme phenotypes following nerve injury.

	GENE		PSNL	Sociability	Anxiety	Depression
Activty	npas4	neuronal PAS Domain Protein 4		-	-	-
	egr1	early growth response protein 1/Zif268	-	-	-	-
	arc	activity-regulated cytoskeleton-associated protein	-	-	-	-
Stress	fkbp5	FK506 binding protein 5	-	-	-	-
	tsc22d3	TSC22 domain family protein 3		-	-	-
	camk1g	calcium/calmodulin-dependent protein kinase IG	-	-	-	-
	nr3c1/gr	nuclear receptor subfamily 3, group C, member 1	-	-	-	#
Inflammation	c1q	complement component 1, q subcomponent, a chain		-	-	-
	il6	interleukin 6		-	-	#
	il1beta	interleukin 1 beta		-	-	#
	gadd45	growth arrest and DNA-damage-inducible, gamma		-	-	#
	gfap	glial fibrillary acidic protein	-	-	#	-
Neuropeptides	crh	corticotropin-releasing hormone		-	-	-
	pdyn	prodynorphin		#	#	-
	bdnf	brain-derived neurotrophic factor	-	-	#	-

Subsequent *post hoc* analysis indicated that nerve injury, but not sham surgery, produced an increase of mechanical and thermal sensitivity of the ipsilateral paw in all phenotypes of sociability-like behaviour. No significant differences were obtained among phenotype groups exposed to nerve injury (Figure 3A, B and C).

The possible emotional manifestations of neuropathic pain were first evaluated by measuring sucrose preference as a relevant model of anhedonic-like responses. No significant effect of group was revealed by one-way ANOVA in the anhedonic state evaluation (Figure 3D) suggesting that neuropathic pain did not modify this behaviour response under the present experimental conditions. The consequences of neuropathic pain in anxiety-like behaviour were evaluated in the elevated zero maze. One-way ANOVA of ratios between time in open arms and total number of entries revealed a significant effect of group ($P<0.05$) and subsequent *post hoc* analysis showed an increase in all PNSL groups, when compared with sham-operated mice. No significant differences of the different phenotype groups after PSNL were revealed (Figure 3E). The cognitive manifestations of neuropathic pain were

evaluated in the novel object recognition test. One-way ANOVA for the discrimination index revealed a significant effect of group ($P<0.05$). A cognitive impairment of the intermediate, low and high sociability-like groups exposed to PSNL was revealed in comparison to the sham group by the subsequent *post hoc* analysis, without significant differences among phenotypes (Figure 3F).

Figure 3.

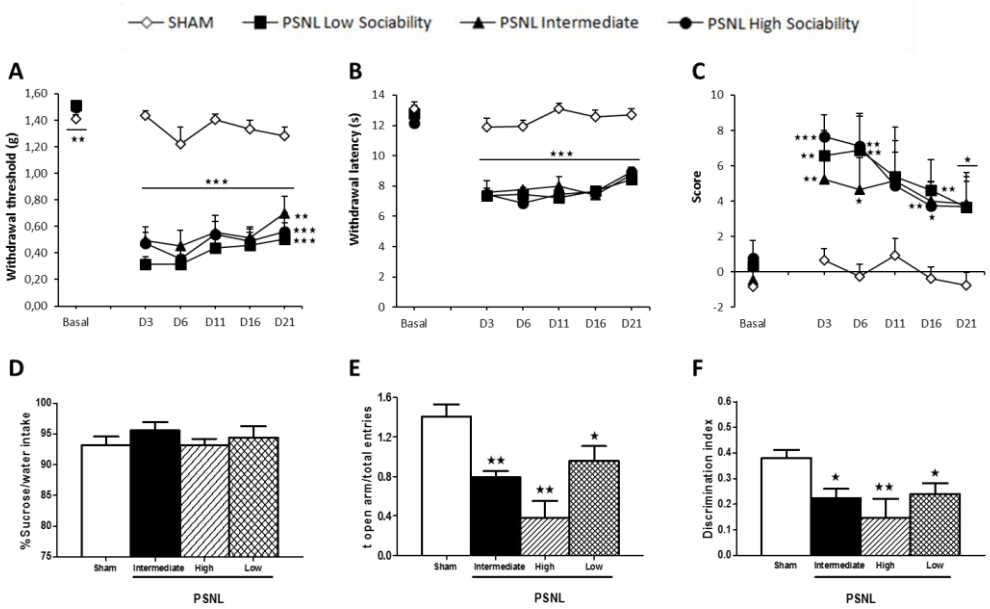

Figure 3. Evaluation of the nociceptive, emotional and cognitive consequences of neuropathic pain on mice displaying extreme sociability-like behavioural phenotypes. (A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) heat hyperalgesia was evaluated in the plantar test and expressed as paw withdrawal latencies, and (C) cold allodynia was evaluated in the cold plate test and expressed as score values (difference in the number of elevations between the ipsilateral and contralateral paws). Nociceptive measurements were performed under basal conditions and on days 3, 6, 11, 16, and 21 after PSNL or sham surgery. (D) Percentage of sucrose preference during 24 hour sessions were evaluated in the monitoring system (Phecomp boxes) on day 10 post-surgery. (E) Ratio of the time expended in open arms to total entries in the elevated zero maze was assessed on day 15 post-surgery. (F) The discrimination index in the novel object recognition was

evaluated on day 20 after PSNL or sham surgery. Values are expressed as mean ± SEM (n= 15 per sham group, and n= 10 per PSNL groups). ★P < 0.05, ★★P < 0.01, ★★★P < 0.001 vs. sham surgery (Fisher's LSD test).

3.2.2. Nociceptive, emotional and cognitive consequences of neuropathic pain according extreme anxiety-like behavioural phenotypes

One-way ANOVA for the ipsilateral responses to mechanical, heat and cold stimulation revealed a significant effect of group (Table 3), while no significant effect was revealed neither for the baseline values nor the contralateral responses (data not shown). Subsequent *post hoc* analysis indicated that surgery increased pain responses in all phenotypes of anxiety-like behaviour. Sham surgery did not modify the nociceptive responses. No significant differences were obtained between extreme phenotype groups after surgery (Figure 4A, B and C).

Regarding the anhedonic state, one-way ANOVA did not reveal significant differences confirming that neuropathic pain did not modify this behavioural response under our experimental conditions (Figure 4D). In the anxiety-like responses produced by

neuropathic pain, one-way ANOVA of the ratio between time in open arms and total number of entries showed a significant effect of group ($P<0.05$). Subsequent *post hoc* analysis revealed an increase of anxiety-like behaviour in PSNL-intermediate and PSNL-high anxiety groups, when compared with sham-operated mice. A significant difference of PSNL-high anxiety and PSNL-low anxiety groups in their anxiety ratio values was also observed (Figure 4E). In the cognitive evaluation, a significant effect of group for the discrimination index was revealed by one-way ANOVA ($P<0.05$). The subsequent *post hoc* analysis demonstrated significant differences in the cognitive manifestation of all groups exposed to nerve injury when compared to sham group, without significant differences among phenotypes (Figure 4F).

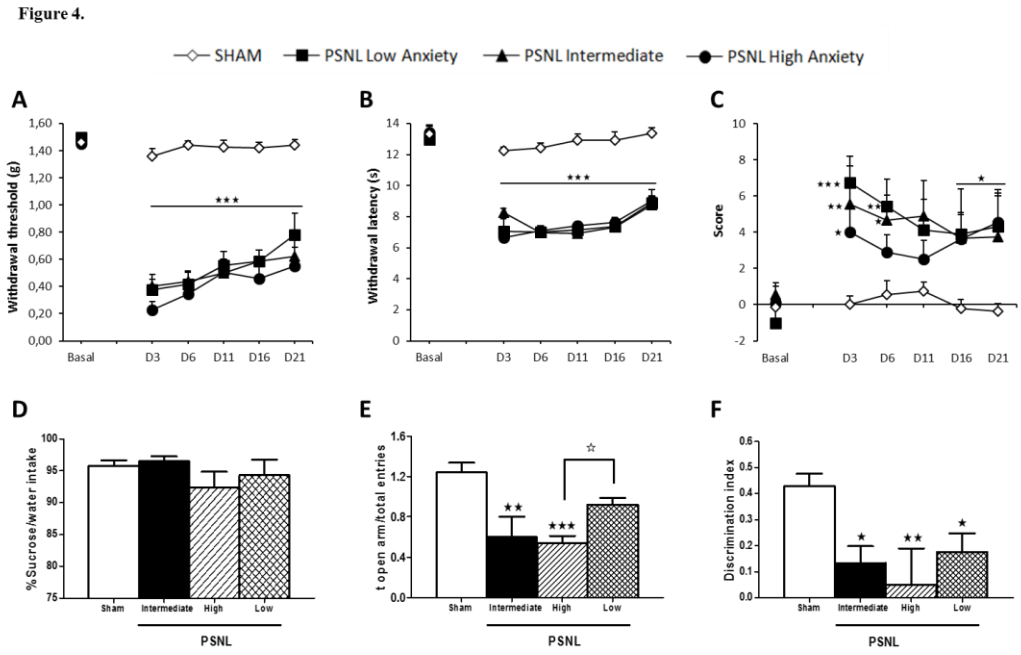

Figure 4. Evaluation of the nociceptive, emotional and cognitive consequences of neuropathic pain on mice displaying extreme anxiety-like behavioural phenotypes. (A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) heat hyperalgesia was evaluated in the plantar test and expressed as paw withdrawal latencies, and (C) cold allodynia was evaluated in the cold plate test and expressed as score values (difference in the number of elevations between the ipsilateral and contralateral paws). Nociceptive measurements were performed under basal conditions and on days 3, 6, 11, 16, and 21 after PSNL or sham surgery. (D) Percentage of sucrose preference during 24 hour sessions were evaluated in the monitoring system (Phecomp boxes) on day 10 post-surgery. (E) Ratio of the time expended in open arms to total entries in the EZM was assessed on day 15 post-surgery. (F) The

discrimination index in the novel object recognition was evaluated on day 20 after PSNL or sham surgery. Values are expressed as mean ± SEM (n= 15 per sham group, and n= 10 per PSNL groups). ★P < 0.05, ★★P < 0.01, ★★★P < 0.001 vs. sham surgery (Fisher's LSD test). P < 0.05 vs. PSNL high extreme PSNL low extreme (Fisher's LSD test).

3.2.3. Nociceptive, emotional and cognitive consequences of neuropathic pain according extreme depressive-like behavioural phenotypes

One-way ANOVA for the ipsilateral responses to mechanical, heat and cold stimulation revealed a significant effect of group (Table 3), while no significant effect was revealed neither for the baseline values nor the contralateral responses (data not shown). Subsequent *post hoc* analysis indicated that all animals exposed to nerve injury increased the mechanical and thermal sensitivity of the ipsilateral paw in all phenotypes of depressive-like behaviour (Figure 5A, B, C).

In the anhedonic state evaluation, no significant effect of group was shown by one-way ANOVA analysis confirming the previous results (Figure 5D). Regarding the anxiety-like behaviour, one-way

ANOVA (ratio between time in open arms and total number of entries) revealed significant effect of group ($P<0.05$). Subsequent *post hoc* analysis showed that all the sciatic nerve injury groups had increased depressive-like behaviour, when compared with sham group, without significant differences among phenotypes (Figure 5E). One-way ANOVA for the discrimination index showed a significant effect of group ($P<0.05$). The subsequent *post hoc* analysis revealed a significant cognitive impairment of all PSNL depressive-like behavioural groups when compared to sham group, without significant differences among phenotypes (Figure 5F).

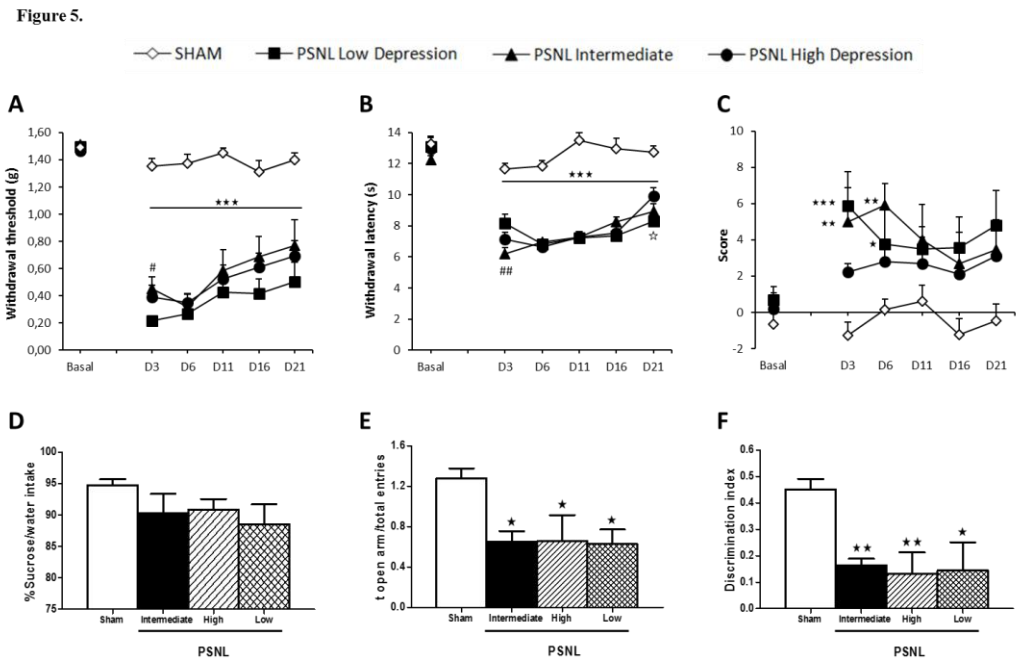

Figure 5. Evaluation of the nociceptive, emotional and cognitive consequences of neuropathic pain on mice displaying extreme depressive-like behavioural phenotypes.

(A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) heat hyperalgesia was evaluated in the plantar test and expressed as paw withdrawal latencies, and (C) cold allodynia was evaluated in the cold plate test and expressed as score values (difference in the number of elevations between the ipsilateral and contralateral paws). Nociceptive measurements were performed under basal conditions and on days 3, 6, 11, 16, and 21 after PSNL or sham surgery. (D) Percentage of sucrose preference during 24 hour sessions were evaluated in the monitoring system

(Phecomp boxes) on day 10 post-surgery. (E) Ratio of the time expended in open arms to total entries in the elevated zero maze was assessed on day 15 post-surgery. (F) The discrimination index in the novel object recognition was evaluated on day 20 after PSNL or sham surgery. Values are expressed as mean ± SEM (n= 15 per sham group, and n= 10 per PSNL groups). ★P < 0.05, ★★P < 0.01, ★★★P < 0.001 vs. sham surgery (Fisher's LSD test). ☆P < 0.05 vs. PSNL high extreme PSNL low extreme (Fisher's LSD test). # P < 0.05, ## P < 0.01 vs. PSNL intermediate PSNL low extreme (Fisher's LSD test).

3.2.4. Analysis of gene expression in relation to extreme behavioural phenotypes and neuropathic pain

Given the importance of the amygdala in the regulation of emotional behaviour and its relevance for the depression, anxiety and social behaviour, we have focused on expression of genes potentially involved in the regulation of the emotional states and in the pathophysiology of neuropathic pain.

Pdyn mRNA levels characterized sociability- ($F_{(2, 80)}$ = 3,582; P<0.05) and anxiety-like phenotypes ($F_{(2, 36)}$ = 4,337; P<0.05). High level of *Pdyn* mRNA in the amygdala was linked to low sociability, high

anxiety and stronger pain sensation while the low *Pdyn* level was correlated negatively (Figure 6A,B,C).

Figure 6.

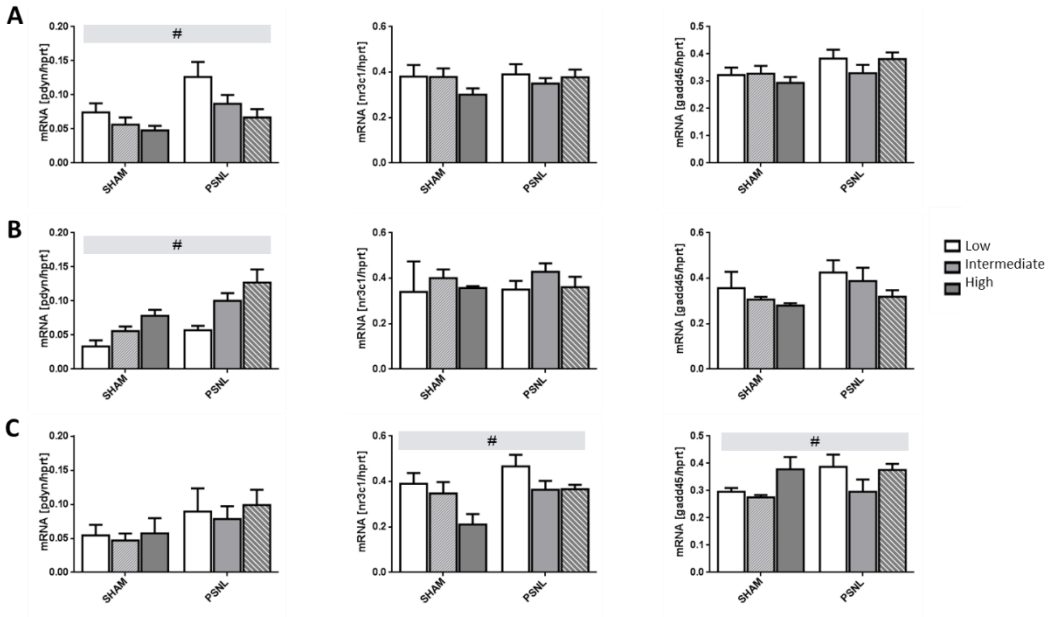

Figure 6. The influence of extreme behavioural phenotypes on selected mRNAs in the amygdala. *Pdyn*, *Nr3c1* and *Gadd45* mRNA levels characterized extreme phenotype groups before and after surgery. Distinct mRNA levels of *Pdyn* gene characterized sociability- and anxiety-like phenotypes while glucocorticoid receptor nr3c1 mRNA along with high Gadd45 mRNA levels was characteristic for the depressive-like phenotype. (A) Sociability-like, (B) Anxiety-like and (C) Depressive-like behaviour. # $P < 0.05$ main effect of the phenotype (two-way ANOVA).

A high level of glucocorticoid receptor *Nr3c1* gene expression was found in the amygdala of low depressive-like behaviour mice, while the high depressive-like mice as well as intermediate group of mice showed a low level of the *Nr3c1 mRNA*. The glucocorticoid receptor *Nr3c1* mRNA and *Gadd45* mRNA levels were characteristic for the depressive-like phenotype ($F_{(2, 41)}$ = 3,414; P<0.05 and $F_{(2, 32)}$ = 3,585; P<0.05), respectively (Figure 6C).

Sciatic nerve injury affected expression profile of genes selected for their role in the neuronal activity *(Npas4),* stress *(Tsc22d3, Nr3c1)*, inflammation *(C1q, I1beta)* and signalling *(Gadd45, Crh, Pdyn)* (Table 3). The aforementioned mRNAs levels were all elevated following partial sciatic nerve injury in the amygdala. In particular, *Pdyn* mRNA level was increased almost two-fold (t = -2.9311, df = 81.865, p-value = 0.004) following nerve injury. Elevated levels of *Pdyn* mRNA in the amygdala were also closely linked to pain perception and anhedonia after nerve injury (Figure 7A,B,C).

Figure 7.

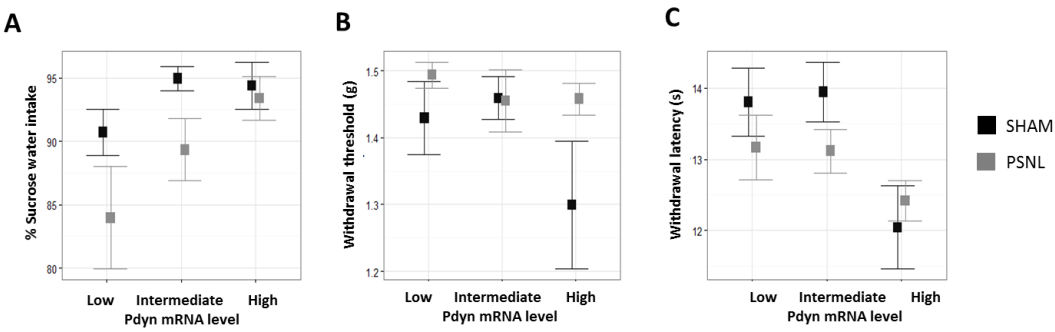

Figure 7. Evaluation of relationship between *Pdyn* expression in the amygdala with anhedonic state, mechanical allodynia and thermal sensitivity in sham and operated mice. High levels of *Pdyn* gene expression was interlinked to stronger mechanical allodynia and increased sucrose preference. Relation between *Pdyn* mRNA levels and (A) Anhedonic state represented as percentage of sucrose preference during 24 hour sessions, (B) Mechanical allodynia evaluated by the von Frey model and expressed as withdrawal thresholds in g and (C) Heat hyperalgesia evaluated in the plantar test and expressed as paw withdrawal latencies. ★ $P < 0.05$ main effect of the surgery # $P < 0.05$ main effect of the *Pdyn* mRNA level (two-way ANOVA).

Nerve injury attenuated sucrose preference. Lower *Pdyn* mRNA level was associated with decreased sucrose preference what was further potentiated by nerve injury. On the other hand, higher

PDYN mRNA level was related to higher sensitivity to painful stimuli.

4. Discussion

In this study a correlation between spontaneous CeA neuronal activity and sociability-like behaviour was revealed. Mice with high sociability-like behaviour showed higher spontaneous CeA activity, while the lowest activity was found in the least sociable mice. The relation between amygdala and sociability has been widely reported, as amygdala has a functional role in social behaviours, aggression and cognition in nonhuman primates (Machado et al. 2008) and rodents (Amaral et al. 2002). The medial amygdala, which relays information to the CeA (Pitkänen et al. 1997), is critical for social male mice behaviour (Meredith et al. 2004; Pereno et al. 2011) and lesions in this area result in reduced territorial aggression and social affiliation (Wang et al. 2013). In our experimental model, the sociability-like behaviour did not influence the manifestations of mechanical and thermal hypersensitivity induced by neuropathic pain. Previous studies have shown

opposite effects of sociability on pain modulation. While the social interaction with conspecifics decreases pain sensitivity (D'Amato and Pavone 2012), the recognition of emotional reactions to the pain of conspecifics can produce hyperalgesia (Langford et al 2006). However, the anxiety and cognitive manifestations of neuropathic pain were influenced by the sociability trait. According to our results, sociability could enhance the development of anxiety and cognitive impairment associated to neuropathic pain, since mice with high sociability trait displayed the highest anxiety levels and the worst cognitive performance. Our findings reveal no correlation between spontaneous CeA and anxiety-like behaviour. In agreement several studies showed that the amygdala, and specially the CeA, has a crucial role in fear, but did not play an important role in anxiety control (Davis et al. 1997, 2010). Data obtained with our experimental model show that the anxiety-like behaviour does not exert a clear influence neither on the mechanical nor thermal hypersensitivity induced by neuropathic pain. Opposite effects of the high anxiety trait have been shown depending on the nociceptive modality (Jochum et al. 2007, Roeska

et al. 2009). In contrast, our results, suggest that anxiety trait is a predisposing factor to the development of anxiety and cognitive impairments associated with neuropathic pain. The highly anxious mice showed the most severe impairments in the anxiety-like behaviour and memory induced by neuropathic pain, while the least anxious animals showed the mildest manifestations. No correlation between the CeA neuronal activity and the depressive trait was observed. Our data revealed that depression trait influenced several nociceptive manifestations of neuropathic pain. Indeed, mice with low depressive-like behaviour enhanced the manifestations of mechanical and thermal allodynia, whereas they showed decreased expression of thermal hyperalgesia. A decrease of mechanical hypersensitivity has been previously reported in rats with depressive-like behaviour in a spinal nerve injury model (Shi et al. 2010b). However, controversial results have been previously obtained in clinical studies have reported both positive (Chiu et al. 2005; Burke et al. 2013) and negative (Bär et al. 2007; Shi et al. 2010a) correlation of depression with pain sensitivity. Nevertheless, our results do not show implication of depression

trait neither on emotional nor cognitive manifestations of neuropathic pain.

The amygdala is involved in the consolidation of memories of emotionally arousing experiences (McGaugh, 2004) like fear, anxiety and social interactions (Kalin et al., 2004; Cassidy and Gutchess, 2012). A relationship between expression of some genes and sociability, anxiety and depression-like behaviour was demonstrated in the present study. A few studies showed that low sociability is related to higher levels of anxiety (Kudryavtseva et al., 2004; Tõnissaar et al.,2008). The highly anxious as well as the least sociable mice showed the highest level of *Pdyn* mRNA. In contrast, low anxiety and high sociability phenotypes were associated with low expression of *Pdyn* mRNA. Thus, our study indicates that *Pdyn* gene expression in the amygdala appears to be associated with anxiety and sociability phenotypes. Moreover, the relationships between gene expression and the phenotypes are remained after sciatic nerve injury and development of neuropathic pain as more anxious and less sociable mice retained its elevated *Pdyn* mRNA level. Bilkei-Gorzo (2014) have recently demonstrated that genetic

deletion of the *Pdyn* gene showed a superior partner recognition ability while pharmacological blockade of kappa opioid receptors (KORs) led to an enhanced social memory in wild-type animals. Another study has suggested that prodynorphin system may play a key role in anxiety (Knoll et al., 2011). Data obtained from different reports however, do not provide a consistent picture of the functions of *Pdyn* in anxiety. Low *Pdyn* expression was reported to be associated with reduced anxiety in rats (Menard et al., 2014), but *Pdyn* deletion increased anxiety-like behaviors, impaired the anxiolytic effect of bromazepam (Femenia 2016). In contrast to the above reports anxiolytic parameters of explorative behaviour in mice lacking *Pdyn* were increased in the open field, the elevated plus maze and the light-dark tests. Consistent with this, treatment of wild-type mice with selective kappa opioid receptor antagonists GNTI or norbinaltorphimine showed similar effects. Furthermore, treatment of *Pdyn* knockout animals with U-50488H, a selective kappa receptor agonist, fully reversed their anxiolytic phenotype (Wittmann et al., 2009).

On the other hand, the present study shown that *Pdyn* mRNA level in the amygdala was not associated with depressive-like phenotype. Interestingly, the depressive-like phenotype was associated with the N*r3c1* mRNA levels. This observation is in agreement with several studies reporting associations of glucocorticoid receptor with depression and depressive disorders. Recent data suggest that higher levels of methylation at the *Nr3c1* promoter may be associated with major depressive disorder (Nantharat et al. 2015). As DNA methylation typically acts to repress gene transcription, our results support the hypothesis that decreased level of *Nr3c1* receptor mRNA is a predisposing factor to the development of depressive-like behaviour. Importantly, low level of *Nr3c1* mRNA marking low depressive-like phenotype was not affected by neuropathic pain. Furthermore, our study indicates that depression-like behaviour appears to be associated with the expression of *Gadd45* gene. High depression trait was associated with a higher expression of the *Gadd45* gene in the amygdala. The gene appears to be involved in synaptic plasticity and memory formation (Sultan et al. 2013).

Sciatic nerve injury altered expression of *Pdyn* and *Gadd45* genes and several genes involved in the neuronal activity *(Npas4),* stress *(Tsc22d3, Crh)* and inflammation *(C1q, I1beta, Il6)* in the amygdala. A few study demonstrated previously that expression of some of the tested genes is altered in the limbic system of rat (Ulrich-Lai et al, 2006, del Rey et al. 2011, Burke et al. 2013). Further studies, however, are required to clarify the links between the altered genes and their functions in the amygdala and pain states.

The results reported in this study highlight the interest of the combinatorial use of behavioural, electrophysiological and genetic approaches. This approach may help in understanding the mechanisms that may explain the inter-individual variation of the neuropathic pain manifestations. This study represents a relevant step towards the development of highly efficient personalized treatments for chronic pain.

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Objective 3

To evaluate the involvement of the dynorphin/KOR system in the behavioural, histological, emotional, cognitive, neurochemical and epigenetic manifestations of chronic osteoarthritis pain in mice.

Article 3.

Involvement of the dynorphin/KOR system on the nociceptive, emotional and cognitive manifestations of osteoarthritis pain in mice

Negrete R, García-Gutiérrez MS, Manzanares J, Maldonado R

Neuropharmacology, 2016

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DISCUSSION

The necessity of a chronic pain model with translational relevance

At the present moment, there is a lack of effective treatment for chronic pain, and the complexity outcomes of pain experience in humans evidences the difficulties for preclinical drug discovery. A research priority is the validation of preclinical animal models that could be translated to the different behavioural manifestations of human pain experience. The aim of our first study was to validate different behavioural outcomes to evaluate the nociceptive, affective and cognitive manifestations of neuropathic pain in mice. For this purpose, we evaluated at different time points after PSNL the nociceptive responses, the anxiety- and depressive-like behaviours, as well as the anhedonic state, the object recognition memory and the operant responding maintained by food. The effects of a chronic treatment with pregabalin, a well-accepted medication to treat neuropathic pain in humans (Verma et al., 2014), was evaluated on these manifestations. In this Thesis, we have also validated these nociceptive, affective and cognitive manifestations of chronic pain in a model of osteoarthritis pain induced by MIA in mice, a well-established rodent model for

osteoarthritis (Guingamp et al., 1997). We demonstrated the development of different emotional and cognitive manifestations associated to chronic pain in both osteoarthritis and neuropathic pain models, such as an increased depressive- and anxiety-like behaviours, and memory deficits.

Our results showed an increased anxiety-like behaviour in the elevated plus maze, and impairment in memory function by the reduction of the discrimination index in the object recognition memory one week after neuropathic pain. Accordingly, both memory impairment and increased anxiety were revealed after the induction of chronic osteoarthritis pain in our model included in the third article of this Thesis (Negrete et al., 2016).

The forced swimming test did not show depressive-like responses during the early stage after neuropathic pain. The increased depressive-like behaviour appeared at later stage after PSNL, suggesting that the depressive symptoms are manifested only once pain persists along time. Indeed, depressive-like behaviour usually appears later than anxiety-like behaviour after neuropathic pain induction (Yalcin et al., 2011). Interestingly, the anhedonic state

related to depressive-like behaviour was increased after neuropathic pain, as revealed by a decrease in the preference for sucrose from the early time points. The reduced consumption and preference for high palatable sweet solutions have been previously used to reveal the anhedonic-like state in different models of chronic pain (Andersen et al., 2009; Bura et al., 2010, 2013). Anhedonia is defined as the inability to feel pleasure (Marbach et al., 1983), and is widely used as robust predictor for pathological depression. In agreement, a pronounced decrease in the preference for sucrose during osteoarthritis was also revealed in the third article of this Thesis (Negrete et al., 2016). However, in our second study, this anhedonic-like state was not significantly revealed in our second study, probably due to the additional behavioural responses that were evaluated at the same time in these selected personality traits mice.

The increased anhedonia observed in the neuropathic pain model seems to be associated with reduced reward responsiveness. Pain and reward play opponent roles that may interact and influence each other. Indeed, a rewarding stimuli is able to reduce pain

sensitivity (Leknes and Tracey, 2008), and pain may impair reward circuitry, as suggested by the increased in the anhedonic-like responses after chronic pain. We have further investigated the relationships between neuropathic pain and reward responsiveness and motivation by using an operant paradigm. Neuropathic pain impaired the operant responding. Indeed, the efforts required to obtain highly palatable food were increased under the fixed ratio (FR) 5 schedule, whereas the responsiveness to obtain standard or highly palatable food under FR1 were not affected. These results suggest that neuropathic pain leads to a loss of gratified responses, probably due to a dysregulation of the mesolimbic dopaminergic pathways responsible for the emotional processing circuit. Moreover, the results found under FR5 were also reflected by the reduced motivation to obtain a reward after neuropathic pain revealed under the progressive ratio (PR) schedules. In this PR schedule, progressively more effort is required to earn each subsequent food pellet. Interestingly, this reduction in the motivation did not depend on the reinforcement itself, since an

analogous decrease in the break point was shown in both standard and highly palatable food.

Concurrently, we demonstrated that nociception and the emotional and cognitive components of chronic pain are not necessarily expressed at the same experimental conditions. Indeed, chronic pregabalin treatment administered (20 mg/kg twice daily) showed antinociceptive effects, as well as improvements in anxiety, depressive-like behaviour, memory alterations and operant responses in our neuropathic pain model. Higher doses of this drug could not be evaluated since they induce important locomotor changes in rodents that would interfere in the behavioural responses (Liu and Chen, 2014). Our results correlate with several studies in animals and humans. Thus, pregabalin has been reported to produce antinociceptive effects in neuropathic pain models in rodents (Baastrup et al., 2011; Gustafsson and Sandin, 2009), and to relief pain perception, anxiety, depression and sleep quality of patients with peripheral neuropathic pain (Verma et al., 2014). Indeed, our results showed that pregabalin treatment significantly increased the percentage of time spent in the open arms of the

elevated plus maze, as well as the discrimination index on the object recognition memory, although this effects remained significantly lower in PSNL mice compared with sham operated mice after chronic pregabalin treatment. These effects to the anxiolytic-like responses of pregabalin in mice (Navarrete et al., 2012) did not reduce the increased immobility time on the forced swimming test after neuropathic pain.

Pregabalin treatment completely abolished the impairments in the operant tasks under FR5 and the anhedonic-like state, probably as a direct consequence of pain relief, although a direct effect of pregabalin on the reward circuitry could not be excluded. Indeed, we have also observed a reduced response during PR schedule in sham operated mice after chronic treatment with pregabalin. These results suggest a possible impairment on motivation produced by pregabalin. The mechanisms by which pregabalin could exhibit those impairing effects have not been explored. However, it has been reported that pregabalin would decrease the release of several neurotransmitters, including glutamate and dopamine in

those brain regions involved in reward processing by reducing calcium currents (Spencer et al., 2014).

The impact of personality traits in the inter-individual variability of chronic pain manifestations

The complexity of chronic pain mechanisms, the occurrence of comorbidities such as affective and cognitive impairments, and the enormous variability in pharmacological responses between patients may contribute to the difficulty of clinicians to palliate chronic pain symptoms. As previously reported, affective, social and cognitive personality traits can widely modulate pain perception (Apkarian et al., 2005; La Porta et al., 2015). This is one of the critical aspects to take into consideration for the validation of preclinical models with translational relevance for the human clinical conditions. In this study, we aimed to evaluate the influence of specific personality traits on the nociceptive, affective and cognitive manifestations of neuropathic pain. For this purpose, we have selected mice displaying extreme phenotypes on social and affective responses, such as anxiety- and depressive-like behaviour

and evaluated the possible influence of these personality traits on the inter-individual variability of pain manifestations after the induction of neuropathic pain. We have also evaluated the possible correlation of the spontaneous and evoked CeA neuronal activity and the behavioural responses. Indeed, increased excitability of the CeA has been demonstrated in models of arthritis (Neugebauer, 2015) and neuropathic pain (Ikeda et al., 2007). Interestingly, our results showed that mice with high social-like behaviour revealed higher spontaneous CeA activity, whereas the lowest neuronal activity was shown in the mice selected for low sociability trait. Accordingly, the AMY has been reported to play a crucial role in social behaviours in nonhuman primates (Machado et al., 2008) and rodents (Amaral, 2002).

Anxiety and cognitive impairments after chronic neuropathic pain were also influenced by the sociability trait. Thus, mice with high social-like behaviour showed high anxiety-like responses and a significant enhancement of the memory impairment after neuropathic pain. However, as other authors previously reported (D'Amato and Pavone, 2012; Langford et al., 2006), social

personality trait did not influence in the nociceptive manifestations of neuropathic pain in our experimental conditions.

Our results also reveal that anxiety personality trait has no correlation with spontaneous CeA activity. Accordingly, it has been reported that the AMY, and specially the CeA do not play an important role in anxiety modulation (Davis et al., 2010). Although anxiety-like phenotype did not modify the nociceptive manifestations of neuropathic pain, this personality trait seems to play a crucial influence in the development of anxiety and cognitive impairments during neuropathic pain. Thus, the high anxiety selected mice showed significantly enhancement of anxiety-like responses and memory dysfunctions after the PSNL.

Finally, our findings revealed that depressive personality trait does not correlate with the spontaneous or evoked CeA neuronal activity. However, some nociceptive responses have been modified in mice selected for these different depressive-like phenotype. Thus, low depressive-like behaviour selected mice showed enhanced mechanical and thermal allodynia, while thermal hyperalgesia was decreased in these mice after the induction of

neuropathic pain. Controversial results have been reported correlating depression and pain sensitivity. While some studies correlate depression and poor sleep with an increased nociceptive threshold in rats (Burke et al., 2013; Shi et al., 2010), others reported a reduced pain perception in depressed patients (Bär et al., 2007; Chiu et al., 2005).

The relationships between the profile expression of specific genes and the social, anxiety and depressive phenotypes have also been evaluated in our study. Our gene profile results suggest the involvement of PDYN gene in the emotional and cognitive manifestations associated to chronic neuropathic pain. Thus, mice selected for high anxiety, as well as mice displaying low social phenotype, showed the highest level of PDYN mRNA expression in the AMY, while low anxiety and high social behaviour selected mice were related to low expression of PDYN in this brain area. Several studies have reported using genetically modified mice that PDYN gene plays an important role in anxiety and social behaviours (Bilkei-Gorzo et al., 2014; Cahill et al., 2014). In addition, pharmacological treatment with KOR antagonists also produced

anxiolytic-like effects in mice (Wittmann et al., 2009). In agreement with the results obtained in neuropathic pain, the results of the third article of the present Thesis using KOR or PDYN KO mice also demonstrate the crucial role of the dynorphin/KOR system in the emotional and cognitive manifestations associated to chronic osteoarthritis pain (see last section of the discussion).

PDYN gene expression in the AMY was not related to depressive personality trait. Interestingly, we demonstrated that depressive-like behaviour modulates the expression of the glucocorticoid nuclear receptor subfamily 3, group C, member 1 (NR3C1) gene. Thus, mice selected for low depressive-like phenotype showed high levels of NR3C1 mRNA expression in the AMY, while high depressive-like mice showed the lowest levels of NR3C1 expression.

Depressive-like phenotype is also associated to the expression of the growth arrest and DNA damage (Gadd45) gene. Indeed, mice selected for high and low depressive trait showed higher expression of Gadd45 in the AMY. Gadd45 has been reported to regulate synaptic plasticity and memory formation and it is over-expressed in the CNS under pathological states (Sultan and Sweatt, 2013).

In addition, our results also revealed the influence of chronic neuropathic pain on the expression profile of other genes in the AMY. Thus, the mRNA expression of several genes involved in the neuronal plasticity (Npas4), stress (Tsc22d3) and inflammation (C1q, l1beta, IL6) in the AMY appears to be altered after the induction of neuropathic pain.

A neurobiological substrate for the comorbidities of chronic pain: Involvement of the dynorphin/KOR system

The endogenous opioid system participates in the modulation of cognitive and affective components of chronic pain (Bodnar, 2015). Interestingly, a particular component of this endogenous opioid system, the dynorphin/KOR system, seems to play a critical role in the nociceptive component of osteoarthritis pain (Shen et al., 2005), and participates in the control of affective and cognitive processes (Cahill et al., 2014; Hang et al., 2015).

In our study, we aimed to identify the involvement of the dynorphin/KOR system in the nociceptive, emotional, cognitive, neurochemical and epigenetic manifestations of chronic

osteoarthritis pain in mice. For that purpose, we have used genetically modified mice deficient in KOR or PDYN gene and the behavioural, histopathological and neurochemical alterations associated with osteoarthritis chronic pain were evaluated after intra-articular MIA administration (Guingamp et al., 1997). The MIA model of osteoarthritis has been widely described in rats (Fernihough et al., 2004; Sagar et al., 2010) and more recently in mice (Burnham and Dickenson, 2013; La Porta et al., 2013). Although most of the chemically osteoarthritis models did not correlate with the pathogenesis of human osteoarthritis (Lampropoulou-Adamidou et al., 2014), the MIA model produces histological alterations similar to those clinically found in the disease (Harvey and Dickenson, 2009; Kobayashi et al., 2003; La Porta et al., 2014). According to previous histopathological studies with this model (La Porta et al., 2013), our results showed cartilage alterations after intra-articular administration of MIA. No significant histological changes in the knee joint were observed when compared mutant mice and their WT littermates.

Our results showed that mechanical allodynia produced by MIA was enhanced in mice lacking KOR or PDYN gene. Accordingly, previous studies have reported that KOR activation produces antinociceptive effects in other models of chronic pain (Obara et al., 2003), and a down-regulation of KOR has been shown in fibroblast-like synoviocytes in patients with osteoarthritis (Harvey and Dickenson, 2009; Kobayashi et al., 2003; Shen et al., 2005). Interestingly, our results reported significant microglial activation by an increased Iba-1 expression in the lumbar section of the spinal cord at later stages of osteoarthritis development. However, no significant alteration was observed in the microglia expression induced by osteoarthritis pain in the different genotypes. These results suggest that the dynorphin/KOR system is not necessary for the increased microglia activation promoted by MIA. In contrast, no significant changes were revealed in the spinal astrocyte activation after osteoarthritis pain in the different genotypes. In agreement to our results, dynorphin KO animals do not show astrocyte activation after peripheral nerve injury (Cahill et al., 2014), although it has been reported that chronic pain may lead to astrocyte activation in the

spinal cord (Raghavendra et al., 2003). This controversial results may be explained due to the critical neuron-glia signal role of the dynorphin/KOR system in chronic pain states (Xu et al., 2007).

The dynorphin/KOR system is involved in sensory process, but also seems to play a crucial role in the development of emotional and cognitive dysfunctions after chronic pain (Cahill et al., 2014; Lalanne et al., 2014). Our results showed that genetic suppression of the dynorphin/KOR system components produced anxiolytic-like responses by increasing the time spent in the open arms of the elevated plus maze after osteoarthritis pain induced by MIA. In addition, a downregulation of CRF gene was revealed in mice lacking KOR or PDYN gene under basal conditions in both AMY and HIP, underlying the role of this system in the modulation of anxiety-like responses. As stated before, CRF and dynorphins are co-expressed in the basolateral and CeA, and CRF induces dynorphin release in the hypothalamus, striatum and spinal cord producing KOR activation (Kang-Park et al., 2015). After osteoarthritis pain, CRF expression was not altered in both KOR and PDYN mutants compared to saline treated mice, correlating with the crucial role of

this dynorphin/KOR system in the regulation of anxiety during chronic pain.

We have also investigated the involvement of the dynorphin/KOR system in the cognitive impairments produced during chronic osteoarthritis pain. As previously reported (La Porta et al., 2015), our results showed cognitive impairment at later stage after osteoarthritis pain, that was significantly ameliorated in mice lacking KOR, but not in PDYN KO mice. These results suggest that the blockade of KOR plays a protective role in the memory dysfunctions associated to osteoarthritis pain.

We have also evaluated the possible epigenetic alterations that could participate in the control of gene expression after chronic osteoarthritis pain. Recently, a growing number of studies provide valuable information about the epigenetic modifications occurring during chronic pain that may guide crucial advances for new treatments (Descalzi et al., 2015; Liang et al., 2015; Tran et al., 2015). Since lysine 9 in histone 3 (H3K9ac) plays an important role in histone acetylation and the regulation of gene promoters activation (Descalzi et al., 2015), we evaluated the acetylation levels

of H3K9ac in the AMY to correlate with osteoarthritis pain manifestations. Our results showed a downregulation of H3K9 acetylation in the AMY at the later stage after osteoarthritis pain in WT and PDYN KO mice. These changes may promote a more condensed chromatin structure, which could prevent gene transcription and finally contribute to the nociceptive, emotional and cognitive manifestations of chronic pain (Descalzi et al., 2015; Liang et al., 2015). In contrast, changes in H3K9 acetylation were significantly attenuated after osteoarthritis pain in mice lacking KOR, suggesting that this epigenetic mechanism could be involved in the positive effects of the blockade of the dynorphin/KOR system on the emotional and cognitive manifestations of chronic pain.

General considerations

In the present Thesis, we have validated behavioural models to evaluate the nociceptive, affective and cognitive manifestations of chronic neuropathic pain and we have validated our model using a first-line therapeutic drug, pregabalin. Our models may represent excellent tools to study the pathophysiological mechanisms underlying the different manifestations of chronic pain. The behavioural manifestations associated to chronic pain mainly depend on the personality traits of the patients, and represent a challenge for the development of highly efficient treatments for chronic pain. Thus, we have also identified the influence of these personality traits on the inter-individual variability on the nociceptive, emotional and cognitive components of neuropathic pain in mice. Our results suggest that specific behavioural traits can be of significance to predict this high vulnerability to chronic pain.

Finally, we have validated the dynorphin/KOR system as a neurobiological substrate of the nociceptive, emotional and cognitive manifestations related to osteoarthritis pain in mice.

Together, we provide new insights to understand the complexity of chronic pain, and an interesting pharmacological target for the management of chronic pain (Figure 27).

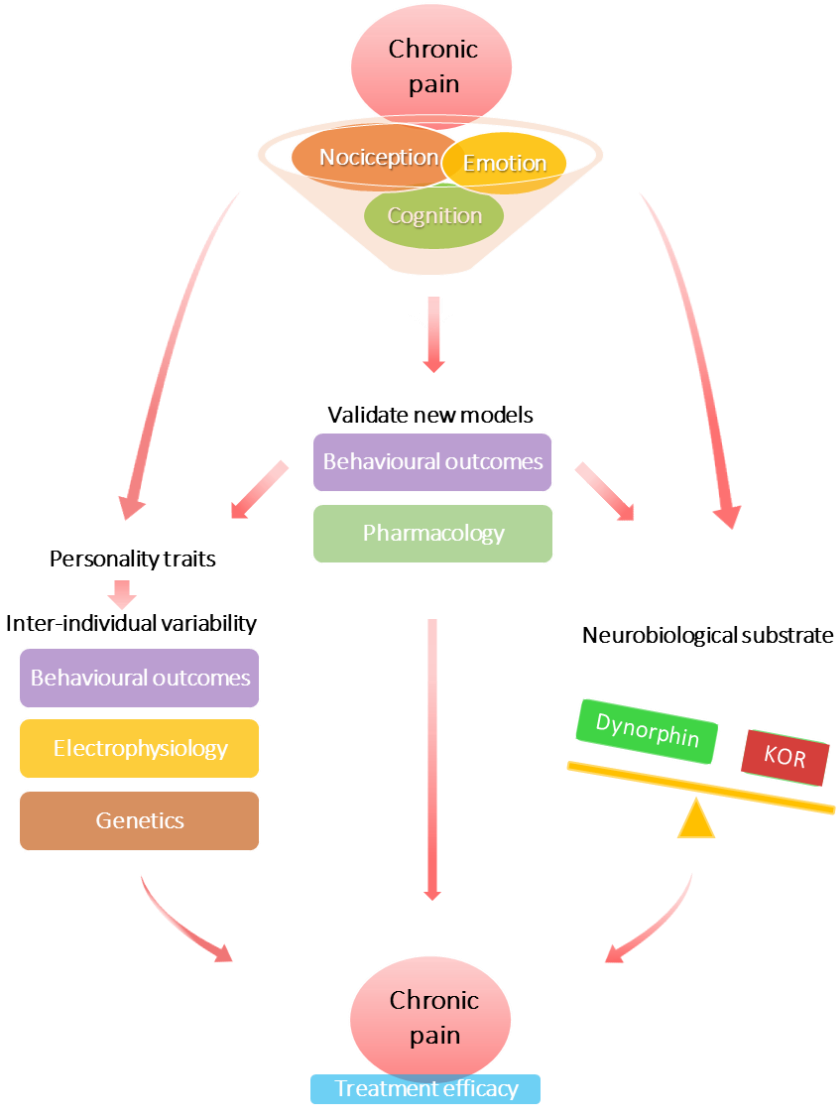

Figure 27. Schematic representation of the present work of this Thesis.

CONCLUSIONS

The main conclusions of the work presented in this Thesis can be summarised as follows:

1. The presence of allodynia and hyperalgesia in mouse model of neuropathic pain is associated with increased anxiety- and depressive-like behaviours, reduced memory functions, the development of an anhedonic-like state and impaired motivation in operant tasks.
2. Depressive-like behaviour appeared at later stages than anxiety-like behaviour and memory deficits in our chronic neuropathic pain model, suggesting that depressive symptoms are manifested only once pain persists during a long period.
3. Reduced responses in operant tasks are shown after chronic neuropathic pain when the efforts required to obtain highly palatable food were increased under the FR5 and PR schedule.
4. Chronic treatment with pregabalin improves nociceptive responses, anxiety- and anhedonic-like behaviour, memory impairment and operant performance during chronic

neuropathic pain, although depressive-like behaviour was not ameliorated in the forced swimming test.

5. Spontaneous neuronal CeA activity was increased in mice selected for high social phenotype and intermediate group for depression, while no differences in this activity were revealed in mice selected for the anxiety personality trait.
6. Mechanical allodynia and thermal hyperalgesia were enhanced after the induction of neuropathic pain in mice selected for low depressive trait.
7. High anxiety trait showed enhanced impairments in the anxiety-like behaviour and memory disruption after neuropathic pain.
8. High anxiety trait as well as low social phenotype mice showed increased levels of PDYN mRNA expression in the AMY, while low anxiety and high sociability phenotypes were associated to low expression of this gene.
9. Low depressive personality trait was associated in mice to high levels of NR3C1 mRNA expression, while high

depressive-like mice showed low levels of NR3C1 expression in the AMY.

10. Mechanical allodynia is enhanced in mice lacking KOR and PDYN gene after chronic osteoarthritis pain.
11. The dynorphin/KOR system does not participate neither in the development of the histological manifestations nor the increased glial activation on the spinal cord promoted by osteoarthritis pain.
12. The pronounced increase in the anhedonic-like state revealed in mice lacking dynorphin/KOR system during osteoarthritis might be influenced by the enhanced nociceptive responses in these genetically modified mice.
13. Mice deficient in KOR and PDYN gene presented an attenuation in the anxiety-like behaviour and reduced levels of CRF expression under basal conditions in AMY and HIP. This downregulation is not altered by chronic osteoarthritis pain exposure.

14. Memory impairment is reduced in KOR-KO mice, but not in PDYN-KO mice, suggesting a cognitive protective role of this opioid receptor during osteoarthritis pain.
15. Osteoarthritis pain down-regulated histone H3K9 acetylation in the AMY. These changes were attenuated in mice lacking KOR, but not PDYN, suggesting that this epigenetic mechanism could be involved in the positive effects of KOR blockade in the emotional and cognitive manifestations of chronic osteoarthritis pain.
16. The dynorphin/KOR system plays a crucial role on the different manifestations associated to osteoarthritis pain in mice, and may be an interesting pharmacological target for the management of these chronic pain manifestations.
17. Together, we provide new insights to better understand the complexity of chronic pain, and we suggest an interesting pharmacological target for the management of chronic pain.

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ANNEX

Annex

Article 4.

Involvement of the endocannabinoid system in osteoarthritis pain

La Porta C*, Bura AS*, Negrete R, Maldonado R

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*Equal contribution

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