Effects of behavioural traits and the endogenous opioid system in neuropathic pain manifestations

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"Lo preocupante no es la perversidad de los malvados sino la indiferencia de los buenos"
Martin Luther King

"La vida es muy peligrosa. No por las personas que hacen el mal, sino por las que se sientan a ver lo que pasa"
Albert Einstein

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gracias por ser cómplices de mis sueños.

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Abstract

Neuropathic pain is a complex disorder that includes nociceptive, emotional and cognitive manifestations. This work was focused on how inter-individual variability and endogenous opioid system may be associated with these manifestations. For this purpose, we first validated behavioural paradigms to measure the emotional and cognitive manifestations. Then, we evaluated the influence of specific behavioural traits as well as the role of preproenkephalin (*Penk*) on neuropathic pain manifestations. We found that time-dependent consequences of neuropathic pain were detected by classical paradigms to evaluate anxiety and depressive-like behaviours, and cognitive function in mice. Amygdalar *Pdyn* and *Gadd45* genes were identified as biomarkers of the influence of anxiety and depression traits on neuropathic pain manifestations. Penk deletion produced a ceiling behavioural effect in anxiety and cognition after neuropathic pain that was related with the maintained amygdalar expression of *Pdyn KOR, Npas4* and *Nr3c1* and with hippocampal decreased expression of *Nr3c1* respectively. In this work, classical behavioural paradigms were capable to detect the influence of behavioural traits and the role of preproenkephalins in nociceptive, emotional and cognitive manifestations of neuropathic pain. These pain manifestations were influenced by anxiety and depression traits. Finally, preproenkephalin was identified as a key component in the development of physiological and behavioural changes induced by neuropathic pain.

Resumen

El dolor neuropático es un trastorno complejo que incluye manifestaciones nociceptivas, emocionales y cognitivas. En este trabajo se estudió cómo la variabilidad interindividual y el sistema opioide endógeno se asocian a estas manifestaciones. Para ello, primero se validaron varios modelos clásicos de comportamiento para evaluar las respuestas emocionales y cognitivas. Después, evaluamos la influencia de los rasgos específicos de comportamiento, así como el papel de la preproencefalinas (*Penk*) en las manifestaciones del dolor neuropático inducido por ligadura parcial del nervio ciático en ratones. Se observó que era posible detectar las manifestaciones del dolor neuropático dependientes del tiempo a través de paradigmas clásicos utilizados para evaluar comportamientos como la ansiedad, la depresión y la función cognitiva. Se identificaron los genes *Pdyn* y *Gadd45* en amígdala como biomarcadores de la influencia de los rasgos de ansiedad y depresión en las manifestaciones del dolor neuropático. La deleción de *Penk* produjo un efecto techo en el desarrollo de ansiedad y déficits cognitivos producidos por el dolor neuropático. Este efecto techo se relacionó con el mantenimiento de la expresión de *Pdyn*, KOR, *Npas4* y *Nr3c1* en la amígdala y con la disminución en la expresión de *Nr3c1* en el hipocampo, respectivamente. En la presente tesis, a través de paradigmas clásicos de comportamiento, se detectó la influencia de rasgos específicos de comportamiento y el papel de las preproencefalinas en las manifestaciones nociceptivas, emocionales y cognitivas asociadas a dolor neuropático. Estas manifestaciones fueron sensibles principalmente

a la ansiedad y depresión. Finalmente, se identificó la preproencefalina como un componente esencial para el desarrollo de los cambios fisiológicos y comportamentales inducidos por el dolor neuropático.

Abbreviations

BLA: Basolateral amygdala
BNST: Bed nucleus of the stria terminalis
CCD: Chronic compression of DRG
CCI: Chronic constriction injury
CeA: Central amydgala
CFA: Complete Freund's adjuvant
CREB: cAMP response element-binding protein
Crh: Corticotropin releasing hormone
CRPS: Complex regional pain syndrome
cVLM: Caudal ventrolateral medulla
DH: Dorsal horn
DOR: Delta opioid receptor
DR: Dorsal rhizotomy
DRG: Dorsal root ganglion
EOS: Endogenous opioid system
ERK: Extracellular signal–regulated kinase
FR1: Fixed-ratio 1
FR5: Fixed-ratio 5
Gadd45: Growth arrest and DNA-damage-inducible 45
GDP: Guanosine diphosphate
GluR1: Glutamate receptor 1
il: Interleukin
KO: Knock-out
KOR: Kappa opioid receptor
LC: Locus coeruleus
MEK: Methyl ethyl ketone
MOR: Mu opioid receptor
mTOR: Mammalian target of rapamycin
N/OFQ: Nociceptin/orphanin FQ
NOP: Nociceptin receptor
Nr3c1: Nuclear receptor subfamily 3, group C, member 1/Glucocorticoid receptor
PAG: Periaqueductal grey matter
Pdyn: Prodynorphin
Penk: Preproenkephalin
PFC: Prefrontal cortex
PR: Partial rhizotomy
PSNL or PSL: Partial sciatic nerve ligation
RVM: Rostral ventromedial medulla
SCI: Spinal cord injury
SNI: Spared nerve injury
SNL: Spinal nerve ligation
TRPV1: Transient receptor potential cation channel subfamily V member 1
VTA: Ventral tegmental área
WDR: Wide dynamic range

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INTRODUCTION

1 What is pain?

1.1 Some conceptual and operational definitions

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey and Bogduk, 1994). Therefore, pain is a multidimensional experience that cannot be defined exclusively in nociceptive terms; it also comprises thoughts, feelings and behaviours that integrate the subjective painful perception. For a better understanding of the IASP pain definition it is important to consider the participation of two components in the pain experience (Bosch and Baños, 2009):

- Nociceptive or sensorial: It is the sensation as consequence of the transmission of painful stimuli from peripheral nerves to the brain.
- Affective or reactive: It refers to the effect of pain in the psychological domain as well as the influence of psychological factors in the appearance of suffering associated to pain.

The contribution of these components varies depending on the individual characteristics and the type of pain. For example, in acute pain the predominant component is the nociceptive while the affective is more important in the chronic state (Bosch and Baños, 2009). Besides this contribution of the type of pain, the individual contribution to the final painful feelings is modulated by the

personality of each patient, which includes previous pain experiences but also the genetic load (Asghari and Nicholas, 2006; Mogil, 1999).

1.2 Classification of pain: a pathophysiological approach

Pain has been traditionally classified using its intensity (mild, moderate or severe), quality (sharp, burning or dull), duration (acute or chronic) or referral (superficial or deep, localized or diffuse). However, these classifications only consider one of the elements of the pain experience, i.e. intensity, quality or duration, and have limited value to understand what pain is and, the most important, its role in the patient's life and how should be treated. In this way, Woolf (2004) proposed an operational classification that considers the physiological value of pain for each individual. In this approach, pain can be divided into two categories: adaptive and maladaptive. Adaptive pain is essential for the survival as it protects the organism in case of injury. In other words, this type of pain would be very important for the body integrity of each individual, as it avoids the continued exposure to injuries. At the end, pain may enhance the survival of living beings as it enhances their withdrawal from dangerous injuries. Therefore, it is an important physiologic process that guarantees the survival of the species. Adaptive pain includes the so-called physiologic and inflammatory pain. The first mediates the needed withdrawal reflex from potentially injuring stimuli, whereas the second helps the body to fully restore the tissues after the injury has occurred. This theory is substantiated by the fact that

those individuals who exhibit insensitivity to painful stimuli have a smaller life expectancy that those with intact pain responses (Hunt and Mantyh, 2001; Julius, 2001; Scholz and Woolf, 2002; Woolf and Salter, 2004).

In contrast with the adaptive pain, the maladaptive pain has no recognized physiological value and it is only a source of suffering. Probably, it is an expression of some unknown pathologic operations of the nervous system that produces pain without any advantage for the individuals that experience it. In this group, Woolf (2004) includes the neuropathic and the functional pain. Most diseases included in this group only produce pain, and suffering without any beneficial physiological consequences. In these patients, pain is not a symptom of a disease: Pain is the disease.

These four types of pain are based in specific neurobiological mechanisms (Woolf and Salter, 2004):

- Nociceptive pain: The nociceptive system is a high-threshold sensory system that mediates the sensory experience of acute pain when it is produced by a noxious stimulus. This system connects the periphery and the cerebral cortex, where the sensation is perceived, through the spinal cord, brain stem and thalamus (Figure 1A). Nociceptive pain mediates the physiological responses to injuries without phenotypic changes and with a short and transient activation.
- Inflammatory pain: Once the tissue is damaged, a mechanism of repair is triggered and, as a part of this

response, pain appears. This adaptive process was already recognized in the ancient Roman medicine as the Celso's signs of inflammation: pain, oedema (consequence of the increase of capillary permeability), redness and heat (both from vasodilation and increased blood flow). These responses are mainly due to the fact that cells from injured tissues release substances that can activate or sensitize sensory neurons as well as from vasodilation effects on small blood vessels. At the same time, while tissues are restored, their cells release chemicals mediators that sensitize and/or cause pain, a phenomenon known as neuronal sensitization. Inflammatory pain decreases when the damage is resolved and these biochemical mechanisms wane and eventually disappear (Figure 1B).

- Neuropathic pain: It is a consequence of neural damage that can affect the peripheral or central nervous system dealing to peripheral or central neuropathic pain. The first appears, for example, in patients with diabetic or AIDS polyneuropathy, post-herpetic neuralgia or lumbar radiculopathy. Central neuropathic pain is common in spinal cord injury, multiple sclerosis or stroke, where the injury appears in spinal cord or encephalic structures as Figure 1C shows.
- Functional pain: This type is produced by an abnormal responsiveness or function of the nervous system. In this situation patients commonly localized the presence of pain in a different place from where the injury occurred.

Conditions as fibromyalgia, irritable bowel syndrome, some forms of non-cardiac chest pain and tension-type headache are included in this classification (Figure 1D).

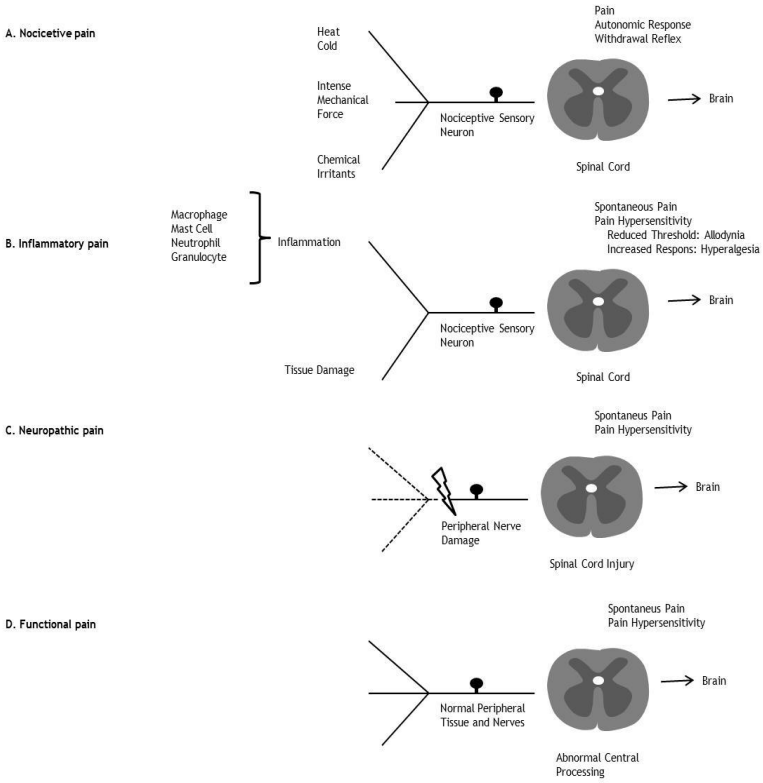

Figure 1. The four types of pain according Woolf's classification (modified from (Woolf and Salter, 2004).

The inflammatory, neuropathic and functional pains share some characteristics. In these syndromes, spontaneous or evoked pain may appear. The first occurs in the absence of any peripheral stimulus whereas the second is a consequence of such stimuli. Evoked pain may also be classified in allodynia and hyperalgesia. Allodynia is the pain due to a low intensity or normal stimulus that does not cause pain in normal circumstances, while hyperalgesia is

an exaggerated and prolonged response to painful stimuli. In the nociceptive type, pain occurs only following a high intensity or noxious stimulus and the spontaneous pain and sensitivity changes do not appear (Merskey and Bogduk, 1994; Woolf and Salter, 2004). These differences have important repercussions in the clinical setting and follow different pathophysiological mechanisms as explained later.

1.3 Neurobiological basis of pain

1.3.1 Peripheral mechanisms

In 1906 Sherrington introduced the concept of *nociceptive nerves* and later the term *nociceptor* to refer to the receptors of pain in sensory neurons (Sherrington, 1911). Nociceptors are located in the terminals of afferent neurons and are responsible of noxious stimuli detection. Such as all primary afferent neurons, neurons that express nociceptors have its cellular body located in the dorsal root ganglion (DRG) with two axons. The first ends into the dorsal horn of the spinal cord whereas the second connect with peripheral organs and constitutes the sensory fibre itself. The activation of the nociceptor is the initial step in a series of sequential mechanisms that finally leads to the sensation of somatic and visceral pain. Nociceptors depolarize and generate action potential in response to noxious stimuli. Nociceptors are specialized on the detection of intense stimuli that represent a risk of tissue injury and therefore they have high threshold activation (Dubin and Patapoutian, 2010). In the other hand, there are several primary afferent sensory neurons that

encode a continuous range of stimuli below the noxious threshold, for example mechanoreceptors for soft touch. Nociceptors are classified according to the stimulus that activates them (thermal, chemical, mechanical). Additionally, different subgroups have been identified: mechanical and heat, heat, mechanical, heat and cold, and mechanical-insensitive and heat-insensitive (Hunt and Mantyh, 2001).

Sensory afferent nerves can also be classified according the diameter and conduction velocity of their axons as well as the size of their cell bodies. Based on axon characteristics they can be defined as (Eilers and Schumacher, 2005; Hunt and Mantyh, 2001):

- Aβ fibres: Neurons with large axons (diameter of 6 μm - 22 μm), heavily myelinated with fast conduction velocity (33 m/s-75 m/s).
- Aδ fibres: Fibres with medium axons (diameter of 2 μm - 5 μm), thinly myelinated with intermediate conduction velocity (5 m/s-30 m/s).
- C fibres: Fibres with small axons (diameter of 0.3 μm - 3 μm), unmyelinated with slow conduction velocity (0.5 m/s-2 m/s).

In the absence of tissue injury, only Aδ and C fibres carry nociceptive signals and Aβ fibres convey non-nociceptive stimuli (such light touch). However, in some circumstances after tissue injury, these stimuli may be felt as painful (Eilers and Schumacher, 2005; Hunt and Mantyh, 2001).

1.3.2 Central mechanisms

From the dorsal horn to the brainstem and limbic system areas, noxious stimuli are conveyed and processed by three neural pathways: spinothalamic, spinomesencephalic and spinoreticular tracts. The dorsal horn contains nociceptive specific neurons, that only respond to noxious stimuli and wide dynamic range (WDR) neurons that are activated by both noxious and innocuous stimuli. The first are mainly located in the superficial layers of the dorsal horn (lamina I) whereas WDR neurons are located predominantly in the deep dorsal horn layers (laminae IV–V). Their axons cross the midline within one or two segments and ascend to brain through the spinothalamic tract. In the lateral spinothalamic tract, most axons originate from lamina I neurons whereas those of the anterior spinothalamic tract originate from deep dorsal horn neurons. These spinothalamic tract neurons project to the lateral nuclei of thalamus, whereas medial nuclei receive input predominantly from lamina I neurons (Brooks and Tracey, 2005; Latremoliere and Woolf, 2009). Cells of these thalamic nuclei project to various cortical areas. Medial and lateral thalamic nuclei and their particular cortical projection targets are called medial and lateral pain systems respectively. Ascending pathways activate neurons in several brain areas involved in the somatosensory component of pain (for example, thalamic nuclei and somatosensory cortex) or the affective component of pain (mainly limbic structures, including the hypothalamus, amygdala and anterior cingulate cortex). The nociceptive system consists of different and partially independent

pathways that ascend in parallel from the spinal cord dorsal horn to the cortex. Descending projections at all levels modulate these pathways (Baron, 2006; Boadas-Vaello et al., 2016).

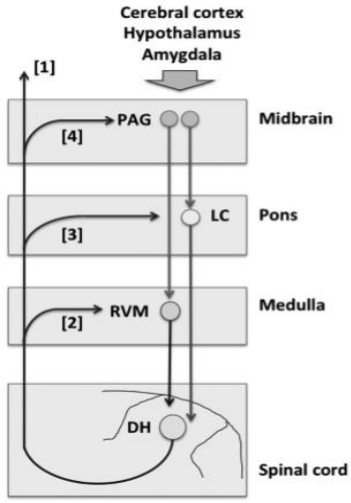

Figure 2. Ascending and descending nociceptive somatosensory pathways Taken from (Boadas-Vaello et al., 2016)**.** Nociceptive neurons of the dorsal horn (DH) project their axons to thalamic neurons through the spinothalamic pathway, [1] the spinobulbar pathway (it projects to neurons of the ventrolateral reticular formation, dorsal reticular nucleus, nucleus tractus solitari and rostral ventromedial medulla (RVM), [2] the spinopontine pathway which projects to neurons of the parabrachial nuclei and locus coeruleus (LC) [3], and the spinomesencephalic pathways that project to neurons of periaqueductal grey matter (PAG) [4]. PAG neurons project their descending axons to neurons located in LC and RVM. RVM and LC neurons directly project their axons to the spinal DH (where the nociceptive input is initially processed). PAG neurons receive input from forebrain structures (cerebral cortex, hypothalamus and amygdala).

Descending pain control arises from a number of supra-spinal sites that include:

- The midline periaqueductal grey matter (PAG) - rostral ventromedial medulla (RVM) system (Heinricher et al., 2009).

- Neurons from PAG do not project directly to the spinal cord. Their main descending projection is received from the RVM that includes the nucleus raphe magnus and the adjacent reticular formation. RVM neurons receive innervation from the PAG and project to the dorsal horn through the dorsolateral funiculus, establishing synapses with superficial and deep layers of neurons of the dorsal horn (Fields and Heinricher, 1985; Heinricher and Ingram, 2010).
- Two types of specialized neurons are found in the RVM: the "ON" and the "OFF" cells. The 'ON' cells are activated just before the withdrawal from a noxious stimulus, while the 'OFF' cells have the opposite function and are pausing during withdrawal from noxious stimulus. It has been suggested that 'ON' cells facilitate, while 'OFF' cells inhibit, pain transmission. In experimental models of neuropathic and inflammatory pain it was demonstrated that descendent pain signalling toward the dorsal horn level in the spinal cord is facilitated throughout the hyperexcitation and sensitization of specific nociceptive and WDR neurons in the spinal cord and in the RVM neurons. In the RVM nucleus a potentiation of the 'ON' and a decrease of the 'OFF' neuron responses are observed. Therefore, 'ON' cells exert a pro-nociceptive effect, whereas 'OFF' cells produce an anti-nociceptive effect (Boadas-Vaello et al., 2016; Zhang and Ko, 2009).

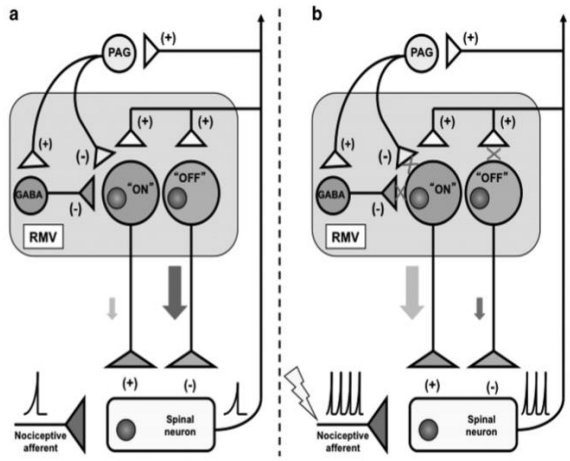

Figure 3. The process of activation and inactivation of 'ON' and 'OFF' neurons from RVM. Taken from (Boadas-Vaello et al., 2016). (a) In physiological conditions, pain sensation travels through the spinothalamic pathway when nociceptors signal depolarizes spinal cord nociceptive neurons. In the brainstem, these action potentials stimulate PAG neurons and 'ON'/'OFF' neurons from RMV. In consequence, PAG neurons stimulate GABAergic interneurons located in RMV and inhibit 'ON' neurons. In this way, 'ON' neurons are inhibited and 'OFF' neurons are excited, producing a decrease in the firing response. (b) After peripheral and/or central nervous system injury, hyperexcitability of spinal cord neurons changes the expression of ion channels and receptors, and consequently 'ON' neurons are insensitive to inhibitory inputs from GABAergic interneurons and PAG neurons, whereas they become more sensitive to the inputs coming from the spinothalamic pathway. In addition, these molecular changes also affect 'OFF' neurons that are insensitive to excitatory inputs from the spinothalamic pathway. Therefore, pain produces a potentiation of 'ON' neuron inputs over spinal cord neurons that produce enhancement of neuronal activity from RVM.

- The dorsal reticular (DRt) nucleus.
    - DRt nucleus stimulation causes hyperalgesia in acute pain, whereas its inactivation induces analgesia in acute

and persistent pain. The descending pronociceptive fibres from DRt nucleus establish synaptic contacts upon lamina I neurons that project back to DRt nucleus. Excitatory synaptic contacts also occur between DRt nucleus projecting spinal cells and spinally projecting dorsal reticular nucleus neurons (Almeida, 1999; Tavares and Lima, 2007).

- The caudal ventrolateral medulla (cVLM).
    - cVLM is one of the main inhibitory components of the endogenous pain modulatory system. Its stimulation induces analgesia in acute pain. The reticular formation known as cVLMlat (the most lateral part of the caudal ventrolateral medulla) seems to be the main region responsible for pain modulation. Neurons of cVLMlat project to the spinal laminae involved in pain transmission (laminae I, IV, V and X). The cVLMlat targets the dorsal horn indirectly through the pontine A5 noradrenergic cell group. Terminals of descending pathways originating in the RVM and other brainstem nuclei (for example, nucleus raphe magnus, A5, A6 and A7 nuclei) interact with afferent fibres, interneurons and projection neurons in the dorsal horn. The main neurotransmitters involved in the descending pathways are serotonin and noradrenaline. The predominant source of serotonin input to the spinal cord came mainly from the nucleus raphe magnus (Ossipov and Gebhart, 1986; Tavares et al., 1996; Tavares and Lima, 2007).

The thalamus mediates the different components of pain, i.e. sensory discriminative (lateral pain pathway) and affective-motivational (medial pain pathway). It has an important role in modulating nociceptive information, central sensitization and hyperexcitability of neurons from the spinothalamic pathway. This modulatory function results not only in an increased stimulation of thalamic neurons via glutamate neurotransmission, but also sensitization via calcium influx (Ab Aziz and Ahmad, 2006; Obara et al., 2013).

1.4 Pathophysiology of pain

1.4.1 Contribution of peripheral sensitization

When a peripheral injury occurs, primary afferent neurons (C and Aδ fibres) develop abnormal activity (Baron, 2006). The main pathological changes that appear in the periphery due to the stimulation of nociceptors are the appearance of ectopic and spontaneous discharges, abnormal nerve conduction, alterations of the ionic channel expression, collateral sprouting of primary afferent neurons, sprouting of sympathetic neurons and nociceptor sensitization (Baños et al., 2003).

Ectopic spontaneous activity and the spontaneous discharges are consequences of an increase in spontaneous firing in the afferent neurons linked to the injury site, which originates from the dorsal root ganglia and along the nerves. This ectopic activity is developed in the injury sensory neurons and in their uninjured neighbours. This abnormal activity is related with the increased expression of

mRNA for voltage-gate sodium channels (VSC) in primary afferent neurons. These neurons selectively expressed the genes that encode VSC Nav 1.8 and Nav 1.9. Also, the up-regulation of the embryonic VSC Nav 1.3 in damaged peripheral nerves is associated with increased electrical excitability. Sodium channel clusters accumulate in the site of the lesion and in the intact dorsal root ganglia. They produce membrane potential oscillations as a consequence of an up-regulation of rapidly repriming III tetrodotoxin-sensitive (TTX-S) channels and down-regulation of tetrodotoxin-resistant (TTX-R) channels (Baños et al., 2003; Baron, 2006).

The up-regulation of some protein receptors in the membrane of primary afferent is also induced by the damage to peripheral nerves. Some of these receptors are marginally expressed under physiological conditions. TRPV1 (Transient Receptor Potential cation channel subfamily V member 1, also known as capsaicin receptor or vanilloid receptor type 1) are the responsible of sensing noxious heat (>43°C). After nerve injury, a TRPV1 down-regulation on damaged afferents and a novel expression of TRPV1 on injured C and A-fibres appears. Recently, it was reported that TRPV1 is also up-regulated in medium and large DRG cells (Ma et al., 2005). A cold and menthol sensitive TRP channel (TRPM) has been identified in the small diameter DRG neurons (Patapoutian et al., 2003). This receptor is normally activated in 8-28°C range. Changes in the expression of this channel after injury might lead to the peripheral sensitization of cold-sensitive C-nociceptors and, as a result, the appearance of cold hyperalgesia. Nerve injury also

triggers the expression of functional α_1 and α_2-adrenoceptors on cutaneous afferent fibres, which could also be involved in the peripheral sensitization. These changes in adrenoceptors produce adrenergic sensitivity in the neurons (Baron, 2006).

The collateral sprouting of primary afferent neurons appears when the fibres of the primary afferent neurons spread in their vicinity and eventually establish new synapses. Sprouting induction is produced as consequence of nerve growth factor action at the level of dorsal root ganglia. Nerve growth factor is associated with the wallerian degeneration and the release of tumour necrosis factor α (TNF-α). Sprouting of sympathetic neurons (noradrenergic perivascular sympathetic postganglionic axons) into the DRG forms baskets around the large diameter neurons that do not transmit pain under physiological conditions. Functional synapses-like structures are established between the terminals of the sprouted neurons and the cell bodies, activating the neurons and having as consequence the aberrant transmission of pain (Baños et al., 2003).

1.4.2 Contribution of central mechanisms

The nociceptor hyperactivity increases the excitability of multireceptive spinal cord neurons, the called central sensitization. This reactive process is the responsible of the amplification and facilitation of the synaptic transfer from the nociceptor to the dorsal horn neurons of the spinal cord. In general terms, the main changes that occur in these neurons are: Increased neuronal activity, expansion of neuronal receptive fields and spread of hyperexcitability to other spinal segments. Several neurochemical

changes follow. Pathologically sensitized C-fibres release glutamate, which acts on postsynaptic N-methyl-D-aspartate (NMDA) receptors, as well as substance P. After peripheral nerve injury, the VCC Nav 1.3 is expressed in the postsynaptic dorsal horn neurons in an abnormal way. The mitogen-activated protein kinase system (MAPK) contributes, among other intracellular cascades, to this central sensitization. One of the consequences of this pathological state is the appearance of pain following innocuous tactile stimuli via Aα and Aβ fibres, a situation known as mechanical allodynia (Baños et al., 2003; Baron, 2006; Woolf, 2004).

Other neurochemical processes also follow the peripheral nerve injury. In absence of this state, the nociceptive transmission in the dorsal horn neurons is modulated by an inhibitory tone of γ-aminobutyric acid (GABA) releasing interneurons. Loss of these cells by apoptosis may appear when a peripheral nerve injury occurs. The reduction of this inhibitory mechanism intensifies the central sensitization. Another intraspinal disinhibition mechanism that involves a trans-synaptic reduction in the expression of the potassium-chloride exporter KCC2 in lamina I neurons has been described. The resulting change in the transmembrane anion gradient causes normally inhibitory anionic synaptic currents to be excitatory. The consequence is that GABA release exerts an excitatory action on lamina I neurons, which increases in turn the central sensitization (Baron, 2006).

Supraspinal brainstem centres have a modulating control function over the dorsal horn neurons. This modulation can be both

inhibitory and potentiating. A loss of function in descending inhibitory serotonergic and noradrenergic pathways may contribute to central sensitization as well as to a transition to chronic pain (Ossipov et al., 2000; Vanegas and Schaible, 2004). Although the focus of the central sensitization has been placed on the dorsal horn, some studies have revealed the presence of sensitized neurons in thalamus and primary somatosensory cortex after partial peripheral nerve injury (Guilbaud et al., 1992).

Another important mechanism that allows the transition from a peripheral injury to chronic pain is the spinal reorganization. Damaged Aβ-fibres sprout into the lamina II of the dorsal horn (normally innervated by C-fibres) and establish functional synaptic contacts with second order neurons (Fig. 4). As a consequence, low-threshold non-noxious inputs can be interpreted as nociceptive by the conscious mind. The consequence of phenotypic changes of Aβ-fibres is the appearance of changes in nociceptors, substance P and CGRP (Bridges et al., 2001).

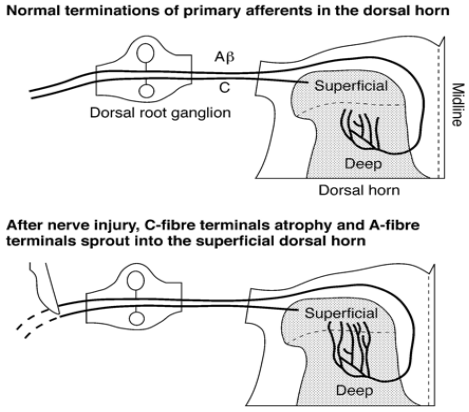

Figure 4. Schematic representation of reorganization of the spinal dorsal horn observed after peripheral nerve injury (Bridges et al., 2001).

1.4.3 Contribution of neuroimmunological mechanisms

Besides these adaptive processes, immunological changes appear after nerve injury. Activated macrophages from the endoneural blood vessels are released into the injured nerve and dorsal root ganglia, and deliver proinflammatory cytokines, mainly TNF-α. At the lesion site, these cell mediators induce ectopic activity in injured and adjacent uninjured primary afferent neurons. In inflammatory neuropathies (vasculitic or HIV neuropathies) deep proximal aching and paroxysmal pain are characteristic clinical symptoms. In nerve biopsy of these patients, an up-regulation of COX_2 and pro-inflammatory cytokines was found. In complex regional pain syndrome (CRPS) patients, increased levels of IL-6 and TNF-α are present in the fluid of artificially produced skin blisters in the affected extremities (Baron, 2006). Macrophage-like cells are rapidly activated after nerve injury. They probably are the main source of cytokines and chemokines that alter cell phenotypic properties or patterns of gene transcription by acting on neurons and supporting glia. These changes in peripheral glia (Schwan cells) produce the release of TNF-α and several growth factors. These signalling substances contribute to the direct activation of neighbouring injured and non-injured sensory fibres. A similar role is developed by astrocytes (Woolf and Mannion, 1999).

2 The case of neuropathic pain

2.1 *Historical aspects*

The concept of pain and theories about it date to ancient civilizations. The first scientific approaches arise with the Greek civilization with Plato, Aristoteles and Empedocles theories (Bosch and Baños, 2009). The Roman physician Galen (second century A.C.) proposed a theory where the brain was the receptor and modulator of sensations, including pain. He also identified the dysfunction of the nervous system as a cause of chronic pain (Bennett, 2010; Bosch and Baños, 2009).

In 1840 Müller postulated the theory of specific energies. In short, this theory established that the external and internal information was conveyed to the brain through nerves, and that the stimulation of every sensory nerve gave a specific sensation. Von Frey contributed with the assumption of the nerve specificity and of the existence of nerve pathways from the periphery to the central nervous system (Serra Catafau, 2007).

In the nineteenth century, the extended use of guns in wars resulted many traumatic nerve injuries suffered by surviving people. In 1864 Silas Weir-Mitchell published several observations of pain associated with peripheral nerve injuries in soldiers during the American civil war. He was the first to use the term “causalgia” to refer to the current CRPS II. However, the special nature of neuropathic pain remained unrecognized until the late 1970s when a specific terminology emerged (Bennett, 2010).

2.2 Definition

Neuropathic pain is defined by the IASP as the pain that is initiated or caused by a primary lesion or dysfunction in the nervous system (Merskey and Bogduk, 1994). It is commonly associated with abnormal sensory signs as spontaneous pain, allodynia and hyperalgesia (Bridges et al., 2001). These sensations are the result of physiological changes in the peripheral and/or central nervous system including spontaneous neuron discharges, alterations of ion channel expression, sprouting of primary afferent neurons, peripheral and central sensitisation, spinal reorganization and changes in inhibitory pain descending pathways (Woolf and Mannion, 1999).

2.3 Classification of neuropathic pain syndromes

Neuropathic pain syndromes are chronic pain disorders that cannot be explained by a single aetiology or specific lesion. In these conditions the relation between aetiology, mechanisms, and symptoms is complex (Fig. 5) One mechanism could be common and responsible of the pain that appears in different diseases. In the other hand, in two patients the same symptom may be caused by different mechanisms. Also, more than one mechanism can operate in a single patient and then change with time. Thus, the prediction of the mechanisms responsible for pain based only in the aetiology or on the distribution and nature of the symptoms is an almost impossible task (Woolf and Mannion, 1999).

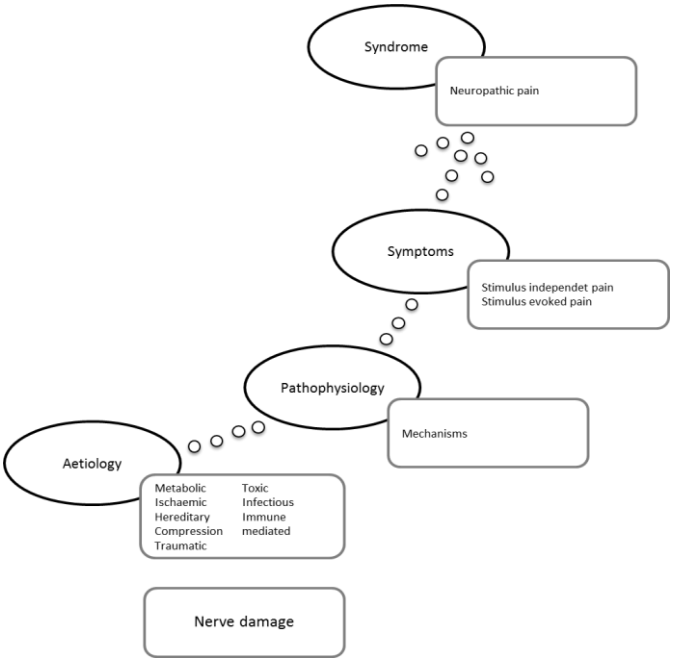

Figure 5. Aetiology, mechanisms, and symptoms of neuropathic pain (Woolf and Mannion, 1999).

Therefore, neuropathic pain classification is a complex issue. However, for diagnosis and treatment purposes, it is commonly classified on the basis of the aetiology of the injury or the anatomical distribution of the pain. Regarding to the aetiology of neuropathic syndromes, the following classification has been proposed (Baron, 2006; Woolf and Mannion, 1999):

- Peripheral focal and multifocal nerve lesions of traumatic, ischemic or inflammatory origin. This includes entrapment syndromes, phantom limb pain, stump pain, post-traumatic neuralgia, postherpetic neuralgia, diabetic mononeuropathy, ischaemic neuropathy or poliarteritis nodosa.
- Peripheral generalized polyneuropathies with toxic, metabolic, hereditary or inflammatory causes. It includes

diabetes mellitus, plasmocytoma, HIV neuropathy, hypothyroidism, hereditary sensory neuropathies, Fabry's disease, Bannwarth's syndrome (neuroborreliosis), vitamin B deficiency and toxic neuropathies (arsenic, thallium, chloramphenicol, metronidazole, nitrofurantoin, isoniazid, vinca alkaloids, taxol, gold).

- Central nervous system injuries. It includes stroke, multiple sclerosis, spinal cord injury, brain infarction (specially the thalamus and brainstem), spinal infarction, syringomyelia and multiple sclerosis.
- Complex neuropathic disorders. It includes CRPS and Sudeck's atrophy. These painful disorders can develop as a disproportionate consequence of trauma and typically affect the limbs. The CRPS are classified in type I and type II. Type I usually develops after trauma in the limbs without obvious nerve injury (bone fracture, surgery). Type II develops after trauma that is associated with a lesion of a large nerve.

2.4 Clinical Picture

Both pathophysiological changes in the central and peripheral nervous system are observed in neuropathic pain. Clinical counterparts of those changes are the appearance of positive (abnormal spontaneous or evoked sensations) and negative (sensory deficits) (Baños et al., 2003). Pain evoked sensations are classified as hyperalgesic, allodynic or dysesthetic and according to the dynamic or static character of the stimulus as well. The condition

depends on the nature of the stimulus, resulting in heat, cold or mechanical hyperalgesia or allodynia. Finally, dysaesthesia is an abnormal and unpleasant sensation, different from pain. Beside these symptoms, there is also a condition within spontaneous pain called paraesthesia, an abnormal not unpleasant sensation that is different from pain. Sensory clinical examination of neuropathic pain symptoms should include touch, pinprick, pressure, cold, heat, vibration and temporal summation.

Table 1 summarizes several sensory signs and symptoms that can be found in painful neuropathies and a brief description of the clinical appropriate tests to assess them. Evaluation is carried out in the area of maximal pain and using the contralateral area as control (Bridges et al., 2001).

Table 1. Signs and symptoms in painful neuropathies and the tests used to assess these symptoms clinically (Bridges et al., 2001).

Symptom/sign	Definition	Assessment	Pathological response
Negative signs and symptoms			
Hypoesthesia	Reduced sensation to non-painful stimuli	Touch skin with painter's brush, cotton swab or gauze	Reduced perception, numbness
Pallhypoesthesia	Reduced sensation to vibration	Apply tuning fork to bone or joint	Reduced perception threshold
Hypoalgesia	Reduced sensation to painful stimuli	Prick skin with single pin stimulus	Reduced perception, numbness
Thermohypoesthesia	Reduced sensation to cold or warm stimuli	Touch skin with objects of 10 ºC (metal roller, glass of water, coolants like acetone) Touch skin with objects of 45 ºC (metal roller, glass of water)	Reduced perception
Spontaneous sensations/pain			
Paraesthesia	Non-painful on-going sensation (ant crawling)	Grade intensity (0-10) Area in cm^2	_

Paroxysmal pain	Shooting electrical attacks for seconds	Number per episode Grade intensity (0-10) Threshold for evocation	_
Superficial pain	Painful on-going sensation, often of burning quality	Grade intensity (0-10) Area in cm^2	_
Evoked pain			
Mechanical dynamic allodynia	Normally non-painful light-pressure moving stimuli on skin evoke pain	Stroking skin with painter's brush, cotton swab or gauze	Sharp burning superficial pain in the primary affected zone, spreading into unaffected skin areas (secondary zone)
Mechanical static allodynia	Normally non-painful gentle static pressure stimuli on skin evoke pain	Manual gentle mechanical pressure to the skin	Dull pain in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Mechanical punctate or pinprick hyperalgesia	Normally stinging-but-not painful stimuli evoke pain	Manual pricking of the skin with a safety pin, sharp stick or stiff von Frey hair	Sharp superficial pain in the primary affected zone, spreading into unaffected skin areas (secondary zone)
Temporal summation	Repetitive application of identical single noxious stimuli is perceived as increasing pain sensation (wind-up-like pain)	Pricking the skin with safety pin at <3 s intervals for 30 s	Sharp superficial pain of increasing intensity
Cold allodynia	Normally non-painful cold stimuli evoke pain	Touch skin with objects of 20 ºC (metal roller, glass of water, coolants like acetone) Control: touch skin with objects of skin temperature	Painful, often burning, temperature sensation in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Heat allodynia	Normally non-painful heat stimuli evoke pain	Touch skin with objects of 40 ºC (metal roller, glass of water) Control: touch skin with objects of skin temperature	Painful burning temperature sensation in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)

Mechanical deep somatic allodynia	Normally non-painful pressure on deep somatic tissues evokes pain	Manual light pressure at joints or muscle	Deep pain in joints or muscles

2.5 Current available treatments and limitations

Neuropathic pain is difficult to treat, as it is generally resistant to available drugs such as opioid and non-steroidal anti-inflammatory drugs (NSAIDs) that are effective in relief nociceptive or inflammatory pain (Baños et al., 2003). Systematic reviews of neuropathic pain treatment have shown that only 60 to 70% of patients achieved moderate pain relief after pharmacological treatment (Collins et al., 2000; Sindrup and Jensen, 1999). Many patients with neuropathic pain do not receive appropriate treatment because of different reasons: low diagnostic accuracy, ineffective drugs and insufficient knowledge about effective drugs and their appropriate use in clinical practice (Finnerup et al., 2016).

There is no single treatment that works for all neuropathic conditions and their underlying mechanisms (Finnerup et al., 2016). Treatment of neuropathic pain comprises pharmacological and non-pharmacological approaches. Non-pharmacological treatments include: complementary therapies (acupuncture), physical modalities (physical rehabilitation), psychological approaches (behaviour modification, relaxation training), spinal cord stimulators and invasive therapies (nerve blocks, ablative surgery, trigger–point injections, epidural steroids, sympathetic blocks), surgical techniques (dorsal root entry zone lesions, cordotomy, sympathectomy) (Chong and Bajwa, 2003). Within the

pharmacological therapies can be mentioned: antidepressants, antiepileptics, antiarrhythmics, topical local anesthetics, capsaicin and opioids (Baños et al., 2003). Most patients receive multiple agents with different mechanisms of action that work together to diminish the peripheral and central manifestations of pain. Current treatments of neuropathic pain present serious side effects, leading to discontinuation of some agents used in the treatment (Namaka et al., 2004).

The evidences of efficacy of the available pharmacological treatments are summarized in the Table 2.

Table 2. Efficacy evidences of the available pharmacological treatments (Attal and Bouhassira, 2015)**.**

Drug	Main mechanisms of action	Common major side effects	Precautions	Other benefits	Efficacy: Level A/B rating[a]
Tricyclic antidepressants					
Nortriptyline Desipramine Amitriptyline	Inhibition of reuptake of monoamines, block of sodium channels, antimuscarinic effects	Somnolence, antimuscarinic effects, weight gain	Cardiac disease, glaucoma, prostatic adenoma, seizure, use of tramadol	Improvement of depression, although at generally higher dosages than in pain (75 mg/h) and sleep (amitriptyline)	A. Diabetic neuropathy, PHN B. Spinal cord injury/central poststroke pain, traumatic nerve injury, cancer neuropathic pain
Serotonin–norepinephrine reuptake inhibitors					
Duloxetine	Inhibition of serotonin and norepinephrine reuptake	Nausea	Hepatic disorder, use of tramadol, hypertension	Improvement of depression and generalized anxiety, improvement of sleep	A. Diabetic neuropathy
Venlafaxine	Inhibition of serotonin and norepinephrine reuptake	Nausea, hypertension at high dosages	Cardiac disease, hypertension, use of tramadol	Improvement of depression and generalized anxiety, improvement of sleep	A. Diabetic neuropathy

Calcium channel α2δ ligands					
Gabapentin	Acts on $\alpha_2\delta$ subunit of voltage-gated calcium channels, which decreases central sensitization	Sedation, dizziness, peripheral edema, weight gain	Reduced dosages in renal insufficiency	No clinically significant drug interactions, improvement of generalized anxiety and sleep	A. Diabetic neuropathy, PHN, cancer neuropathic pain B. Spinal cord injury pain
Pregabalin	Acts on $\alpha_2\delta$ subunit of voltage-gated calcium channels, which decreases central sensitization	Sedation, dizziness, peripheral edema, weight gain	Reduced dosages in renal insufficiency	No clinically significant drug interactions, improvement of generalized anxiety and sleep	A. Diabetic neuropathy, PHN, spinal cord injury
Topical therapy					
Lidocaine 5% plasters	Block of sodium channels	Local erythema, itch, rash	None	No systemic side effects, potential effect on allodynia	A. PHN
Capsaicin patches 8%	TRPV1 agonist	Pain, erythema Elevated blood pressure due to initial increase in pain	None	No systemic side effects-potential effects on burning pain, itch, and allodynia	A. HIV neuropathy and PHN
Opioid agonists					
Tramadol	MOR agonist and inhibition of monoamine reuptake	Nausea and vomiting, constipation, dizziness, somnolence	History of substance abuse, suicide risk, use of antidepressant in elderly patient	Rapid onset of analgesic effect, effect on inflammatory pain	A. Diabetic neuropathy, phantom pain B. Spinal cord injury
Morphine Oxycodone Methadone Levorphanol	MOR agonists (oxycodone may also cause KOR antagonism)	Nausea and vomiting, constipation, dizziness, somnolence	History of substance abuse, suicide risk, risk of misuse on long-term use	Rapid onset of analgesic effect, effect on inflammatory pain	A. Diabetic neuropathy, PHN, phantom pain

Abbreviations: HIV, human immunodeficiency virus; PHN, postherpetic neuralgia; TRPV1. [a] Level A: good scientific evidence from several Class I trials (upper-class); Level B: some scientific evidence from Class II trials (lower-class trials).

2.6 Experimental models for the study of experimental neuropathic pain

Although the neuropathic pain has been reported for over 150 years, until recently there was no proper animal model to study these conditions (Qu and Ma, 2011). The first neuropathic pain model was reported 38 years ago by Wall et al (Wall et al., 1979). They established the neuroma model by completely transecting the sciatic nerve. More recently, it has been developed an increased use and reliance on animal models to study of fundamental biological processes and causes of disease (Mogil, 2009). These animal models are essential for a better understanding of sensory and psychological complexities of pain. The study of pain relies extensively on preclinical animal models for the discovery of novel targets and the development of better analgesics. However, the major challenge is the translation of discoveries made in animal models to realized pain therapies in humans (Burma et al., 2017). The experimental models of neuropathic pain developed included central nervous system and peripheral nerves injuries (Figure 6).

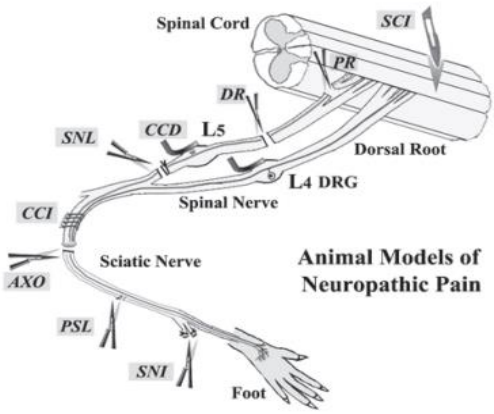

Figure 6. Schematic representation of some animal models of central or peripheral neuropathic pain. *Abbreviations* SCI: spinal cord injury, PR: partial rhizotomy, DR: dorsal rhizotomy, CCD: chronic compression of DRG, SNL spinal nerve ligation, CCI chronic constriction injury to the sciatic nerve, AXO neuroma or axotomy–autotomy, PSL partial sciatic nerve ligation (also named PSNL), SNI spared nerve injury.

2.6.1 Models bases on the injury of central nervous system

Preclinical models of injury to central nervous system to produce central neuropathic pain are scarce. Most of them produce injury at the spinal cord by trauma, neurological disease, or infection. The spinal cord injury models range from contusion to hemisection of the spinal cord to excitotoxic and ischemic damage to the cord. Consistent phenotypes of behavioural hypersensitivity are produced in all these models and they give powerful tools to understand the mechanisms of develop and maintenance of chronic pain after spinal cord injury. Models of neurological disease are not usually used as models of pain; the exception is the multiple sclerosis. There are two models of multiple sclerosis to study nociceptive responses. Migraine and HIV-induced central neuropathies models became as the result of the central nervous system infection. They provide information about the mechanisms responsible of pain facilitation after an infecting agent is introduced in the central nervous system. In Table 3, the principal animal models used in research of central neuropathic pain are summarized (Hains and Vera-Portocarrero, 2011).

Table 3. Experimental models based on central nervous system injury

Model	Reference
Mechanical injury	
Contusion	
MASCIS impactor	(Basso et al., 1996)
Infinite Horizons impactor	(Scheff et al., 2003)
Hemisection	(Christensen et al., 1996)
Posterior rhizotomy	(Basbaum and Wall, 1991)
Dysesthesic syndrome	(Levitt et al. 1991)
Chemical injury	
Excitotoxic	
Quisqualic acid	(Yezierski et al., 1998)
Laser-induced ischemia of spinal cord	(Hao et al., 1991)
Multiple sclerosis	
Experimental autoimmune encephalomyelitis (EAE)	(Paterson, 1966)
Intracerebral inoculation of Daniels (DA) strain of Thelier's murine encephalomyelitis virus (TMEV)	(Rodriguez et al., 1983)
Infection	
Endotoxin lipopolysaccharide	(Perry and Andersson, 1992)
HIV-1 virus glycoprotein (gp120)	(Sundar et al., 1991)

2.6.2 Models based on the injury of peripheral nervous system

The most common experimental model of peripheral neuropathy is traumatic nerve injury (full or partial) by ligation, transection, or compression. The nerves used in these procedures are sciatic nerve, distal branches of the sciatic nerve, infraorbital nerve or trigeminal nerve roots. Metabolic and chemically induced neuropathies have also been used to imitate peripheral neuropathic pain associated with chemotherapy, anti-HIV therapy, and diabetes (Burma et al.,

2017). Models of animal neuropathic pain by the injury to peripheral nerves are mentioned in Table 4.

Table 4. Experimental models based on peripheral nervous system injury

Model	Reference
Mechanical injury	
Nerve section	(Wall et al., 1979)
Chronic constriction injury of sciatic nerve	(Bennett and Xie, 1988)
Partial sciatic nerve ligation	(Shir and Seltzer, 1991)
Spinal nerve ligation	(Ho Kim and Mo Chung, 1992)
Neuropathy induced by fixed diameter polyethylene cuffs on sciatic nerve	(Mosconi and Kruger, 1996)
Physical injury	
Peripheral cryogenic nerve lesion	(Deleo and Coombs, 1991)
Laser induced ischemia of sciatic nerve	(Kupers et al., 1998)
Metabolic neuropathy	
Sterptozotocin induced diabetic neuropathy	(Ahlgren and Levine, 1993)
Chemotherapy-induced neuropathy	
Vincristine administration	(Authier et al., 1999)
Paclitaxel administration	(Polomano et al., 2001)
Cisplatin administration	(Authier, 2003)
Neuroinflammation induced neuropathy	
Tumour necrosis factor administration	(Wagner and Myers, 1996)
Nerve growth factor administration	(Ruiz et al., 2004)
Complete Freund's adjuvant administration	(Eliav et al., 1999)

The mechanical methods are the most commonly used to induce peripheral neuropathic pain. Within them are those that injure part of a major nerve (chronic constriction injury or CCI and partial sciatic ligation or PSNL models) or its branches (spinal nerve ligation or SNL and spared nerve injury or SNI models). In these

models, the intact nerve fibres, which are adjacent to the injured ones, play a critical role in the development of evoked pain symptoms such as hyperalgesia and allodynia (Qu and Ma, 2011).

The PSNL or Seltzer model is produced by the unilateral ligation of one third to one half of the sciatic nerve at the upper-thigh level with a single ligature. The ligation is performed where the sciatic nerve is not fasciculated into its main distal components. Thus, partial injury eliminates sensory fibres. Nociceptive manifestations of PSNL start hours after surgery and last for up to 7 months after the nerve injury. Animals with PNSL developed tactile-defensive behaviour of the injured hind paw and also often licked it, suggesting a sign of spontaneous pain. Bilateral touch-evoked allodynia and mechanical hyperalgesia, as well as ipsilateral thermal hyperalgesia and cold allodynia, are observed in the injured animals. In the PSNL model, animals showed a decrease in withdraw thresholds to von-Frey filaments stimulation (mechanical allodynia), which is mediated principally by myelinated A-fibres. The PSNL is a model difficult to standardize and, as a consequence, behavioural effects produced by surgery are highly variable due to the differences in location and size of the ligation between individual animals (Qu and Ma, 2011).

2.6.3 Limitations of experimental current models

The main problem that has led to questioning the utility and veracity of animal models in the discovery of new therapies is the failure translation from laboratory to clinical side. There are several

examples of targets that were very promising at the preclinical stage and that fail to translate into real therapies (Burma et al., 2017).

Many of the existing limitations of basic pain research have been improved by technological advancements. Video-based behavioural algorithms are being develop for the quantification of spontaneous behaviours and allowed the evaluation of animals in their home cages over extended periods of time. Another advantage is the reduction of subjectivity by the observer. Finally, automation helps to minimize the differences related to human–animal interactions (Chesler et al., 2002a, 2002b; Jourdan et al., 2001).

Animal models in which the aetiology of the pain is endogenous, and not induced by the experimenter, will be the most useful. Genetic approaches applied to certain species have produced strains of mutants that are susceptible to the endogenous development of painful disease. There is evidence of neuropathic pain studies in this engineered mutants that showed better results that chemical o mechanical induce pain models (Burma et al., 2017; Ueta et al., 2005).

The prevalence of certain types of chronic pain conditions varies across different age demography. Some types of chronic diseases associated with pain (diabetic neuropathy for example) are more common in the geriatric population. Despite of that, the preclinical studies rarely use older animals. There is an important age-dependent divergence in cellular and behavioural pain responses that is necessary to consider (Chakour et al., 1996; Jones et al., 2001; Schmader, 2002).

It is well established that genetics has influence on pain processing and response to analgesics. An interesting interstrain variability in the development of neuropathic pain manifestations after peripheral nerve injury has been described in some animal models (Sorge et al., 2012; Zhou et al., 2001).

Recently there is an emerging realization that sexual dimorphism has an enormous impact on the development and presentation of chronic pain. Most pain studies have been carried out exclusively in male rodents. There are some theories of divergent mechanisms that may be account for sexual dimorphisms in pain. Thus, there is a complex relation between sex and genotype, and the interaction between these two variables greatly impacts the pain phenotype (Mogil, 2012; Sorge et al., 2015).

2.7 Interaction with psychological states

Chronic pain is a complex disorder often associated with emotional, cognitive and social deficits. Neuropathic pain is frequently associated with affective disorders, such as anxiety and depression. Several reports have established the association of pain with cognitive deficits, including memory, learning and decision making impairment (La Porta et al., 2016). It is also known that pain perception can be modulated by social factors as interaction with conspecifics, isolation housing and sexual behaviour (D'Amato and Pavone, 2012). There is an overlapping between brain areas involved in cognition, mood and anxiety disorders with pain modulation, suggesting a reciprocal modulatory effect (Buhle and Wager, 2010; Price and Drevets, 2012). Nociceptive, emotional,

cognitive and social alterations could aggravate each other leading to an impairment of the quality of life of patients with neuropathic pain(La Porta et al., 2016). Hereby, there are increasing evidences that pharmacological management of pain comorbidities may significantly reduce pain (Liu and Chen, 2014).

Clinical research of pain interaction with affective, cognitive and social components is largely phenomenological, whereas animal models are the best source of the precise aetiology and pathogenesis of pain and its comorbidities. Preclinical research in animals is necessary and important for increasing the understanding of the underlying mechanisms but also for promoting the development of therapeutic approaches to pain treatment (Liu and Chen, 2014).

2.7.1 The importance of social interactions in chronic pain

Chronic pain has important social consequences in terms of health care costs, working incapacity and loss of productivity. The negative impact is not limited to the physical functioning and ability to develop daily living activities; it also limits social interactions and family relationship. Pain can produce functional o social disability. The first comprises all aspects of disturbance in physical and functional activities of every day; while the social disability refers to the inadequacy in fulfilling social or interpersonal relations including sexuality and self-care (Gheldof et al., 2006). Performance in workplace is also affected in patients with chronic pain, and the disparity between self-expectations and current

performance can affect self-confidence (Chapman and Gavrin, 1999).

In the other hand, pain perception can be decreased or potentiated by social factors. There are animal studies exploring for example the interference of social isolation, social interaction or "empathy" and pain. Social isolation has a significant effect on weight gain and mechanical sensitivity in SNI mice. Isolated animals gain less weight than grouped mice; in contrast, grouped SNI mice gained more weight than isolated and SNI mice. This difference in body weight gain produced by social isolation between neuropathic and healthy animals is an indicator of impaired wellbeing. Recently, it was also demonstrated that isolation increase sensitivity to Von Frey filaments (Pitzer et al., 2016). Empathy from an evolutionary perspective is defined as the ability to discern emotional signals from others, with the objective of species survival, facilitating behaviours relevant to reproduction and escaping from predators. It was observed that the interaction with conspecific animals with neuropathic pain produced increased nociceptive responses (in the writhing test) and induced anxiogenic-like responses in the cage mates (in the elevated plus maze and the open field test). Interestingly, responses related to depression and corticosterone levels were unaltered (Baptista-de-Souza et al., 2015).

An overlapping between brain areas involved in social and pain regulation is a notable possibility. Recently, it was demonstrated that social rejection and physical pain share a common representation in somatosensory brain systems (Kross et al., 2011). Social development is also highly associated with personality, and it

has been reported an association between personality traits and the volume of different brain regions. They found that neuroticism was positively associated with increased volume in a region of the cingulate cortex linked to pain responses (DeYoung et al., 2010).

2.7.2 The role of depression

The psychiatric morbidity most frequently reported associated with chronic pain is depression. Prevalence ranges from 4.7% to 22.0% in population-based studies and from 1.5% to 100% in clinical studies (Knaster et al., 2012). Depressive patients report more often severe and enduring pain than non-depressed patients, while patients with chronic pain tend to exhibit more depressive symptoms (Kroenke et al., 2011).

The relationship between pain and depression has been extensively demonstrated in preclinical research. Animal models of neuropathic pain have been evaluated in different depressive-like behaviour paradigms, demonstrating a negative effect of pain in the development of depression. In rats injected with the complete Freund's adjuvant it was observed a lower sucrose preference and longer immobility time in the forced swimming test (Kim et al., 2012; Shi et al., 2010b). A model-related difference in the depressive-like behaviour response was observed in neuropathic pain. Contradictory results were obtained in the forced swimming test and in the tail suspension test. SNL increased the immobility time in the forced swimming test (Steru et al., 1985a), while with the PSNL model no differences were observed between the operated and the sham animals (Hasnie et al., 2007b). Recently, it

has been reported that the depressive-like behaviour appears in the late phase of the neuropathic pain development and that the forced swimming test is more sensitive than other experimental paradigms (tail suspension or anhedonia) to detect this disorder in rodent models (Fukuhara et al., 2012; Yalcin et al., 2011; Zeng et al., 2008).

It is believed that pain and depression interact each other reciprocally. Pain can induce depressive-like behaviour in animals and in the same way; depression can also induce or enhance pain-related behaviours. Decreasing muscle pressure threshold and tactile allodynia in rats (i.e., clinical symptoms in fibromyalgia) were observed with the repeated subcutaneous administration of reserpine, a classical model of depression (Nagakura et al., 2009). These results suggested that depression could affect pain-related behaviours. The effect of depression in pain is controversial; there are studies that demonstrate an enhancement of nociceptive responses in depressive-like behaviour models, while others demonstrate the opposite influence. In two animal depression models, the chronic unpredictable mild stress (CUMS) and olfactory bulbectomy, differential influences on evoked and spontaneous nociceptive behaviours were observed. Withdrawal latency to noxious heat stimuli was increased in the CFA model compared with sham groups. In the same model, an enhancement in formalin-induced spontaneous licking behaviours was also reported (Shi et al., 2010c; Wang et al., 2010). In the chronic unpredictable mild stress model, the paw withdrawal threshold to mechanical stimulation was elevated in SNL or sham animals (Shi et al.,

2010b). Contradictory results were also reported in the effect of depression on experimentally evoked pain. Kyoto rats are a genetic variation of the Wistar strain that has been developed as preclinical model of depression. CCI produced an exacerbated mechanical allodynia in Wistar-Kyoto rats (Wang et al., 2012; Zeng et al., 2008). However, in rats undergoing unpredictable mild stress an enhancement of CCI evoked contralateral cold allodynia (but not mechanical allodynia) and place escape/avoidance responses were observed (Bravo et al., 2012). Finally, enhanced nociceptive responses under physiological and inflammatory pain/neuropathic pain conditions were obtained in the olfactory bulbectomy model in the Wistar-Kyoto rats (Burke et al., 2010, 2013). Possible explanations for the discrepancy in pain responses due to depressive effect include the use of different models and tests, different time point measurement, and experimental variables.

2.7.3 The role of anxiety

Anxiety is a common symptom in chronic pain patients. The prevalence of anxiety is approximately the double in persons with chronic pain when compared with healthy population (35% vs. 17% respectively). Anxiety is under diagnosed and remains untreated as patients usually initially report somatic symptoms (pain), rather than anxiety (Atkinson et al., 1991; McWilliams et al., 2003; Polatin et al., 1993). Anxiety and panic disorders share common physical features with pain, such as muscle tension and autonomic arousal symptoms, suggesting a linkage between the conditions.

Treating efficiently these common symptoms may affect positively pain and anxiety at the same time (Wittchen et al., 2002).

A reciprocal relation between anxiety and pain has been demonstrated by several reports in animal and human research. In a population of patients with chronic pain (49% neuropathic pain, 21% nociceptive pain, 5% visceral pain and 25% idiopathic pain) a mood disorder was detected in 45% and, specifically, anxiety disorders in 25%. Pain intensity is associated with psychiatric morbidity (anxiety and depression), although pain duration is weakly related (Knaster et al., 2012).

There are several animal studies elucidating the mechanisms of neuropathic pain related with the anxiety-like behaviour using different animal models. Increased levels of anxiety-like behaviour in PSNL mice were detected in the elevated plus maze and in the light dark box tests (Bilkei-Gorzó et al., 1998). An altered opioid function in the amygdala and increased astrogliosis in the cingulate cortex were found to be responsible of the anxiety-like behaviour enhancement (Narita et al., 2006a, 2006b). This enhanced anxiety-like behaviour induced by neuropathic pain is sensitive to the administration of imipramine, milnacipran, paroxetine and pregabalin (Matsuzawa-Yanagida et al., 2008; La Porta et al., 2016).

In animal models of neuropathic pain related to HIV infection, such as HIV-1 glycoprotein 120 treatment or systemic delivery of the anti-retroviral agent zalcitabine or stavudine (d4T), it was reported a persistent mechanical hypersensitivity accompanied by anxiety-like behaviour in the open field. Evidences of analgesic efficacy has

been described in these models using different treatments, such as amitriptyline, morphine, gabapentin, the cannabinoid receptor agonist WIN 55,212-2 and the palmitoylethanolamide analogue palmitoylallylamide (L-29) (Huang et al., 2013; Wallace et al., 2007).

Evidence of anxiety-like behaviour related with pain development has been shown in other animal models of pain such as CCI (Roeska et al., 2008), spinal nerve transection, varicella zoster virus -induced postherpetic neuralgia (Hasnie et al. 2007), sciatic nerve cuffing (Benbouzid et al., 2008; Yalcin et al., 2011) and incisional pain (Dai et al., 2011; Li et al., 2010). Taking into account all these evidences, it seems that the unanimous conclusion is that chronic pain of certain severity and duration will produce comorbid anxiety-like behaviour in animals regardless of the specific aetiology and site.

Anxiety will also exert negative influence upon pain perception (Arntz et al., 1994; Ploghaus et al., 2001). Anxiety could enhance different components and measures of pain (ratings of intensity, unpleasantness and pain threshold). Symptoms close to those manifested in humans with anxiety disorders are replicated in a model social defeat. This model consists of a 30 min protected confrontation followed by a 15 min physical confrontation (repeated for 4 days). After these 4 four days, the development of anxiety-like behaviour was confirmed by the decrease of sweet-water consumption and reduction in body weight. The defeated rats with higher prevalence of anxiety-like behaviour showed an enhanced

nociceptive behaviour measured by the formalin test (Andre et al., 2005).

2.7.4 Effects on cognition

Chronic pain patients often complain of cognitive changes, particularly in the ability to concentrate and remember. The assessment and treatment of pain in persons with cognitive impairments is a challenge. People with memory, language, and speech deficits and consciousness impairments are often unable to communicate clearly about their pain and symptoms. Hospitalized patients with cognitive impairments, particularly dementia, have problems with analgesic treatment compliance (Buffum et al., 2007).

Experimental evidences in several animal models of chronic pain substantiate these and other cognitive alterations. Neuropathic pain can exert a negative impact on the attention processing in rodents as demonstrated by Low et al. (2012) in the visual non-selective, non-sustained attention task with spared nerve injured rats (Low et al., 2012). Lack of influences of chronic pain in aversive memory formation was reported by two different studies, with two different pain models and tests to evaluate de cognitive performance. The first used the SNL model and the mouse passive avoidance test; the second evaluated the SNL and CFA models in a novel air-puff passive avoidance paradigm (Moriarty et al., 2012; Suzuki et al., 2007). However, cognitive impairment due to PSNL surgery was detected in recognition memory. This cognitive dysfunction was improved by the increasing of extracellular glycine by the systemic

administration of a selective glycine transporter 1 inhibitor; suggesting this via as a possible therapeutic target for the treatment of chronic pain with cognitive disturbances (Kodama et al., 2011). Deficits in working memory and short-term memory (eight-arm radial maze test and novel object recognition test respectively) were also reported by Ren et al. (2011) in PSNL mice and rats (Ren et al., 2011).

Cognitive impairment produced by PSNL procedure was associated with hippocampal abnormal function (Mutso et al., 2012). It was shown that SNL triggers a cascade of events in hippocampus such reduced ERK expression and activation, decreased neurogenesis and altered short-term synaptic plasticity. Also in the hippocampus, the disregulation of BDNF expression might be responsible for the spatial memory deficit developed in rats with spinal nerve transection (Hu et al., 2010).

The peripheral diabetic neuropathy is related with a variety of functional and structural disorders including cognitive deficits (Croxson and Jagger, 1995; Helkala et al., 1995). Putative changes in water maze learning and hippocampal synaptic plasticity have been reported in the STZ-diabetic rats (Biessels et al., 1996, 1998; Kamal et al., 2000). In a study with hyperglycemic rats it was found impairment in spatial learning and hippocampal long-term potentiation expression in the CA1 field of the hippocampus. This deficit was only observed in severely hyperglycemic rats. It was observed that the early treatment with systemic insulin prevent the behavioural and synaptic dysfunctions, whereas later insulin

administration failed to reverse established deficits in maze learning and only restored CA1-LTP partially (Biessels et al., 1996, 1998).

The studies regarding the relationship between pain and the effect on the cognitive function are fewer than those related with anxiety and depression. Despite of this, the cognitive impairment produced by neuropathic pain is well described. Some brain regions involved in cognition also have a role in pain modulation, including the prefrontal cortex, which is involved in decision-making and executive function (Miller and Cohen, 2001), and the anterior cingulate cortex, which is involved in selective attention and memory (Moriarty et al., 2011; Seminowicz and Davis, 2007). Recently, another question was raised: is the cognitive component related with the development or chronicity of pain? Attal et al. (2014) have observed that patients with constitutively limited attention resources or cognitive flexibility are at greater risk of suffering from chronic pain after surgery (Attal et al., 2014). Limited cognitive flexibility may reflect individual premorbid variations of the corticolimbic circuitry (Leber et al., 2008; Miller and Cohen, 2001). Patients with chronic pain exhibit changes in neuroplasticity and brain morphology, particularly in the dorsolateral prefrontal complex (Apkarian, 2004; Mutso et al., 2012). Impairment of cognitive flexibility could be responsible for the incapacity to elaborate adaptive coping strategies or cognitive regulation of emotions to deal with chronic pain (Solberg Nes et al., 2009). However, other individual differences as gene polymorphisms and personality traits should be considered to influence cognitive flexibility (Williams, 2010).

3 Endogenous opioid system

3.1 General aspects

3.1.1 Brief history

Opium is found in poppy seeds (*Papaver somniferum*). It has been consumed since the Antiquity to relieve pain and to get euphoria. The main alkaloid of opium, morphine, was isolated by Serturner in 1805. This discovery initiated the modern opioid pharmacology. In the 1960s, it had become apparent that opioid drugs were likely to exert their actions at specific receptor sites. But it was not until 1973, that three different groups published almost simultaneously the experimental evidence of the existence of an opioid receptor. The first team was Terenius group at Uppsala (Terenius, 2009), followed shortly by two other groups: Pert and Snyder (1973) at Baltimore and Simon et al. (1973) at New York (Magistretti and Allaman, 2013; Ohio Attorney General's Office, 2014).

In 1975, the first endogenous molecules that bind opioid receptors were isolated from the pig's brain: met- and leu- enkephalins (Hughes et al., 1975). The next year it was discovered another endogenous peptide with morphine-like action, which was named with abbreviation of endogenous morphine: endorphin (Simantov et al., 1976). In the following years, three families of endogenous opioid peptides precursors named pre-proenkephalin (*Penk*), pre-prodynorphin (*Pdyn)* and proopiomelanocortin (*Pomc*) were identified (Goldstein et al., 1979; Guillemin et al., 1976; Hughes et al., 1975; Li and Chung, 1976). *Penk* (Comb et al., 1982; Gubler et

al., 1982; Noda et al., 1982), *Pdyn* (Kakidani et al., 1982) and *Pomc* (Nakanishi et al., 1979) encoding genes were isolated in the early 1980's.

Currently, three different opioid receptors, mu (MOR), delta (DOR) and kappa (KOR) have been identified. The MOR was originally defined and characterised pharmacologically on the basis of its high affinity for, and sensitivity to, morphine (Lord et al., 1977; Martin et al., 1976). The DOR was established using the mouse vas deferens preparation (Lord et al., 1977). Finally, the KOR was first proposed on the basis of *in vivo* studies in dogs with ketocyclazocine and related drugs (Martin et al., 1976). The DOR was the first opioid receptor to be sequenced in 1992 (Evans et al., 1992; Kieffer et al., 1992), MOR (Chen et al., 1993a; Thompson et al., 1993; Wang et al., 1993) and KOR (Chen et al., 1993b; Li et al., 1993; Meng et al., 1993) were cloned in the following years. A timeline of these discoveries is summarized in Figure 7.

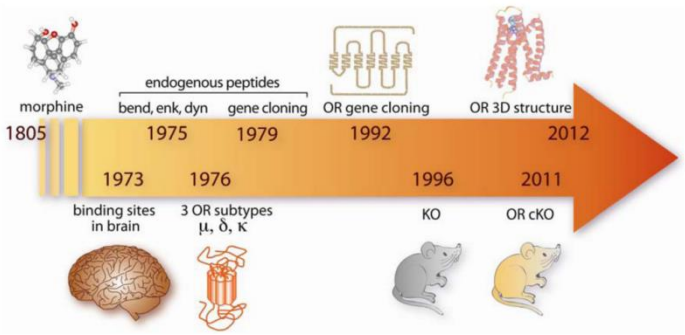

Figure 7. Timeline discoveries in endogenous opioid system research (Klenowski et al., 2015). Morphine is the most active alkaloid extracted from opium and was the first opioid isolated (1805). Opiate action on the nervous system is through the activation of specific receptors (1973). These opioid receptors are stimulated by a family of endogenous neurotransmitters: β-endorphin, enkephalins and dynorphins (1975). Based on receptor

pharmacology others subtypes of opioid receptors were further described (1976). In late 70's and early 80's gene cloning for peptide precursors occurred (1979), the turn of opioid receptors arrived in early 90's (1992). The first cKO mice available were those animals lacking the MOR and enkephalins (1996). Mice with a DOR deletion restricted to primary afferent nociceptive neurons, was the first cKO mouse for the opioid system (2011). The 3D crystal structure of all three receptors was elucidated recently (2012). Abbreviations: OR: opioid receptor, KO: knockout mouse, cKO: conditional knockout mouse.

3.1.2 Biology of opioid receptors

The existence of three opioid receptors is now accepted. Although they were originally named mu, delta and kappa, in 1996 the International Union of Pharmacology (IUPHAR) renamed the DOR to OP1, the KOR to OP2 and the MOR to OP3. In 2000 this nomenclature was again changed to the current classification: DOR, KOR and MOR (Pathan and Williams, 2012).

More specific studies that include pharmacologic, molecular biology and thermodynamic approaches have suggested subtypes of each of the opioid receptors: three for MOR, two for DOR and three or more for KOR. The relevance of these subtypes has not been clarified yet. Additionally, for explain some actions of β-endorphins has been postulated another receptor, epsilon. The sigma receptor was once classified as an opioid receptor based on it homology and role on pain. The opioid receptor-like protein shares a 50 to 60% homology with opioid receptors; interestingly its activation causes hyperalgesia and analgesia (Peppin and Raffa, 2015). As one possible explanation of some pharmacological responses of opioids that are not consistent with the activation of classical receptors, the

formation of heterodimers was proposed; in particular, DOR/KOR and DOR/MOR type. These heterodimers represent a new functional structure with different properties to the original receptor (Jordan and Devi, 1999).

Opioid receptors belong to the receptors group coupled to G protein. These receptors are seven transmembrane spanning proteins that couple to inhibitory G proteins. The G protein has two subunits: the Gα and Gβγ. All opioid receptors display similar cellular responses following receptor activation, even when the activation of each of them might cause different functional effects. When an agonist activates a receptor, the Gα and Gβγ subunits dissociate and trigger various intracellular effector pathways. When an opioid agonist binds to an opioid receptor coupled to G-protein on the transmembrane portion of the receptor, the α subunit of the G-protein exchange its bound guanosine diphosphate (GDP) molecule with intracellular guanosine triphosphate (GTP). This phosphorylation allows to the α-GTP complex to dissociate away from the βγ complex and they are free to interact with target proteins. In the case of antagonist action on the receptor, the adenylate cyclase becomes inhibited producing a reduction in intracellular cyclic adenosine monophosphate (cAMP) levels. cAMP intracellular reduction levels hyperpolarized the neurons affecting the neurotransmitter release (Al-Hasani and Bruchas, 2011). Also, the activity of protein kinase A (PKA) is reduced, which results in a decreased phosphorylation of intracellular effectors. Receptor activation also induces a change in the signalling pathways of phospholipase C and MAPK. The MAPK

family is composed of 12 to 15 gene products including extracellular signal-regulated kinases 1 and 2 (ERK1/2), JNK1–3, and p38 (α,β,γ,δ) stress kinase (Al-Hasani and Bruchas, 2011). These changes produce the activation of the transcription factors, such as CREB, that leads to the transmission of the signal inside the nucleus and the transcription of specific genes (Ligeza et al., 2008).

Additionally, these G protein complexes interact with a number of ion channels, producing activation of potassium conductance and an inhibition of calcium conductance. The dissociated Gβγ subunit binds directly to the calcium channel and inhibits calcium conductance causing a reduction in Ca^{2+} currents. The binding of Gβγ subunit reduces voltage activation of channel pore opening. The acute administration of opioid agonists reduced Ca^{2+} content in synaptic vesicles and synaptosomes, with compensatory up-regulation of vesicular Ca^{2+} content (Zamponi and Snutch, 1998, 2002). This interaction between opioid receptors and potassium and calcium channels has been demonstrated in a wide range of systems, from neurons in the hippocampus, locus coeruleus, and ventral tegmental area to the dorsal root ganglia, demonstrating that these channels are highly conserved opioid receptor substrates and represent one of the most important targets for opioid receptor modulation (Al-Hasani and Bruchas, 2011).

The desensitization, sequestration, sorting and ultimately assisting in determining opioid receptor fate is regulated by arrestin molecules. These key proteins bind to the receptor when is phosphorylated. The principal arrestins that regulates the opioid receptors are the arrestin-2 and arrestin-3 (also called β-arrestin 1

and β-arrestin 2, respectively) and this interaction depends on the model system and agonist treatment procedure (Bohn et al., 1999, 2000).

In 1994 a fourth G protein coupled endogenous opioid like receptor was found and was named the nociceptin receptor (NOP). After this discovery, its endogenous ligand nociceptin/orphanin FQ (N/OFQ) was isolated from brain extracts. This endogenous ligand derived from the polypeptide precursor pre-pro-nociceptin. Something remarkable of this receptor is its lack of response to the classical opioid antagonist (naloxone). The NOP receptor shows 80% homology to the KOR receptor in its classical amino acid sequences of G-protein opioid receptors. At a cellular level, N/OFQ produces similar actions to the classical opioid receptors. For these reasons, it has been classified as the fourth opioid receptor. The IUPHAR considers the NOP receptor to be a non-opioid branch of the opioid receptor family (Magistretti and Allaman, 2013).

The opioid system plays a central role in nociception and analgesia and also regulates numerous physiological functions. Opioid receptors are broadly expressed throughout peripheral and central nervous systems regulating the previously mentioned functions. Opioid receptors are expressed in the cortex, limbic system, and brain stem. There is an overlapping of binding sites for the three opioid receptors in most structures. The expression of each receptor varies in accordance with the brain structure (Le Merrer et al., 2009a).

MOR is the opioid receptor with the highest expression in the amygdala (except in the central nucleus of the amygdala), thalamus,

mesencephalon, and some brain stem nuclei. A moderate density of MOR is found in periaqueductal gray matter and raphe nuclei. These brain regions have a role in the descending inhibitory control of pain and analgesia. Other physiological functions regulated by MOR include respiratory and cardiovascular functions, intestinal transit, feeding, mood, thermoregulation, hormone secretion and immune functions. In the basal anterior forebrain, including the claustrum and endopiriform cortex, olfactory tubercle, striatum (caudate putamen and nucleus accumbens), preoptic area, hypothalamus, and pituitary, KOR is the most ubiquitous. This receptor has been implicated in the regulation of nociception, diuresis, feeding, neuroendocrine and immune system functions. The DOR is the most common receptor in the olfactory tract (olfactory bulb, anterior olfactory nucleus, medial amygdala) and in the cortices (whole neocortex) and in some regions of the amygdala (basolateral, cortical, and median nuclei), and is also highly expressed in the striatum. In the spinal cord, DOR are present in dorsal horn where they play a role in mediating the analgesic effects of DOR agonists. The functional roles of DOR are less clearly established than for MOR; in addition to analgesia, DOR may have a role in gastrointestinal motility, mood and behaviour as well as in cardiovascular regulation (Goody et al., 2002; Mansour et al., 1987, 1994, 1995b; Simonin et al., 1998).

MOR and KOR coexist in most structures, whereas the distribution of DOR is more restricted (low expression in the hypothalamus, thalamus, mesencephalon, and brain stem). The sites of opioid receptor expression (mRNA) generally match the distribution of

binding sites (protein), suggesting that many neurons synthesizing opioid receptors are local neurons (Le Merrer et al., 2009a).

Opioid receptors are also located in the spinal cord; approximately 60% of spinal opioid receptors are MOR, while 21% are DOR and 19% KOR. MOR are mainly expressed in the gelatinous substance and to a lesser extent in other structures such as the laminae III, IV, V and VIII. The KOR are also mainly expressed mostly in the substantia gelatinosa. The DOR are preferentially located in the lamina I of the dorsal horn. Most of MOR, DOR and KOR are expressed presynaptically in the spinal cord. However, they are also located postsynaptically on the plates of the ventral region of the spinal cord (Stein, 1993).

The modulation of physiological functions is also dependent of the opioid receptor expression in peripheral tissues. They are located at the level of sensory and sympathetic nerve fibres of the skin and joints, in the plexuses of the intestine, urinary bladder and vas deferens, in endocrine cells and in the immune system (Stein, 1993).

3.1.3 Biology of endogenous ligands

The opioid peptide precursors are encoded by three genes: pre-proopiomelanocortin, pre-proenkephalin, and pre-prodynorphin. Precursors undergo post-translational modifications to produce multiple active peptides. These peptides share the “opioid motif” that is an N-terminal sequence of Tyr-Gly-Gly-Phe-(Met or Leu), followed by various C terminal extensions yielding peptides ranging from 5 to 31 residues in length (Kieffer, 1995). β-endorphin is

encoded by pre-proopiomelanocortin plus the non-opioid peptides adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), and β-lipotropic pituitary hormone (β-LPH). Pre-proenkephalin encodes two copies of met-enkephalin and a single copy of leu-enkephalin. Pre-prodynorphin encodes three opioid peptides, all of them beginning with the leu-enkephalin sequence: dynorphin A, dynorphin B, and neoendorphin (McNally and Akil, 2002) .

The relationship between the opioid peptides and their receptors is complex. It is clear that high-affinity interactions between each one of the precursor and receptor families is possible. The endogenous opioids met-enkephalin, leu-enkephalin, extended forms of met-enkephalin including metorphamide and BAM-18, β-endorphin, and truncated forms of dynorphin (e.g. dynorphin-1-9 and shorter dynorphin peptides) have affinity for MOR, although these endogenous peptides are not fully selective for MOR. Two putative natural ligands, endomorphin 1 and endomorphin 2, appear to mediate their effects exclusively through the MOR, also have been reported to be present in brain although no gene, precursor protein, or other mechanism for their endogenous synthesis has been identified (Zadina et al., 1997). Enkephalins are generally considered the preferred endogenous ligands for DOR (Lord et al., 1977). The affinity of enkephalins for MOR is similar to that of morphine, whereas their affinity for DOR is about tenfold higher (Dhawan et al., 1996). Dynorphins A and B and α-neoendorphin appear to be the endogenous ligands for KOR, although shorter

peptides derived from prodynorphin have comparable affinities at MOR and KOR (Mansour et al., 1995a).

The most abundant and distributed opioid precursor is *Penk*, mainly found in the thalamus, where its distribution overlaps with MOR. *Pdyn* is present in most brain structures, with the highest concentration in the nucleus accumbens (NAc) and the lowest concentration in the thalamus. *Pomc* shows a more restricted distribution and it is absent from cortical structures except for the amygdala (Le Merrer et al., 2009a; Roques et al., 2012).

Penk-expressing cell bodies are also the most abundant in the brain. *Pdyn* cell bodies are also widespread, with a more presence in the hypothalamus overlapping with high KOR density. In contrast, *Pomc* cell bodies are highly restricted and only detected in three regions of the brain: the arcuate nucleus of the hypothalamus, nucleus tractus solitarius (nucleus tactus solitarius, brain stem), and pituitary (anterior lobe and intermediate lobe). Neurons from the arcuate nucleus and nucleus tactus solitarius project mostly to limbic, mesencephalic, and brain stem subcortical regions, where high *Pomc* mRNA levels are detected (Le Merrer et al., 2009a).

3.2 Pain modulation by the opioid system

Physiological analgesia is "a form of pain relief induced by endogenous effectors that stimulate the same targets as natural or synthetic opiates" (Roques et al., 2012). In the case of endogenous opioid system, the targets are the opioid receptors and the endogenous ligands the effectors responsible to activate them. Analgesia mediated by the opioid endogenous system relates with

the modulation of ascending and descending pain pathways. MOR, DOR and KOR expression is confirmed in several structures involved in the ascending (dorsal root ganglia, spinal cord, trigeminal nucleus) and descending (central gray area, pontine, nucleus gigantocellulare, intermediate reticular nuclei) pain pathways (Mansour et al., 1988). In the periaqueductal gray region and the nucleus locus coeruleus, opioid receptors activate descending fibres through blockade of GABAergic neuronal inputs, which in turn regulate other descending pathways as noradrenergic neurons (Pan et al., 2004; Vaughan et al., 1997). The systemic or local PAG administration of opioids increased the off cells activity. In the other hand, the on cells in the rostral ventromedial medulla (RVM) are a cell population that are directly inhibited by opioids through MOR. On cells are also activated by cholecystokinin (CCK) via a CCK2 receptor. An overlapping of CCK2 receptors and MOR is found in the RVM neurons (Al-Hasani and Bruchas, 2011).

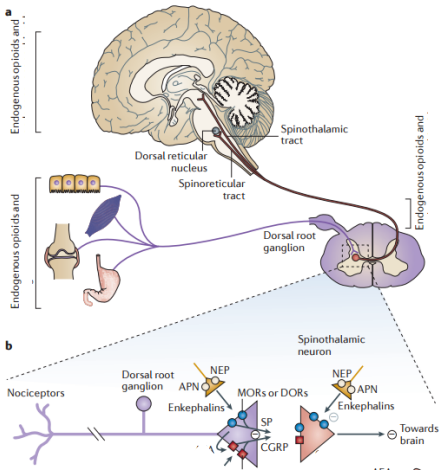

Figure 8. Opioid system and pain modulation. Modified from (Roques et al., 2012). a) Endogenous opioids are present at three levels of pain control: central nervous system, spinal cord and peripheral organs. At the periphery, endogenous opioids are in epithelial cells of the intestine and kidney, in the joints, lung, and skin and in immune cells that surround nerve fibres as oligodendrocytes and Schwann cells. In the spinal dorsal horn, MOR and DOR are mainly located at the presynaptic end of afferent fibres. DOR and MOR are expressed in areas involved in the control of pain and emotions such as the periaqueductal grey, thalamus, cortex and limbic system. b) Opioid receptors are synthesized in the dorsal root ganglion and transported to the spinal afferent terminals. Stimulation of opioid receptors by enkephalins from interneurons inhibits the release of pro-nociceptive peptides such as substance P (SP) and calcitonin gene related peptide (CGRP). Enkephalins are also released near the spinothalamic neurons, where they block the transfer of nociceptive inputs to the brain via an increase in K^+ conductance and subsequent hyperpolarization.

Opioid ligands are also involved in the modulation of pain. Neurons containing enkephalin are widespread throughout the central and peripheral nervous systems. These neurons are in supraspinal circuits in the limbic system structures (hippocampus, septum, bed nucleus of the stria terminalis) suggesting a possible involvement in the emotional response to pain. (Drolet et al., 2001)Also, they have been found in the spinal cord nociceptive pathway (Guindon and Beaulieu, 2009; Joseph and Levine, 2010; Maldonado et al., 1994; Stein et al., 2003). Enkephalins have an inhibitory role and antinociceptive activity within the CNS (Labuz et al., 2007). Enkephalins are very susceptible to proteolytic action, so their central antinociceptive effect is weak and short-lasting. There are some drug treatment studies proposing deactivation of enkephalinases to produce stronger and *Pomc* is expressed in

nucleus arcuatus of the hypothalamus. Nerve ramifications originating in this nucleus terminates in brain areas that have been implicated in nociception such as the periaqueductal grey matter. It is suggested that activation of β-endorphinergic fibres and the posterior enhance release of peptide may mediate stress-induced analgesia (Millan et al., 1983). β-endorphin descending neurons from nucleus tractus solitarius also input spinal cord neurons (Bronstein et al., 1992). Some β-endorphin containing neurons have been found in the dorsal spinal cord indicating a functional role in spinal antinocipetion (Gutstein et al., 1992). β-endorphin has been implicated in antinociception at central or peripheral level. The effect of β-endorphin in hyperalgesia reduction is associated with peripheral tissue inflammation, which can be result of the local interaction opioid receptors localized on peripheral afferent nerve terminals (Przewlocki and Przewlocka, 2005).

Prodynorphin neurons are distributed in the limbic system and in descending pathways from supraspinal structures to the spinal dorsal horn. Although dynorphin has been found in lamina I projection neurons, it seems that the main source of spinal dynorphins is the spinal interneurons. Dynorphins are present in cutaneous nerves overlapping with markers for sensory neurons as the genes related with calcitonin (Hassan et al., 1992). In morphine "tolerant rats" the elevated levels and subsequent release of spinal dynorphins enhance the release of CGRP from primary afferents. This enhancing effect of dynorphins may modulate some aspects of opioid-induced abnormal pain and the expression of antinociceptive tolerance to morphine (Gardell et al., 2002). Dynorphins have the

capability to produce antinociceptive actions in the spinal cord via both opioid and non-opioid receptor mechanisms (Obara et al., 2003; Ossipov et al., 1996; Wang et al., 2001).

Endomorphins are found in the central nervous system in regions that modulate nociception and highly express MOR (spinal cord and thalamus). The presence of endomorphins in nociceptive pathways at spinal and supraspinal levels was also reported (Martin-Schild et al., 1998). At the spinal cord, endomorphins are localized in the primary nerve endings and co-localized in a subset of substance P and MOR-containing primary sensory afferents. (Martin-Schild et al., 1998; Pierce et al., 1998; Zadina et al., 1999). Endomorphins at the spinal cord could be critical regulators of pain perception because of its action on MOR at pre and postsynaptic level. These peptides are released in response to painful and traumatic stimuli and act as endogenous analgesics with an antinociceptive potency comparable to morphine in acute pain models (Stone et al., 1997).

Pain produces multiple adaptations in the nervous, endocrine, and immune systems. As inflammation is an essential component of painful syndromes including arthritis, neuropathic pain, cancer, wounds, and postoperative pain, the influence of opioid receptors on painful peripheral inflammation and nerve damage has been widely studied (Stein et al., 2009). It was observed that the systemic or local application of MOR, DOR and KOR agonists produced more analgesic effects in inflamed than in those non-inflamed tissues of animals and humans (Epstein and Stein, 1995). This observation raises the hypothesis that their antinociceptive effects can be mediated by those receptors located on peripheral sensory

neurons. The peripheral tissue inflammation can induce differential regulation of opioid receptor types and their mRNAs in DRG neurons (Busch-Dienstfertig and Stein, 2010; Stein and MacHelska, 2011). The cytokine production in the inflamed tissue may be responsible of this up regulation and also may be related to cytokine-induced binding of transcription factors to opioid receptor gene promoters (Jeanjean et al., 1995; Mousa et al., 2007). Cytokines and growth factors produced within the inflamed tissue stimulate the axonal transport of opioid receptors resulting in an increased density and antinociceptive functionality of opioid receptors on peripheral nerve terminals (Zhou et al., 1998). The receptors affinity remain unchanged during this processes while the responsible of the up regulation of opioid binding sites is the increase in the number of neurons expressing receptors and the number of receptors per neuron (Zollner et al., 2003). All this changes in the number of opioid receptors in peripheral sensory nerve terminal is only observed after long inflammation periods. During inflammation signalling pathways are also altered, G protein coupling of opioid receptor is augmented (Zollner et al., 2003). The decrease in extracellular pH observed in inflammation increase opioid agonist efficacy probably by alter the interaction of the adenylyl cyclase with G proteins (Rasenick and Childers, 1989; Selley et al., 1993). As a consequence, an enhanced inhibition of cAMP, Ca^{2+}, Na^{+}, TRPV1, and/or ASIC currents occurs, as well as the opening of GIRK channels producing neuronal hyperpolarization (Cai et al., 2014; Nockemann et al., 2013; Spahn et al., 2013).

3.3 The opioid system in neuropathic pain

Expression of peripheral opioid receptors in sensory neurons is influenced by the nerve injury (MacHelska, 2011; Stein and MacHelska, 2011). In different animal models of neuropathic pain the mRNA levels of the three opioid receptors are usually modified in the dorsal root ganglion. However, opioid mRNA levels are not always correlated with protein expression in dorsal root ganglion cell bodies. The expression of MOR is modified and varies in the different animal models of neuropathic pain. Expression of MOR was found to be down regulated or unchanged in diabetic neuropathy, nerve transection, and sciatic nerve ligation models. While after partial sciatic nerve ligation, contradictory (decreased or increased) MOR expression has been reported. In the chronic constriction injury model the expression of MOR was found unchanged or increased (Hall et al., 2001; Mousa et al., 2013). MOR up regulation was shown at the nerve injury site and in hind paws innervated by damaged saphenous nerves (MacHelska, 2011). It was observed that the administration of opioid agonist at the injury site or at the peripheral nerve terminal enhances antinociceptive activity in the nerve constriction model (Labuz and MacHelska, 2013; Schmidt et al., 2012).

DOR control the development of some nociceptive manifestations of neuropathic pain as thermal hyperalgesia and mechanical allodynia in a model of sciatic nerve crush (Mika et al., 2001). Additionally, the role of DOR in neuropathic pain was confirmed in DOR KO mice, as in these animals the different behavioural manifestations of neuropathic pain are enhanced. In some areas

related to the control of nociceptive transmission, as the spinal cord, a down regulation of DOR after a nerve injury was found (Stone et al., 2004). DOR are also implicated in the inhibition of glutamate synaptic transmission in spinal cord during neuropathic pain and, in this way, DOR reduce dynorphin sensitization and inhibit the ascending noxious stimuli (Glaum et al., 1994). DOR regulate the tonic spinal regulation by inhibiting the release of substance P (Zachariou and Goldstein, 1996). In the C-fibres of the dorsal root ganglion neurons the interaction between protachykinin (a substance P precursor) and DOR is responsible of the transport of these receptors to the plasma membrane, which would modulate pain transmission (Guan et al., 2005).

Some observations suggest that enkephalins may be associated with neuropathic pain. In the late phase of chronic constriction injury of sciatic nerve was observed an increase of met-enkephalin. It was demonstrated that local release of proenkephalin-derived peptides produced an antinociceptive action in neuropathic pain (Przewlocki and Przewlocka, 2005). Enhancing the extracellular concentrations of enkephalins can reduce or block the noxious stimuli at their origin (Basbaum et al., 2009; Maldonado et al., 1994; Tegeder et al., 2003). In neuropathic pain, a high expression of opioid receptors is found on both sides of the nerve injury (Hassan et al., 1993). The recycling of MOR preserves the antinociceptive effect of continuously available enkephalins, thus preventing the peripheral opioid tolerance (Zöllner et al., 2008). Various mechanisms during inflammation or nerve injury are responsible of the enhanced availability of enkephalins. One of these mechanisms is the

migration of immune cells containing opioids from surrounding blood vessels. This is enhanced by the expression of endothelial adhesion molecules and triggered by neuropeptides such as substance P, which is released from noxiously stimulated nerve terminals (Labuz et al., 2006, 2009). Other mechanism is mediated by the release of chemokines, CRF, interleukins, leukotrienes and protons by membrane disruption of the damaged tissue (Labuz et al., 2006; Mousa et al., 2007). The interaction of these molecules with lymphocyte receptors or ion channels produces the enkephalins release (Schäfer et al., 1997). Finally, enkephalins produce peripheral desensitization, when are issued from inflamed keratinocytes and stimulate nerve fibre by binding to opioid receptors reducing or eliminating the transfer of noxious inputs to the spinal cord (Hassan et al., 1993; Rittner et al., 2001; Stein et al., 2003).

Little is known about the involvement of the proopiomelanocortin system in neuropathic pain. A decrease of β-endorphin in the brain and spinal cord of rats was found after sciatic nerve section (Hughes and Smith, 1993). In rats after chronic constriction injury of the sciatic nerve the enhancement of proopiomelanocortin expression elevated β-endorphin levels and increased pain threshold appear (Lin et al., 2002). β-endorphin levels in the cerebrospinal fluid were reduced in patients with diabetic polyneuropathy compared with those suffering other kinds of pain (Almay et al., 1978).

Melanocortins are involved in axonal regeneration following peripheral nerve injury and in the recovery of sensory and motor functions after crush or transection of the sciatic nerve in rats

(Edwards et al., 1984). The density of MC4 receptor for melanocortins is altered in spinal cord and in dorsal root ganglia after nerve injury (Beltramo et al., 2003). This may indicate an important role of the spinal MC4 receptor in both post- and presynaptic modulation of the nociceptive processing in neuropathy. Administration of melanocortin receptor antagonists into the cisterna magna or intrathecally produced antiallodynic effect in neuropathic rats (Przewlocki and Przewlocka, 2005).

In neuropathic pain, dynorphins levels are generally increased. Several studies in different animal models have demonstrated the increase of dynorphins after nerve injury at local spinal interneurons and in projecting neurons. The chronic constriction of the sciatic nerve was associated with an enhancement of dynorphin in superficial laminae I and II as well as in laminae V-VII on the ipsilateral side to the injury (Bian et al., 1998; Obara et al., 2003; Vanderah et al., 2000). In a spinal nerve ligation model at L5/L6 level the increase of dynorphins were found at local site, surrounding the spinal damaged area (ipsilateral dorsal parts of L4-L6 segments) (Bian et al., 1998). Not only peptide levels are increased after spinal cord, the prodynorphin mRNA levels are also modified. The appearance of neuropathic pain may be correlated with the up-regulation of the spinal dynorphins after nerve injury. The intrathecal or direct spinal cord administration of dynorphin A1-17 induced mechanical allodynia, cold allodynia and an enlargement of receptive fields and facilitated C-fiber-evoked reflexes in mice and rats (Laughlin et al., 1997; Vanderah et al., 2000). It was also observed that nociceptive manifestation as cold

and tactile allodynia were induced by dynorphin A1-17 via a non-opioid mechanism (Vanderah et al., 2000). Dynorphin A2-17, that is devoid of opioid activity, may damage spinal cord an produce hind limb paralysis when administered intrathecally to rats at high doses (Faden and Jacobs, 1984; Long et al., 1988; Millan et al., 1983). It can be concluded that dynorphin effects at least are partially mediated via non-opioid mechanisms and that these actions are pronociceptive and possibly excitotoxic.

The chronification of neuropathic pain produced by an injury is associated with a decrease of endomorphins in the primary afferents. This was observed in the ipsilateral side of the spinal cord in a sciatic nerve injury model (Smith et al., 2001).

3.4 Opioid system regulation of behaviour

Additional to the well-known action of opioid in pain, there is some evidence that opioids can modify emotional responses. Opioid peptides and receptors are expressed in brain areas associated with reward, motivation, learning and stress (Le Merrer et al., 2009a). To explore the role of the endogenous system in anxiety, depression and cognition several pharmacological and genetic strategies have been used.

3.4.1 Anxiety

The integration of aversive states and defensive behaviour is carried out in brain areas as the amygdala, hypothalamus and midbrain structures (as dorsal PAG and superior and inferior colliculi)

(Brandão et al., 1999; Dell-Ben and Graeff, 2009; LeDoux, 2003). The amygdala and hypothalamus are the main structures involved in the control of fear and anxiety. The enkephalin modulation of fear and anxiety responses is mainly conducted in the amygdala. In the central amygdala a high density of encephalin-immunoreactive fibres are found; in turn, the bed nucleus of stria terminalis (BSNT) and other amygdalar nuclei project enkephalin afferents to the central amygdala. A high percentage (40%) of central amygdala neurons express postsynaptic MOR (Zhu and Pan, 2004). When mice displaying high and low anxiety behaviours are compared, animals with high anxiety levels expressed lower density of enkephalins mRNA in the central and basolateral amygdala and increased enkephalins cell counts in the central, medial and basolateral amygdala (Hebb et al., 2004). This suggests that the release of enkephalins by neurons of the amygdala inhibits the projecting cells of the central amygdala through postsynaptic MOR (Colasanti et al., 2011). In other studies, regarding the role of opioid peptides as endorphins and proenkephalins in anxiety, it was observed that endorphins deficient mice did not show any alteration in levels of anxiety, while the KO strains of proenkephalins have increased responses in anxiety-evoking environments. Also, these animals were more responsive in fear condition paradigms and aggressive behaviour models (König et al., 1996b; Ragnauth et al., 2001). Consequently, a positive modulation of DOR in mood states was observed in DOR mutant mice as they display anxiogenic and depressive-like responses (Le Merrer et al., 2009a).

A complex relationship between the norepinephrine, CRF and DOR for the regulation of anxiety is established. In the nervous system norepinephrine and CRF are important mediators of behavioural responses. In the amygdala, the norepinephrine and the corticotropin-releasing factor are crucial in the mediation of behavioural and emotional consequences of stress responses as anxiety (Reyes and Van Bockstaele, 2017). The delta opioid system is implicated in the control of emotional behaviours and the amygdala appears to be an important site of action for the anxiolytic effects of delta agonists. The met and leu-enkephalin, reduce anxiety through activation of DOR (Primeaux et al., 2006). The role of the endogenous opioid system in anxiety has been explored trough genetically modified mice. Emotional responses were evaluated in mice deficient of Oprm, Oprd1 and Oprk1. In this study the activity of Oprd1-encoded receptors seemed to be related with decreased levels of anxiety (Filliol et al., 2000a). Increased anxiety levels were found in deficient pre-proenkephalins deficient mice (König et al., 1996b). These results evidence a positive modulation of anxiety states by the activation of DOR by endogenous pre-proenkephalin derived peptides. The phenotype of enhanced anxiety was specific to the DOR as it was not observed in MOR and KOR KO mice. Stress induced anxiety was attenuated by the activation of DOR in the CeA, suggesting that this receptors interact with the CRF neurons in the amygdala to produce this effect (Nieto et al., 2005; Reyes and Van Bockstaele, 2017). In the limbic circuitry a co-localization of CRF and DOR in neurons of the basolateral and central amygdala exists. These neurons are in close

proximity with norepinephrine afferents suggesting a possible modulating impact of norepinephrine in the amygdalar opioid-CRF circuitry that may be critical for the regulation of anxiety-like behaviours (Reyes and Van Bockstaele, 2017).

Opioid peptide precursors such as pro-opiomelanocortin and pro-enkephalins are synthesized in the hypothalamic infundibular nucleus and in many hypothalamic nuclei respectively. Opioid receptors are also highly expressed in the hypothalamus, and KOR are found in most hypothalamic nuclei and DOR in the ventromedial hypothalamus. The paraventricular nucleus of the hypothalamus through its CRF efferent releases ACTH, and projects directly to preganglionic sympathetic and parasympathetic neurons. They control in this way the stress system. Additionally, the paraventricular nucleus receives afferents from limbic and midbrain structures that are involved in control of emotions and defensive behaviours (Colasanti et al., 2011). The gene expression of enkephalins in the paraventricular nucleus of the hypothalamus is increased under acute stress situations. Enkephalins are involved in the regulation of sympathetic, cardiovascular and respiratory neural control systems, relating these aspects enkephalins may have a role in stress adaptation of the organism. As a result, the release of enkephalins in the hypothalamus may decrease the stress response by attenuating some physiological responses. Enkephalins also regulate the synthesis and release of CRF in the hypothalamus (Drolet et al., 2001). There is a possible role of hypothalamic dynorphinergic neurons in control of the stress and anxiety responses observed in predynorphin deficient mice. This KO

animals exhibited a diminished expression of CRF mRNA in the periventricular hypothalamus and amygdala (Wittmann et al., 2009).

In the midbrain, opioid pathways modulate the expression of panic-like behaviours. The direction of this modulation depends on the type of opioid receptors that is activated: MOR and DOR display, respectively, an anxiolytic and anxiogenic profile. The effect of opioids was observed in rats evaluated in the elevated plus maze before agonist and antagonist MOR and KOR microinjections in the dorsal PAG. Low doses of opioid agonist produced anxiolytic effects and high doses of morphine caused anxyogenic effects (Motta and Brandão, 1993). Moreover, MOR agonists cause anti-aversive effects and KOR agonists produce the contrary effect (aversive states) (Bals-Kubik et al., 1989; Bechara and Van der Kooy, 1987; Mucha and Herz, 1985). In the midbrain tectum of rats high concentrations of MOR and moderate densities of KOR was revealed, and this receptor expression and the lower affinity of KOR to morphine could be an explanation for the need of high doses of morphine for activating KOR (Mansour et al., 1988). It was also observed that the systemic administration of opioids has an overall inhibitory effect, leading to the reduction of fear and anxiety responses (Colasanti et al., 2011).

In paradigms of anxiety and depression, strains of the three opioid receptor KO mice were evaluated in order to determine de role of these receptors in mood regulation. It was revealed that KOR are not tonically involved to control anxiety and depression since the KOR mutant mice do not show any behavioural alteration. In the

other hand, a contribution of MOR and DOR in these mood disorders was confirmed showing opposite phenotypes (Kieffer et al., 2000).

3.4.2 Depression

The opioid system is involved in the regulation of brain sites that modulate the pathogenesis of depression. Mood disorders are regulated trough the dopaminergic mesolimbic system (DMS) (Nestler and Carlezon, 2006). KORs are located on presynaptic terminals of DMS neurons in the NAc (Svingos et al., 2001). Depressive-like behaviours, dynorphins and KOR signalling are increased when the transcription factor cAMP and the response element-binding protein CREB are overexpressed in the NAc (Knoll and Carlezon, 2010). Enkephalin release is also involved in DMS pathway. Several animal studies have suggested that a low enkephalin tone in the NAc is associated with stress-induced anhedonia (Bertrand et al., 1997). It has been suggested a DOR control of mood, since the action of antagonists potentiate antidepressant-like effects produced by DOR activation (Cordonnier et al., 2005). The decrease activity in serotonergic (5-HT) or noradrenergic (NA) neurons is related with the appearance of depressive disorders (Krishnan and Nestler, 2010). The interaction between 5-HT and NA has been established by pharmacological studies. Weak MOR agonists (codeine) reduced the depressive-like behaviour in the tail suspension test (Berrocoso and Mico, 2009) and the effect of tricyclic antidepressants measured in the forced swimming test is antagonized by naxolone (Devoize et al., 1984).

MOR controls the activity of 5-HT neurons. Morphine is proposed to activate serotoninergic and dopaminergic neurons through a disinhibitory mechanism, with local GABA interneurons in the DRN expressing MOR (Tao and Auerbach, 2002a). While the chronic effect of morphine is the opposing and led to a compensatory up-regulation of the GABA tone on 5-HT neurons, resulting in decreased 5-HT activity (Jolas et al., 1999).

Molecular adaptations as the transiently desensitization of the main autoreceptor that controls 5-HT neuron activity (5-HT1A receptor) after morphine withdrawal treatment were identified in several rodent models of depression. DOR and KOR regulate the activity of 5-HT neurons (Tao and Auerbach, 2002b). In studies of acute social defeat, the phosphorylation of KOR and p38a kinase in the DRN is triggered. When p38a is phosphorylated the 5-HT transporter (SERT) translocation to the plasma membrane is promoted, which increases the reuptake of the neurotransmitter. The KOR/p38a signalling in the DRN involved in the prolonged mood-related deficit is not completely elucidated yet. NA neurons express MOR and, therefore, are sensitive to endogenous opioid modulation. In contrast, dopaminergic and serotoninergic neurons are not. Thus, morphine is capable to increase 5-HT and dopamine release, but decreased NA release in the forebrain (Rossetti et al., 1993).

In humans, an increase of MOR expression in frontal and temporal cortex and in caudate nuclei was found in post mortem analysis of suicide victims (Gabilondo et al., 1995). This finding suggests that depression and suicide may be associated with MOR activity. Neuroimaging techniques have enabled the study of MOR in

patients with various depressive mood states. In a study with women during neutral and sadness states it was observed a decrease in MOR neurotransmission in the rostral anterior cingulate, ventral pallidum, amygdala, and inferior temporal cortex (Zubieta et al., 2003). In women with a diagnosis of major depressive disorder an increased neurotransmission of MOR in the posterior thalamus was detected compared to healthy controls. Finally, it was observed a decreased MOR activity in the rostral anterior cingulate in healthy controls under sadness condition but not in depressed patients. An increased MOR neurotrasmission in the left inferior temporal cortex of depressed women has been reported (Kennedy et al., 2006). All these data suggested that acute (sadness state in healthy women) and chronic (major depressive disorder) negative emotional states are associated with opposing activities of MOR (Lutz and Kieffer, 2013).

3.4.3 Cognition

DOR are found in brain regions involved in learning and memory formation such as hippocampus and striatum. The first has an important role in memory formation and retrieval of spatial and contextual associations. Hippocampal DOR inhibits presynaptic neurotransmitter release and increase excitation of pyramidal cells in CA1, CA3 and dentate gyrus regions, reducing the evoked and spontaneous GABA-mediated inhibitory postsynaptic potentials. In the hippocampus, the inhibition of GABA release mediated by DOR disrupts memory retrieval and the activation of GABA receptors impairs memory acquisition and consolidation in contextual

learning tasks. The internalization of DOR in the CA1, CA3 and dentate gyrus regions in mice was observed when animals were re-exposed to a context previously paired with morphine, and this reaction was probably due to the release of enkephalin (Faget et al., 2012). The involvement of DOR in impaired performance in contextual and spatial learning tasks was confirmed in KO mice. All these results suggested an involvement of DOR in the hippocampus in the acquisition and consolidation of declarative memory (Klenowski et al., 2015).

Motor learning, operant learning and the habitual performance of these learned behaviours are modulated in the striatum and in associated basal ganglia circuits. Also, the connections established between the ventral striatum, the basolateral amygdala and the hippocampus contribute to declarative memory formation. The ventral striatum comprises the nucleus accumbens core and shell. Limbic and cortical inputs to the ventral striatum contribute to motivational learning. The nucleus accumbens has a key role in motivational learning associated with goal-directed actions and decision-making. DOR are mainly expressed at nucleus accumbens presynaptic level, contributing to cognitive behaviour by inhibitory and dopaminergic inputs into the nucleus accumbens. It was recently confirmed also postsynaptic expression of DOR in GABA projection neurons and cholinergic interneurons within the nucleus accumbens shell (Bertran-Gonzalez et al., 2013; Scherrer et al., 2006). MORs are expressed at the extrasynaptic membrane of dendrites of cholinergic cells and GABA medium-sized spiny neurons. These neurons form synapses that receive excitatory and

GABA input to modulate the activity of nucleus accumbens neurons. The expressions of MOR in nucleus accumbens cells modulate the presynaptic release of GABA (Svingos et al., 1997).

The striatum is the largest nucleus of the basal ganglia involved in learning and memory. Around the 95% of the striatum neurons are GABA medium-sized spiny neurons; these neurons receive convergent glutamate afferents from the cortex and thalamus, as well as dopaminergic afferents from the substantia nigra (Graybiel, 2008). GABA medium-sized spiny neurons of striatum are classified based on the differential expression of dopamine receptors in D1 and D2 (Blomeley and Bracci, 2011). The GABA medium-sized spiny D1 receptor neurons define the direct pathway and express dynorphins, while the D2 receptor neurons contribute to the indirect pathway and express enkephalins (Britt and McGehee, 2008; Gerfen, 1992; Gertler et al., 2008). Currently is accepted that D1 pathway mediates action initiation and the D2 pathway inhibits alternative actions (Cui et al., 2013).

Opioids have the ability to modulate the activity between neurons within the striatum. For example, MOR can inhibit the cortical-induced excitation of neighbouring GABA medium-sized spiny neurons probably thought the release of enkephalin from these striatopallidal neurons (Blomeley and Bracci, 2011). The reduction in cholinergic interneuron excitability that resulted in local inhibition may be mediated by MOR action (Ponterio et al., 2013). Recently, it was demonstrated the importance of corticostriatal circuits in the dorsal striatum in the development of goal directed to habitual learning (Gremel and Costa, 2013).

Dynorphins have a role in the signalling of activation and suppression of memory formation. The relation between these opioid peptides and the cognitive abilities was observed in a human genetic study (Kölsch et al., 2009). However, the influence of the decrease in KOR on memory is controversial. There are reports of improvement, deficit and no changes (Colombo et al., 1993; Fanselow et al., 1991; Jamot et al., 2003; Magnusson et al., 2009). A positive effect of KOR and dynorphins in social memory has been reported with the use of KOR antagonists and in animals with genetic deletion of prodynorphins (Bilkei-Gorzo et al., 2014).

4 Use of genetically modified mice in neuropathic pain

4.1 General aspects

The homologous recombination has allowed researchers to target genes and generated strains of mice deficient of opioid genes. These animals are powerful tools that allow the identification of molecular targets of prototypic opioid agonists or antagonists. The use of genetic modified animals let explore the implication of each receptor and peptide in many opioid-controlled behaviours and to discover functions that have remained unexplored. Regarding neuropathic pain, there are few studies with opioid mutant mice. Nowadays, there are available mice lacking every component of the opioid system. The mutant strains could be lacked of only one component of the opioid system or could have multiple mutations

generating mice lacking two or more opioid receptor genes (Kieffer and Gavériaux-Ruff, 2002).

In general, opioid receptor genes are organized similar: there are three exons that contain the coding regions of opioid receptors. In the exon 1 the coding region for the extracellular domain and transmembrane domain I is localized, the transmembrane domains II–IV are in exon 2 and exon 3 encode transmembrane domains V–VII followed by the cytoplasmic C-terminal. The MOR gene is a little different in the three coding regions and it has a fourth coding exon with twelve codons (Kieffer and Gavériaux-Ruff, 2002).

4.2 Opioid system knockout mice

4.2.1 Opioid receptor deficient mice

4.2.1.1 MOR KO mice

Currently, it has been reported the generation of MOR-deficient mice by three techniques: the deletion of exon 1 (Pintar et al., 1999; Sora et al., 1997), the insertion of a neomycin cassette in exon 2 (Matthes et al., 1996) or the deletion of exons 2 and 3 (Loh et al., 1998).

With the objective to evaluate whether MOR are critical for the anti-allodynic action of long-term nortriptyline treatment, mice lacking these receptors were evaluated (Matthes et al., 1996). It was observed that MOR does not have a critical role in the physiological control of neuropathic allodynia in the sciatic nerve cuffing model. The intensity of allodynia was similar between MOR deficient mice and their wild-type littermates before and after the induction of the

neuropathy. After chronic treatment with nortriptyline, the MOR KO and wild-type mice recovered from their neuropathic allodynia, the delay for therapeutic onset was unaffected by the presence of absence of MOR. This study revealed that while MOR mediate the acute analgesic effect of morphine in a neuropathic pain condition, they are not necessary for the effect of chronic antidepressant drug treatment. However, DOR appear to be critical for nortriptyline relief of neuropathic allodynia (Bohren et al., 2010).

4.2.1.2 DOR KO mice

Two groups have reported the generation of mice deficient DOR (Filliol et al., 2000a; Zhu et al., 1999). To generate these strains, exon 1 or exon 2 is deleted by homologous recombination.

The involvement of DOR in neuropathic pain was evaluated in a study with mice lacking DOR. Different behavioural manifestations of neuropathic pain were enhanced in these KO animals. It was observed that the partial sciatic nerve ligation enhances thermal hyperalgesia and mechanical allodynia in male and female DOR KO and that thermal allodynia was only exacerbated in males. The results of this study suggested a new potential therapeutic use of DOR agonists (Nadal et al., 2006).

4.2.1.3 KOR KO mice

There is only one report of mice lacking KOR (Simonin et al., 1998). In this case the first coding exon is deleted.

A study to determine whether the KORs participate to the action of nortriptyline in neuropathic pain using genetic and pharmacological approaches was performed. The authors did not find differences neither in basal mechanical sensitivity nor after sciatic nerve cuffing between KOR mutant and wild type mice. It was observed that KOR does not play a critical role in the establishment or the maintenance of neuropathic allodynia. They also found that KOR are not necessary for the antiallodynic action of tricyclic antidepressants and as a result they confirmed the critical role of DOR for the relief of neuropathic mechanical allodynia following tricyclic antidepressant treatment (Megat et al., 2015).

Finally, mice lacking all three opioid receptor genes have also been obtained in a two-step strategy. Breeding of mice lacking MOR exon 2 (Matthes et al., 1996) with mice lacking KOR exon 1 (Simonin et al., 1998) is produced MOR/KOR double mutants, while breeding mice lacking MOR exon 2 with exon 1 DOR-deficient mice (Filliol et al., 2000a) resulted in a MOR/DOR double mutant. Also, triple mutants were obtained from MOR/KOR and MOR/DOR mattings (Simonin et al., 2001).

4.2.2 Opioid peptide deficient mice

4.2.2.1 Proopiomelanocortin (Pomc)

Two strains of Pomc KO mice have been produced: one with a specific deletion of the opioid peptide β-endorphin and a second one without the complete *Pomc* gene. A mouse with specific deletion of β-endorphin is produced by introducing a stop codon in exon 3 of

the *Pomc* gene (Rubinstein et al., 1996). In these mice only the β-endorphin peptide was absent, while the β-melanocyte stimulating hormone (β-MSH) and the ACTH were unchanged. Another mutant mouse lacking the whole coding region of the *Pomc* gene was reported (Hochgeschwender et al., 1999).

4.2.2.2 Preproenkephalin (Penk)

For the *Penk* gene there are described two strains of KO mice. In both cases, the enkephalin-coding region (5 part of exon 3) is targeted. In the first mutant the exon resulted truncated resulting in an unexpected partial duplication of exon 3 (König et al., 1996b). The gene KO proved successful since enkephalin could not be detected in the homozygous mutant. The second is lacking the 5 part of exon 3 (Ragnauth et al., 2001).

4.2.2.3 Preprodynorphin (Pdyn)

Two mice lacking the *Pdyn* gene are available. The first strategy comprises the deletion of the whole coding region (exons 3 and 4); this produces a loss of *Pdyn* mRNA in homozygous mutants (Sharifi et al., 2001). The second strategy consisted in the deletion of exon 3 and part of exon 4, which produced mutant animals where dynorphin peptides were absent (Zimmer et al., 2001).

Pdyn KO mice had normal responses to acute non-noxious stimuli and a mild increased sensitivity to some noxious stimuli. In a study with *Pdyn* KO mice it was observed that dynorphins are not required for the initiation of neuropathic pain. After spinal nerve

ligation, both wild type and KO mice had decreased thresholds to innocuous mechanical and to noxious thermal stimuli. In day 10 after surgery KO mice return to baseline pain values, while in wild type neuropathic pain was sustained. Up regulation of lumbar dynorphins was found in wild type on day 10 but not in day 2 after surgery. Interestingly, the density of MOR, DOR and KOR and G-protein activation were not different between wild type and KO mice. These levels remained unchanged after nerve injury. This study confirmed that the up regulation of spinal dynorphin is pronociceptive and is required for the maintenance of persistent neuropathic pain, and that there are different processes for the initiation and for the maintenance of the neuropathic pain (Wang et al., 2001).

OBJECTIVES

Objectives

- To validated different behavioural outcomes to measure the emotional and cognitive manifestations of neuropathic pain induced in mice by partial sciatic nerve ligation.
- To evaluated the influence of specific behavioural traits on the nociceptive, emotional and cognitive manifestations of neuropathic pain.
- To explore the role of the preproenkephalins on the nociceptive, emotional and cognitive manifestations of neuropathic pain.

RESULTS

Article 1

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

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* These authors contributed equally to this work.

Objectives

To validated different behavioural outcomes to measure the emotional and cognitive manifestations of neuropathic pain induced in mice by a partial sciatic nerve ligation.

Material and methods

Swiss albino male mice underwent a partial sciatic nerve ligation or sham surgery. These animals were evaluated in three experimental sequences to determine at different time points nociceptive, emotional and cognitive manifestations of neuropathic pain. Von Frey, plantar and cold plate test were used to evaluated the nociceptive responses. In the elevated plus maze was assessed the anxiety-like behaviour and the depressive-like behaviour in the forced swimming test. The anhedonic state was determined in metabolic boxes. Finally, the cognitive component was evaluated as measure of long-term memory in the object recognition memory test and by the operant responding maintained by food. Moreover, the effects of a chronic treatment of pregabalin at dose 20 mg/kg twice daily were evaluated on these pain manifestations.

Results

Allodynia and hyperalgesia in neuropathic pain mice was associated with increased anxiety- and depressive-like behaviours, reduced memory functions, development of an anhedonic state and impaired motivation to obtain food in the operant task. Chronic pregabalin treatment improved the nociceptive, anxiety-like and anhedonic responses, as well as the memory deficit, but did not modify the depressive-like alterations and the decreased motivation in these mice.

Conclusions

Some emotional manifestations of chronic pain do not necessarily resolve when pain is relieved and underline the relevance to evaluate multiple behavioural responses associated with chronic pain, including the affective-motivational and cognitive behaviours, to increase the predictive value of preclinical drug discovery.

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SUPPLEMENTARY RESULTS

Evaluation of the cognitive manifestations associated with chronic neuropathic pain in relevant operant and non-operant behavioural mouse models.

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1. Introduction

Neuropathic pain is defined by the International Association for the Study of Pain as the pain caused by a lesion or disease of the somatosensory nervous system. The pain experience consists of three dimensions: sensory, affective and cognitive (Liu and Chen, 2014). Currently, there is an increasing interest in elucidating the effects of chronic pain in cognition that can be summarized in two issues. First, the negative effects of pain may exacerbate the suffering of patients and could produce anxiety, depression and restriction of activities, leading to a decrease in quality of life of patients. Second, the cognitive impairment in patients produced by chronic pain could restrict patients' effectiveness in communicating their symptoms and making in this way the choice of treatment difficult. Additionally, as cognitive symptoms are not assessed, they are not adequately considered in the therapeutic plan (Kreitler and Niv, 2007). Many patients suffering from chronic pain reported cognitive problems, mainly related to memory and attention. The most common cognitive problems reported are forgetfulness, difficulty in finishing tasks, attention deficits, memory disturbances, difficulty in handling everyday tasks and even daily conversations (McCracken and Iverson, 2001; Munoz and Esteve, 2005). Despite this high frequency in the clinical field, there are few studies in animals trying to elucidate and evaluate the cognitive function in chronic pain models (Liu and Chen, 2014). In the present study, we characterized the consequences of neuropathic pain exposure on different cognitive functions using operant and non-operant behavioural models of learning and memory in mice.

2. Materials and methods

2.1 Animals

Swiss albino male mice (Charles River, Lyon, France) were used in this study. Mice were 8-12 weeks old at the beginning of the experiments and were housed individually with free access to water and food, except in specific moments described in the experimental protocol. The housing conditions were maintained at 21±1º C and 55±10% relative humidity. A light/dark reverse cycle (light off at 08:00 AM and on at 08:00 PM) was used. 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 (Comité Etico Experimental Animal, Instituto Municipal de Asistencia Sanitaria/Universitat Pompeu Fabra). All the experiments were performed under blind conditions and the treatments randomized between groups.

2.2 Drugs and treatments

Pregabalin (generously provided by Laboratorios Dr. Esteve, Barcelona, Spain) was dissolved in physiological sterile saline solution (0.9%) and administered intraperitoneally (ip) twice daily at the dose of 10 mg/kg. Control mice received the ip administration of physiological sterile saline solution (0.9%). The pregabalin treatment started one week after the partial sciatic nerve ligation (PSNL) surgery when the majority of pain-related alterations were already established. The duration of the treatment was until day 23

after neuropathic pain induction when the last pain measure was performed.

2.3 Neuropathic pain induction

PSNL was performed to induce neuropathic pain, as previously described (Malmberg and Basbaum, 1998). Mice were anaesthetized with isoflurane 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. (9–0) non-absorbable virgin silk suture (Alcon® Surgical Inc., TX, USA), leaving the rest of the nerve "undamaged". Control mice underwent sham surgery, consisting in the same procedure used for PSNL, but in this case the sciatic nerve was not ligated.

2.4 Nociceptive behaviour

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation through the up–down paradigm, as previously reported (Chaplan et al., 1994). Heat hyperalgesia was assessed by evaluating the hind paw withdrawal latency in response to radiant heat with the plantar test apparatus (Ugo Basile, Italy), as previously reported (Hargreaves et al., 1988). Cold allodynia was assessed with the hot/cold plate analgesia meter (Columbus, USA), as previously described (Bennett and Xie, 1988). The number of elevations of each hind paw was recorded for 5 min on the cold surface of the hot/cold plate analgesia meter, which was maintained at a temperature of

5 ± 0.5º C. A score was calculated for each animal as the difference of number of elevations between ipsilateral and contralateral paws (Bura et al., 2013).

2.5 Cognitive behaviour

Novel object recognition memory (NOR) was performed in the V-maze (Panlab, Spain) to measure cognitive performance. This task consists of three sessions: habituation, training and test. On day 1, mice were habituated for 9 min to the V-maze. On the second day, mice were put back in the maze for 9 min, two identical objects were presented and the time that mice spent exploring each object was recorded. Mice were again placed in the maze 24 h later for 9 min, one of the familiar objects was replaced with a novel object and the total time spent exploring each of the two objects (novel and familiar) was computed, and 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. A higher discrimination index is considered to reflect greater memory retention for the familiar object (Puighermanal et al., 2009).

2.6 Operant behaviour maintained by food

2.6.1 Operant chambers

Training session of food-maintained operant behaviour were performed in mouse operant chambers (model ENV-307A-CT, Med Associates Inc., Georgia, VT, USA), as previously described (Bura et al., 2013). The chambers were equipped with two retractable

levers, one randomly selected as the active and the other as the inactive. Pressing the active lever resulted in a pellet delivery together with a stimulus-light for 2 s (associated cue), while pressing the inactive lever had no consequences. A food dispenser equidistant between the levers permitted the delivery of food pellets. The beginning of each session was signalled by turning on a house light placed on the ceiling of the box for 3 s, which was then turned off during the remaining duration of the session. The active and inactive levers were counterbalanced between animals. A time-out period of 10 s was established after each pellet delivery where no cues were presented and no reward was provided following an active response. The session was finished after 100 reinforces were delivered or after 1 h, whichever occurred first.

2.6.2 Food pellets

Standard precision food pellets of 20 mg were used (TestDiet, IN, USA). The standard pellet formula was similar to the standard maintenance diet provided to mice in their home cage: 24.1% protein, 10.4% fat, 65.5% carbohydrate (sucrose content was 3.09% of the total quantity of carbohydrates), with a caloric value of 3.30 kcal/g.

2.7 Experimental protocol

The nociceptive responses were evaluated in the von Frey and plantar models before PSNL surgery (day -1) and on days 3, 6, and 23 post-surgery (Figure 1). The cold plate test was not performed on day 3 to minimize the habituation of animals to this test. Mice were

single housed in a room with the reversed light/dark cycle for 7 days before the beginning of the experiments to reduce the possible stress induced by the isolation of mice during each daily 1 h session in the operant chamber. The reversed light-dark cycle, in which mice are naturally more active, was essential to allow an appropriate acquisition of the operant tasks. The nociceptive responses were assessed in the von Frey and plantar models before (day -1) and on days 3, 6 and 23 after PSNL (Figure 1). On day 6 (three days before starting the operant responding sessions) mice were food-deprived to maintain the 85% of their initial weight adjusted for growth. This food restriction was maintained during the training period and when the novel object recognition test was performed, until day 22 after PSNL. In the whole experiment water was available ad libitum. Chronic pregabalin (10 mg/kg) or saline treatment started on day 7 and lasted until day 23 after PSNL. Mice were tested 30 min after drug administration. The operant responding sessions were divided in two phases. During all the experiment, standard pellets were delivered as reward when the mouse selected the active lever. In the first phase (day 9 to 14 after surgery) mice were trained during 5 days on a fixed-ratio 1 (FR1) schedule of reinforcement. On a FR1 schedule the food pellet is delivered after one active lever selection. As a measure of motivation, on day 14 mice were exposed to a progressive ratio (PR) schedule. The response requirement to earn one pellet was escalated according to the following series: 1-5-12-33-51-75-90-120-155-180-225-260-300-350-410-465-540-630-730-850-1000.

The PR session lasted 3 h or until mice stopped responding for at

least 1 h. The breaking point was determined in each animal as the last response ratio completed. The second phase of training and the NOR test started on day 14. As the NOR test was performed in three days, the habituation was carried out on day 14, the training on day 15 and the test on day 16 to evaluate long-term memory. In the second phase, mice were trained for 5 additional days (days 15-19) on FR5 and a second PR was performed on day 20. A FR5 means that after 5 active lever selections the mouse will receive one pellet of food. A second NOR test was performed from day 20 to day 22.

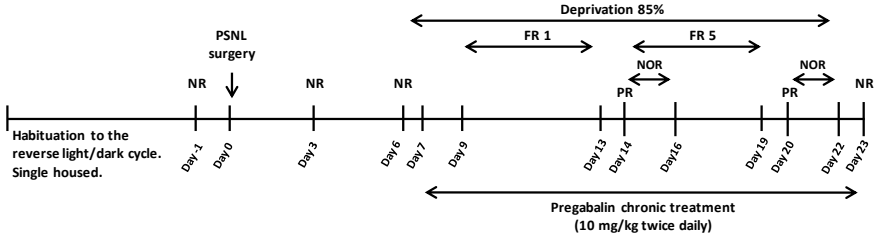


Figure 1. Experimental sequence to evaluate the consequences of neuropathic pain on different cognitive functions using operant (food-maintained operant behaviour) and non-operant (novel object recognition test) behavioural cognitive models after pregabalin chronic treatment in mice exposed to PSNL. PSNL, partial sciatic nerve ligation; NR, nociceptive responses; NOR, novel object recognition test; FR1, fixed ratio 1; FR5, fixed ratio 5; PR, progressive ratio.

2.8 Statistical analysis

For nociceptive responses, data obtained in the von Frey, plantar test and cold plate were analysed by three-way repeated measures ANOVA (surgery and treatment as between-subject factors and day as within-subject factor). Data of PR and active responses in the operant responding paradigm were analysed by two-way (treatment

and surgery) or repeated measures four-way ANOVA (treatment and surgery as between subject factors, and day and phase as within subject factors), respectively. *Post hoc* analysis (Fisher's LSD) was performed after ANOVA when appropriate. Data obtained in the NOR test were analysed by a two-way ANOVA (surgery and treatment). STATISTICA 6.0 software (StatSoft, Inc., OK, USA) was used. The differences were considered statistically significant when the P value was below 0.05.

3. Results

3.1 Mechanical allodynia (von Frey stimulation model)

Three-way ANOVA for the ipsilateral responses to mechanical stimulation revealed a significant effect of surgery ($F_{(1,43)}$=228.19; P<0.001), treatment ($F_{(1,43)}$=6.88; P<0.05) and day ($F_{(3,129)}$=6.29; P<0.001), and interaction between these factors ($F_{(3,129)}$=10.06; P<0.01). No significant effects were revealed for the contralateral responses. Subsequent post hoc analysis indicated that the baseline values for ipsilateral and contralateral hind paws were similar in all mouse groups before surgery. Nociceptive responses were not modified by sham surgery. In contrast, PSNL significantly decreased the withdrawal threshold only in the ipsilateral paw. Saline treatment did not modify any of the nociceptive responses, whereas pregabalin treatment significantly increased the withdrawal threshold in the ipsilateral paw of the PSNL mice. Therefore, chronic pregabalin treatment significantly improved the mechanical allodynia induced by PSNL in the ipsilateral paw (Fig. 2a).

3.2 Heat hyperalgesia (plantar test)

Three-way ANOVA for the ipsilateral responses in plantar test revealed a significant effect of surgery ($F_{(1,44)}$=378.24; P< 0.001), treatment ($F_{(1,44)}$=4.92; P< 0.05) and day ($F_{(3,132)}$=77.54; P< 0.001), and interaction between these factors ($F_{(3,132)}$=12.45; P<0.001), whereas no significant effects were revealed for the contralateral responses. Subsequent *post hoc* analysis indicated that the baseline values for both ipsilateral and contralateral hind paws were similar in all mouse groups before PSNL or sham surgery. Sham surgery did not modify the nociceptive responses. In contrast, PSNL significantly decreased the withdrawal latency in the ipsilateral, but not the contralateral paw. Saline treatment did not modify any of the nociceptive responses, whereas pregabalin treatment significantly increased the withdrawal latency only in the ipsilateral paw of the PSNL group. However, the ipsilateral withdrawal latency in PSNL mice was still significantly different from sham mice after pregabalin treatment. Therefore, chronic pregabalin treatment significantly improved the thermal hyperalgesia induced by PSNL in the ipsilateral paw (Fig. 2b).

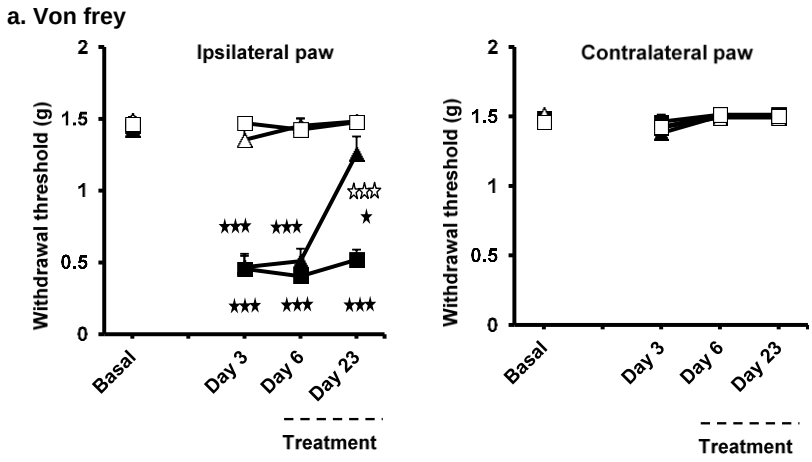
3.3 Cold allodynia (cold plate test)

Three-way ANOVA for the score value revealed a significant effect of surgery ($F_{(1,43)}$=33.02; P<0.001) and day ($F_{(2,86)}$=25.26; P<0.001), and interaction between these day and surgery ($F_{(2,86)}$=26.76; P<0.001). Subsequent post hoc analysis indicated that the baseline score values were similar in all mouse groups before pain induction. Sham surgery did not modify these nociceptive responses, but

PSNL surgery significantly increased the score value. Saline treatment did not modify any of the nociceptive responses. Chronic treatment with pregabalin reduced the cold allodynia induced by PSNL **(Fig. 2c)**.

3.4 Food-maintained operant behaviour

Four-way ANOVA for the active responses in the operant task revealed only a significant effect of surgery ($F_{(1,41)}$= 6.46, P<0.05), whereas no significant effects were revealed for the inactive responses. In this analysis was revealed significant decrease in the active responses in both saline and pregabalin PSNL groups compared to sham. Two-way ANOVA for the breaking point revealed no significant effect of surgery or treatment or interaction between these factors in any of the PR performed at the end of each of the two training phases (Figure 3b,c). Therefore, PSNL impaired the operant responses in the FR5 and chronic pregabalin treatment did not affect motivation for food.



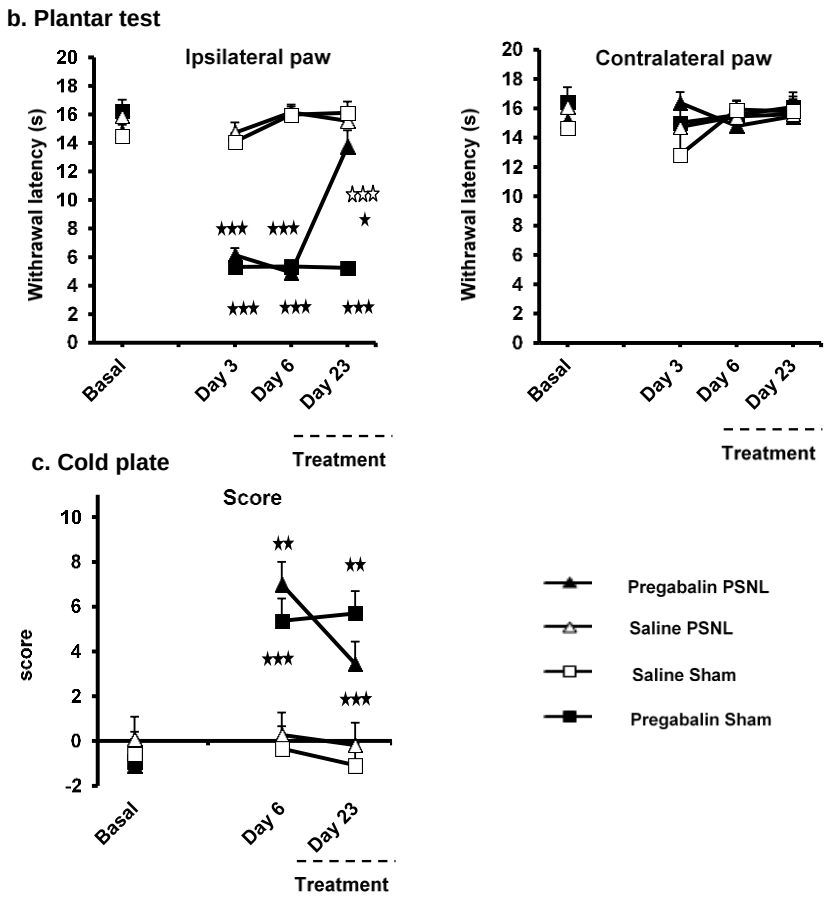


Figure 2. Effects of pregabalin on nociceptive behaviour of mice exposed to PSNL. (a) Mechanical allodynia evaluated by the von Frey model (withdrawal thresholds, g) and (b) Heat hyperalgesia evaluated in the plantar test (paw withdrawal latencies, s) were measured under basal conditions and on days 3, 6, and 23 after PSNL or sham surgery. c) Cold allodynia evaluated in the cold plate test, score values: difference in the number of elevations between the ipsilateral and contralateral paws was measured on basal conditions and on day 6 and 23 after PSNL. From day 7 to 23, mice were chronically treated with pregabalin (10 mg/kg, twice daily) or saline. The dotted line indicates the test days in which the behavioural responses were evaluated under pregabalin or saline chronic treatment. Data are expressed as mean ± SEM (n= 12 per group). ★★ P <0.01, ★★★ P <0.001 vs. sham surgery (Fisher's LSD test). ☆☆☆ P <0.001 vs. saline treatment (Fisher's LSD test). PSNL: partial sciatic nerve ligation.

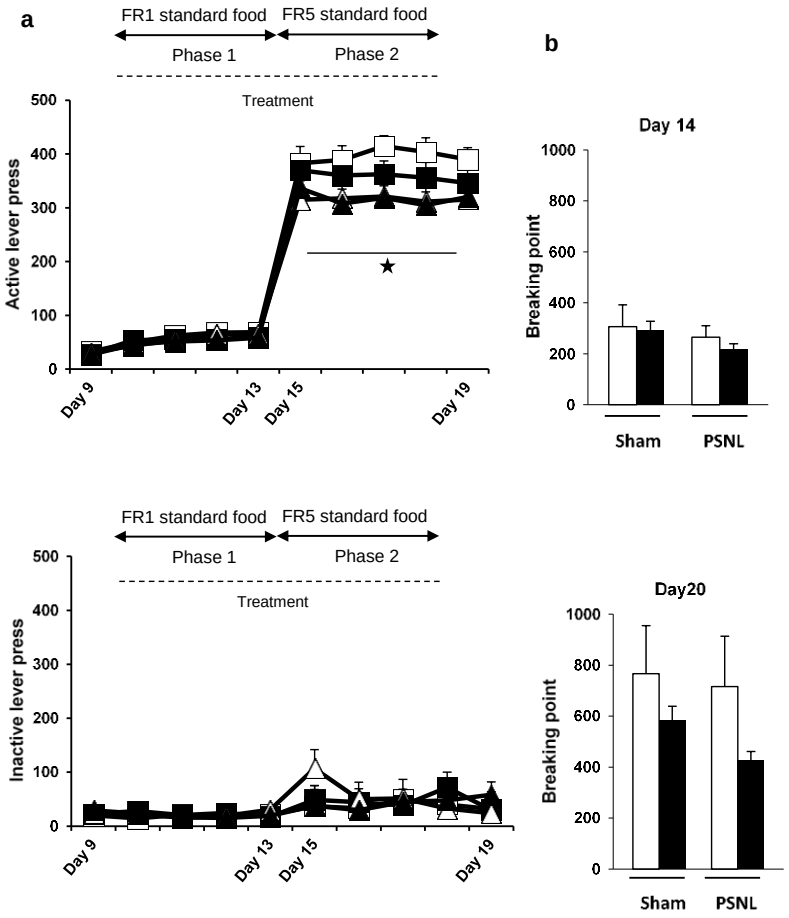


Figure 3. Effects of pregabalin on the cognitive component of neuropathic pain measured in an operant behavioural model in mice. a) Active and inactive lever presses were evaluated after PSNL or sham surgery under FR1 (phase 1, days 9-13) and FR5 (phase 2, days 15-19) with standard pellets. (b) Breaking point achieved in the PR sessions at the end of phases 1 (day 14) and 2 (day 20) in PSNL or sham mice. From day 7 to 27, mice were chronically treated with pregabalin (10 mg/kg, twice daily) or saline and the behavioural responses evaluated 30 min after the treatment administration. The dotted line indicates the test days in which the behavioural responses were evaluated under pregabalin or saline chronic treatment. Data are expressed as mean ± SEM (n=12 per group). ★ $P<0.05$ vs. sham surgery (Fisher's LSD test). PSNL, partial sciatic nerve ligation; FR1, fixed ratio 1; FR5, fixed ratio 5; PR, progressive ratio

3.5 Novel object recognition test

Two-way ANOVA for the discrimination index data obtained in the first NOR at day 16 showed a significant effect of surgery ($F_{(1,41)}$=6.04, P<0.05) (Figure 4a). This significant effect of surgery was not found in the two-way ANOVA analysis performed for the discrimination index data obtained at day 22 after surgery (Figure 4b).

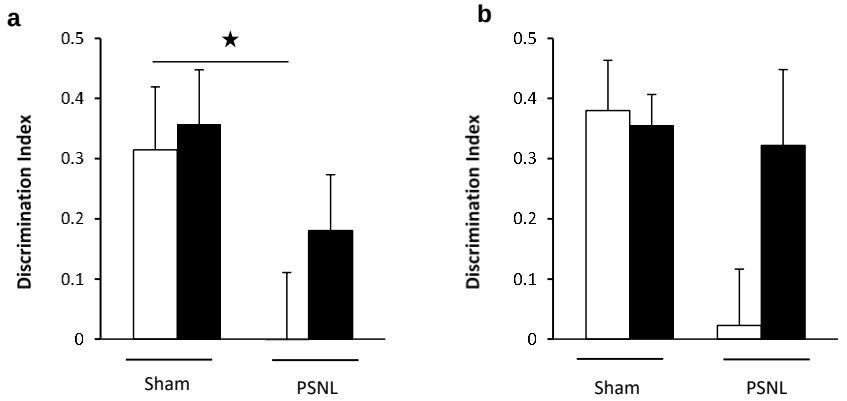


Figure 4. Effects of pregabalin on the cognitive component of neuropathic pain measured in a non-operant cognitive model in mice. The NOR discrimination index was evaluated at two-time points a) on day 16 and b) on day 22 after the PSNL or sham surgery. The effects of the chronic pregabalin (10 mg/kg, twice daily) or saline treatment (from day 6 to 22) were evaluated in a non-operant cognitive model. Data are expressed as mean ± SEM (n= 12 per group). ★P<0.05 vs. sham surgery (Fisher's LSD test). PSNL, partial sciatic nerve ligation; NOR, novel object recognition test.

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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, Bilecki W, Bargiela A, Gonçalves L, Dickenson AH, Przewlocki R, Baños JE and Maldonado R. Submitted to *European Journal of Pain*. * These authors contributed equally to this work

Objectives

To identified the influence of sociability, anxiety and depressive traits in the inter-individual vulnerability to develop the nociceptive, emotional and cognitive manifestations of neuropathic pain.

Material and Methods

250 swiss albino mice were evaluated in the sociability, elevated plus maze, light dark box, tail suspension and forced swimming test to identify the animals displaying extreme phenotypes on social and emotional responses. Locomotor activity was evaluated to eliminate animals with abnormal basal behaviour. First, electrophysiological study was performed to find a possible correlation between the spontaneous and evoked CeA neuronal activity and the behavioural responses. In another group of extreme phenotype mice, neuropathic pain was induced to evaluate the possible influence of the behavioural traits on the inter-individual variability of pain manifestations. 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. In this part of the emotional and cognitive evaluation was used the sucrose preference, zero maze and object recognition test. Finally, brain samples of amygdala were isolated at day 41 after surgery from animals used for the behavioural study. Transcriptional modifications in this area were examined.

Results

In accordance with the behavioural responses obtained, mice were classified in groups of high, intermediate and low sociability, anxiety and depressive-like behaviour. Spontaneous CeA neuronal activity was only related with the sociability-like behaviour. The anxiety-like behavioural was relates with several manifestations of neuropathic pain. An increased expression of *Pdyn* was observed in the PSNL sociability and anxiety-like behaviour selected mice. Modification in *Nr3c1* and *Gadd45* expression were associated with the depressive trait.

Conclusion

Anxiety trait is an inter-individual vulnerability factor that aggravates neuropathic pain manifestations with *Pdyn* mRNA as a concomitant biomarker in the amygdala. Depressive trait conferred some resistance to the development of neuropathic pain and may be attributable to the expression of the *Gadd45* gene in the amygdala.

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

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Keywords: neuropathic pain, behavioural traits, anxiety, depression, sociability, biomarkers, amygdala.

Abstract

Chronic neuropathic pain is a complex disorder often associated with emotional and cognitive deficits that may interact with nociception. There is high inter-individual variability in the manifestations of neuropathic pain, which can relate to personality traits of the patient. We aim to identify the influence of different behavioural traits in the 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 association among the spontaneous and evoked neuronal activity in the central amygdala and their behavioural traits. In a second step, neuropathic pain was induced to these extreme phenotype mice to evaluate the possible influence of the behavioural traits on pain manifestations and gene expression profiles in the amygdala. Our results showed an association of the spontaneous central amygdala neuronal activity with the sociability-like behaviour, but not with emotional-like responses. The data showed that the anxiety-like behavioural trait was associated with behavioural and neurochemical changes related to the emotional manifestations of neuropathic pain. Gene expression analysis identified *Pdyn* and *NR3C1* genes as potential molecular markers of anxiety- and depressive-dependent susceptibility to chronic pain, respectively. Overall, these results suggest that anxiety-like behaviour can be of relevance to predict the inter-individual vulnerability to the emotional 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 et al., 2016). Several reports have established its association with emotional disorders, such as anxiety and depression (Neugebauer et al., 2004; La Porta et al., 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 interact 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 therapeutic approach for the treatment of these patients should consider these three factors of chronic pain.

The manifestations of neuropathic pain show a high inter-individual variability that depends on multiple factors, including the personality traits of patients (Asghari and Nicholas, 2006). It has been well documented that emotional, cognitive and social personality traits can modulate pain perception (D'Amato and Pavone, 2012; Rhudy et al., 2008). 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 (Ikeda et al., 2007; Neugebauer et al., 2004, 2009). The amygdala plays a key role in the formation of fear-related memories and emotional processing (Phelps and LeDoux, 2005a). The amygdala contains several nuclei, including the lateral (LA), basolateral (BLA) and central (CeA) nuclei, which are important for sensory processing (Neugebauer et al., 2009). Strong neuronal responses to peripheral stimuli have been reported in the CeA, which has been defined as the 'nociceptive amygdala' (Neugebauer et al., 2004). Increased excitability of CeA neurons has 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 and Wager, 2007).

In this study, we evaluated the influence of sociability, anxiety-like and depressive-like behavioural traits on the nociceptive, emotional and cognitive manifestations of neuropathic pain. We selected mice displaying extreme phenotypes on social and emotional responses, and analyzed the possible relationship between the spontaneous and evoked CeA neuronal activity and their behavioural traits. Neuropathic pain was induced to evaluate the influence of the behavioural traits on the inter-individual variability of pain manifestations. Gene expression profiles in the amygdala were also studied to elucidate the molecular mechanisms associated with the manifestations of chronic neuropathic pain.

2. Materials and methods

2.1. Animals

Swiss albino male mice with an 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 to avoid potential bias with other behavioural tests (Table 1). Those displaying high, low and intermediate social and emotional responses were selected according to the criteria exposed in Table 1. Spontaneous and evoked CeA neuronal activities were recorded in mice selected for 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). 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 cm 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, although allowing 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 tests were used to determine the extreme phenotypes; the elevated zero maze was performed after sciatic 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) that was elevated 40 cm above the floor. The percentage of entries and time spent in the

open arms were 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., 2000b). 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 et al., 1985b). Mice were suspended by their tails with tape, in such a position that escape or

hold on to nearby surfaces were not allowed 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 test. Mice were placed in a narrow (17.5 x 12.5 cm) Plexiglas cylinder containing water to a depth of 15 cm (22±0.2 °C) (Porsolt et al., 1977a). 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 an 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

The novel object recognition test was performed 20 days after nerve injury as previously described (Puighermanal et al., 2009) 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 that the animal spent exploring the novel (Tn) and familiar (Tf) object divided by the total time of object exploration: (Tn-Tf)/(Tn+Tf).

2.4. Electrophysiological procedures

Extracellular recordings were made from single neurons in the right CeA after the behavioural test used to select extreme phenotypes mice. Parylene coated tungsten electrodes were applied (A-M Systems, USA) using the following stereotaxic coordinates (Paxinos and Watson, 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, 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 and Basbaum, 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 sequence.

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 avoid 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 (QiagenInc.) in tissue samples from amygdala. The qPCR reactions were performed using isoform specific TaqMan® probes (BDNF-Mm01334042_m1, C1qa-Mm00432142_m1, FKBP 5-Mm00487401_m1, Gadd45g-Mm00442225_m1, GFAP-Mm0125 3033_m1, HPRT1-Mm01545399_m1, Il1b-Mm00434228_m1, Il6-Mm00446190_m1, Nr3c1-Mm00433832_m1, PDYN-Mm0045757 3_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 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 (Table 3). Prism 6 (GraphPad) was used for statistical analysis. Differences were considered to be statistically significant when $p < 0.05$. Data were expressed as mean ± SEM.

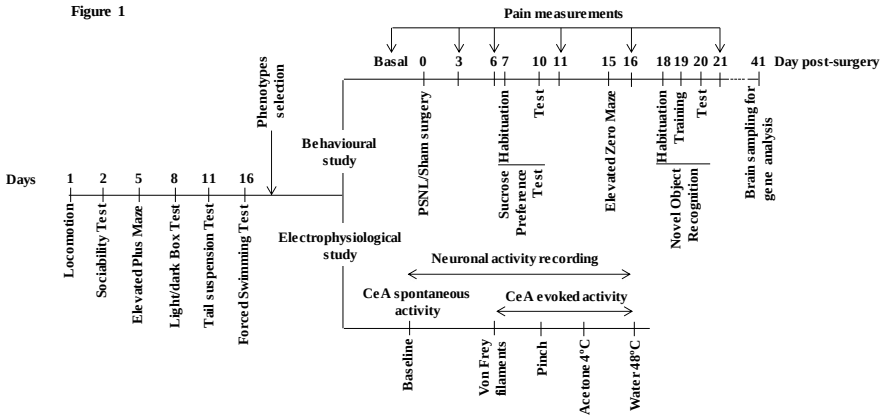

Figure 1. Experimental sequence to select mice displaying extreme phenotypes on different behavioural responses, for the assessment of electrophysiological correlates and for the evaluation of the nociceptive, affective and cognitive behaviours in mice exposed to neuropathic pain.

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 to avoid a bias of this abnormal behaviour. After excluding these animals (n=21), mice were classified into three phenotypes (high, low and intermediate percentiles) for each behavioural trait, using the criteria defined in Table 1. Each behavioural trait was analyzed 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: sociability-, anxiety- and depressive-like behaviour (25 mice per each high, low and intermediate percentile). Ten animals per group were used for electrophysiological studies, and 15 mice per group were evaluated for establishing nociceptive, emotional and cognitive manifestations of neuropathic pain, and to provide tissue samples for identification of biomarkers. 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	Below 10th 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 sociability, anxiety and depressive-like behavioural phenotypes

Electrophysiological data were obtained from selected mice displaying extreme phenotypes. CeA neurons of mice presenting a high sociability phenotype 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 selected considering the anxiety-like phenotype showed no significant differences among low, intermediate and high percentile (Figure 2B). Neurons from the intermediate percentile of the depressive-like phenotype 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 groups displaying extremes phenotypes. Neurons were individually analyzed and separated according to any change observed in the activity after application of stimuli. 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.

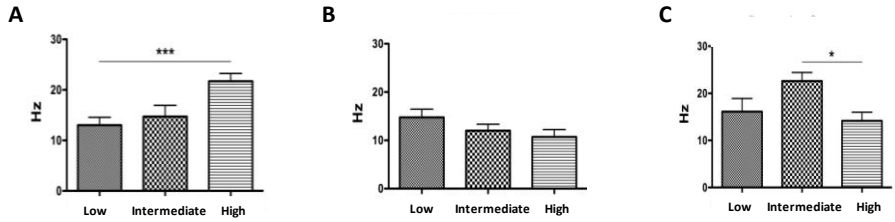

Figure 2. Electrophysiological evaluation of mice displaying extreme phenotypes. Central amygdala (CeA) spontaneous activity of mice in low, intermediate and high phenotypes for (A) sociability-like, (B) anxiety-like and (C) depressive-like behaviour. ★P < 0.05, ★★★P < 0.001 between groups

3.3. Nociceptive behaviours

3.3.1. Nociceptive, emotional and cognitive manifestations of neuropathic pain on mice displaying different sociability-like behavioural phenotypes

Series of 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 surgery (Table 2). No significant effect was revealed neither for the baseline values nor the contralateral responses (data not shown). 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).

Table 2. One way ANOVA values for nociceptive evaluation

		Behavioural trait								
	Day after surgery	Sociability-like			Anxiety-like			Depressive-like		
		Error	*F*	*P*	Error	*F*	*P*	Error	*F*	*P*
Mechanical allodynia	Baseline	35	5.05			N/S			N/S	
	3	36	77.32		36	59.08		37	67.81	
	6	37	16.28		36	76.44		36	63.89	
	11	32	36.96	<0.001	35	40.34	<0.001	34	39.93	<0.001
	16	32	26.31		37	35.49		33	15.7	
	21	32	14.04		32	13.82		33	12.5	
Heat hyperalgesia	Baseline		N/S			N/S			N/S	
	3	25	17.64		19	19.65		23	30.79	
	6	36	62.04		38	87.06		39	68.58	
	11	37	55.01	<0.001	38	86.77	<0.001	41	70.72	<0.001
	16	38	50.11		39	46.45		38	27.97	
	21	36	29.86		39	27.14		38	22.44	
Cold allodynia	Baseline		N/S			N/S			N/S	
	3	33	7.81	<0.001	37	5.81	<0.01	34	6.81	<0.01
	6	35	5.5	<0.01	37	4.04	<0.05	38	4.15	<0.05
	11		N/S			N/S			N/S	
	16	34	4.74	<0.01	32	2.96	<0.05		N/S	
	21	32	3.48	<0.05	32	2.98			N/S	

Degrees of freedom in all cases: 3, N/S= No significant, P value for ANOVA model.

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 between groups 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 surgery ($P<0.05$) and subsequent *post hoc* analysis showed an increase in all PSNL groups, when compared with sham-operated mice. No significant differences among 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 surgery ($P<0.05$). 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).

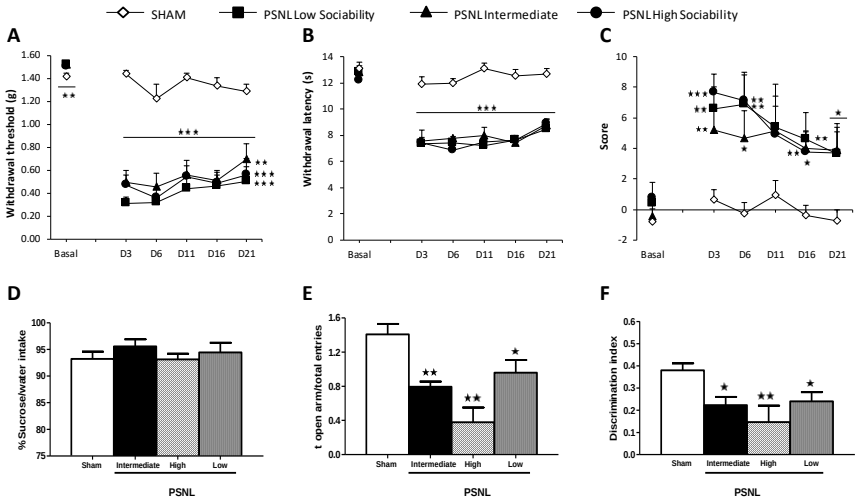

Figure 3. Evaluation of the nociceptive, emotional and cognitive consequences of neuropathic pain on mice displaying different sociability-like behavioural phenotypes. (A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) heat hyperalgesia was evaluated 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 was 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 manifestations of neuropathic pain on mice displaying different anxiety-like behavioural phenotypes

One-way ANOVA for the ipsilateral responses to mechanical, heat and cold stimulation revealed a significant effect of surgery (Table 2), 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 surgery ($P<0.05$). 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 surgery 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).

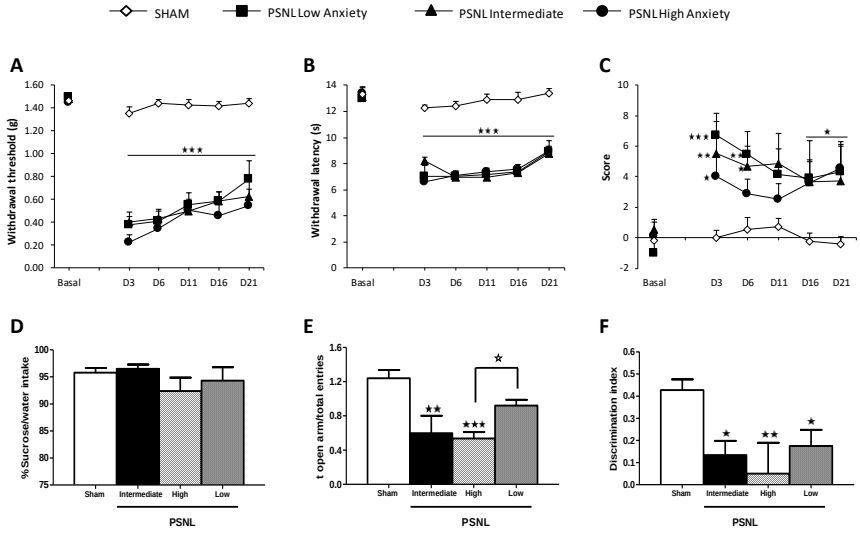

Figure 4. Evaluation of the nociceptive, emotional and cognitive consequences of neuropathic pain on mice displaying different anxiety-like behavioural phenotypes. (A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) heat hyperalgesia was evaluated 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 was 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 manifestations of neuropathic pain on mice displaying different depressive-like behavioural phenotypes

One-way ANOVA for the ipsilateral responses to mechanical, heat and cold stimulation revealed a significant effect of surgery (Table 2), 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). Significant differences ($P<0.05$) in the mechanical allodynia between the low and intermediate depressive phenotypes were found on day 3 (Figure 5A). In thermal hyperalgesia, significant differences between the low and intermediate depressive phenotypes were observed on day 3, and on day 21 between the low and the high depressive phenotypes (Figure 5B).

In the anhedonic state evaluation, no significant effect of surgery was shown by one-way ANOVA 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 surgery ($P<0.05$). 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 surgery ($P<0.05$). 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).

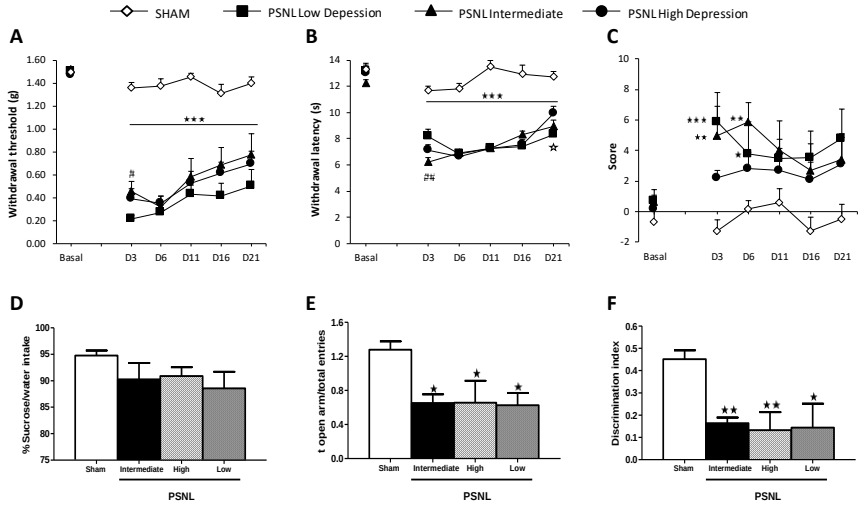

Figure 5. Evaluation of the nociceptive, emotional and cognitive consequences of neuropathic pain on mice displaying different depressive-like behavioural phenotypes. (A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) heat hyperalgesia was evaluated 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 was 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 relevance of the amygdala in the regulation of emotional behaviour including depressive, anxiety and social behaviour, we focused on this brain structure to evaluate the expression of genes potentially involved in the regulation of the emotional states during neuropathic pain (Table 3).

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	-	-	#	-

Arrows indicate elevated or decreased mRNA level in PSNL mice.
★P < 0.05, ★★P < 0.01 vs. sham surgery (t-test). # P < 0.05 main effect of the phenotype (two-way ANOVA).

Pdyn mRNA levels were differentially expressed in extreme 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 strong nociceptive manifestations, while the low *Pdyn* level was negatively associated to the same behavioural manifestations (Figure 6A, B). The glucocorticoid receptor *Nr3c1* mRNA and *Gadd45* mRNA

levels were differentially expressed in the extreme depressive-like phenotypes ($F_{(2, 41)}$ = 3,414; P<0.05 and $F_{(2, 32)}$ = 3,585; P<0.05) (Figure 6C). 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 lower level of the *Nr3c1 mRNA.*

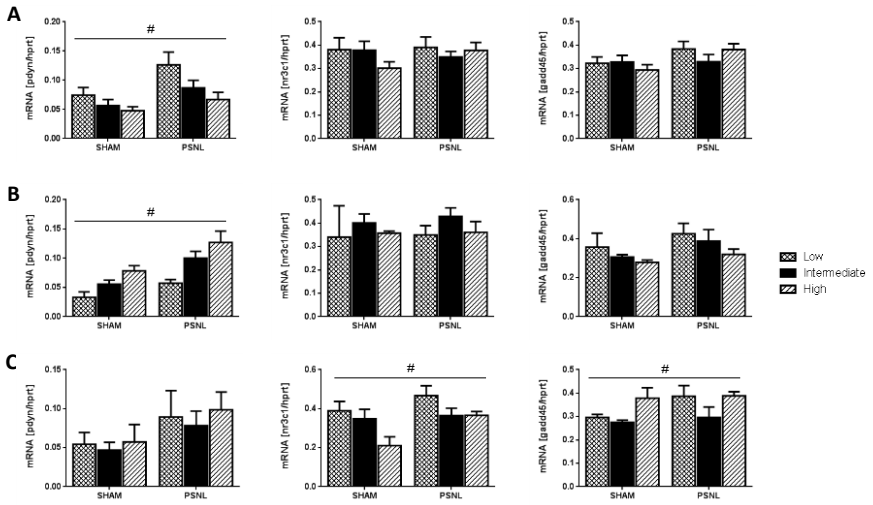

Figure 6. The influence of extreme behavioural phenotypes on selected mRNAs in the amygdala. *Pdyn*, *Nr3c1* and *Gadd45* mRNA levels characterized extreme phenotype groups before and after surgery. Distinct mRNA levels of *Pdyn* gene characterized sociability- and anxiety-like phenotypes while glucocorticoid 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).

Sciatic nerve injury modified the expression profile of genes involved in neuronal activity *(Npas4),* stress *(Tsc22d3, Nr3c1),* inflammation *(C1q, I1beta)* and intracellular signalling *(Gadd45,*

Crh, Pdyn) (Table 3). The aforementioned mRNA 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, d.f. = 81.865, p-value = 0.004) following nerve injury.

4. Discussion

An association between spontaneous CeA neuronal activity and sociability-like behaviour was revealed in this study. 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, 2002). 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 decreased pain sensitivity (D'Amato and Pavone, 2012), the recognition of emotional reactions to the pain of conspecifics could produce hyperalgesia (Langford et al., 2006). Emotional and cognitive manifestations of neuropathic pain were not affected by sociability trait. No correlation between spontaneous CeA and anxiety-like behaviour was shown in the present study. In agreement, several studies showed that the amygdala, and specifically the CeA, had 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 showed that mice displaying high anxiety trait developed more severe impairment in the anxiety-like behaviour following the development of neuropathic pain, while the least anxious animals showed the mildest manifestations. Furthermore, no association between the CeA neuronal activity and the depressive trait was observed. Our data suggested that low levels of depression trait increase the vulnerability to develop enhanced manifestations of neuropathic pain. Indeed, mice with low depressive-like behaviour enhanced mechanical allodynia on day 3 and thermal hyperalgesia on day 21 after nerve injury. Further, this same tendency of the effect of depression trait on mechanical and cold allodynia was observed on the other evaluated days. These results are in agreement with previous animal and clinical studies, which had reported a decrease of mechanical hypersensitivity in rats with depressive-like behaviour in a spinal nerve injury model (Shi et al., 2010a) and a reduction of thermal pain sensitivity in the skin of depressed patients (Bär et al., 2007). Our results do not show an interaction between the depression trait on emotional and 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 (Cassidy and Gutchess, 2012; Kalin et al., 2004). Altered gene expression profiles in the amygdala of mice displaying extreme phenotypes of sociability, anxiety and depression-like behaviour were shown in the present study. We also observed changes in the amygdala gene expression profiles after nerve injury in already established state of pain. Some studies have showed that low sociability is related to higher levels of anxiety (Kudryavtseva et al., 2004; Tõnissaar et al., 2008). In agreement, the highly anxious and the least sociable mice showed the highest level of *Pdyn* mRNA, whereas low anxiety and high sociability phenotypes were associated with low expression of *Pdyn* mRNA. Therefore, *Pdyn* gene expression in the amygdala appears to be associated with anxiety and sociability phenotypes. Moreover, the sciatic nerve injury increases *Pdyn* gene expression levels, while the relationships among this gene expression and the phenotypes remained after the development of neuropathic pain since more anxious and less sociable mice retained its elevated *Pdyn* mRNA level. Genetic deletion of the *Pdyn* gene has been reported to enhance partner recognition ability, while pharmacological blockade of kappa opioid receptor (KOR) enhanced social memory in wild-type animals (Bilkei-Gorzo et al., 2014). Other studies have suggested that the prodynorphin system may play a key role in anxiety (Knoll et al., 2011). However, data obtained from different studies 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 (Ménard et al., 2014), but *Pdyn* deletion increased anxiety-like behaviours and impaired the anxiolytic effect

of bromazepam (Femenía et al., 2011a). In contrast, 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 these results, wild-type mice treated with the selective KOR antagonists GNTI or norbinaltorphimine showed similar effects. Furthermore, treatment of *Pdyn* knockout animals with U-50488H, a selective KOR agonist, fully reversed their anxiolytic phenotype (Wittmann et al., 2009).

On the other hand, the present study showed that *Pdyn* mRNA levels in the amygdala were not associated with depressive-like phenotype. Instead, the depressive-like phenotype was associated with enhanced *Nr3c1* mRNA levels. This observation agrees with several studies reporting the association 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, high level of *Nr3c1* mRNA marking low depressive-like phenotype was not affected by neuropathic pain. Furthermore, our study indicates that depressive-like behaviour was associated with a higher expression of the *Gadd45* gene in the amygdala. An enhanced *Gadd45* expression was also revealed after sciatic nerve injury. However, the increase of *Gadd45* expression after PSNL was more evident in the low depressive group, reaching similar expression levels to the high

depressive mice. The previously association of this gene to synaptic plasticity and memory formation (Sultan and Sweatt, 2013) could explain the absence of behavioural differences in the cognitive component of neuropathic pain in the depressive selected mice. Sciatic nerve injury also altered the expression of several genes involved in the neuronal activity *(Npas4),* stress *(Tsc22d3, Crh)* and inflammation *(C1q, I1beta, Il6)* in the amygdala. Some studies have previously reported that the expression of some of these genes is altered in the limbic system of rats exposed to neuropathic pain (Burke et al., 2013; del Rey et al., 2011; Ulrich-Lai et al., 2006). Further studies, however, are required to clarify the links between these altered genes in the amygdala and pain states.

In summary, we have identified anxiety trait as an inter-individual vulnerability factor that aggravates emotional manifestations of neuropathic pain along with *Pdyn* mRNA as a concomitant biomarker in neuropathic pain in the amygdala. Our results also suggest that depressive trait conferred some resistance to the development of neuropathic pain and may be attributable to the expression of the *Gadd45* gene in the amygdala. The results reported in this study highlight the virtue of the combinatorial use of behavioural, electrophysiological and genetic approaches. This approach may help in the understanding of mechanisms that may explain the inter-individual variation of the neuropathic pain manifestations. This study may represent a relevant step towards the development of more efficient personalized treatments for chronic pain.

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Article 3

Nociceptive, emotional and cognitive manifestations of neuropathic pain in pre-proenkephalin knockout mice

Lara-Mayorga I.M., Bargiela A., Kieffer B., Przewlocki R., Baños J.E. and Maldonado R.

Objectives

To evaluate the involvement of pre-proenkephalin (Penk) on the nociceptive, emotional and cognitive manifestations of neuropathic pain.

Materials and methods

Three strains of Penk KO were evaluated in two experimental sequences. In the first, constitutive Penk KO ($Penk^{-/-}$) mice were used. In the second, two new lines of Penk KO mice obtained using the Cre-lox system were used. Penk KO mice were produced under the constitutive cytomegalovirus (CMV) promoter (Penk $CMV^{-/-}$). Conditional mutants in inhibitory GABAergic neurons of forebrain (Penk $Dlx^{-/-}$) were generated using the promoter of distal-less homeobox 5/6 (DLX5/6). Neuropathic pain was induced by a partial sciatic nerve ligation (PSNL) to evaluate pain sensitivity, anxiety-like behaviour, long-term memory and depressive-like behaviour in these mice. Nociceptive responses were assessed in the von Frey, plantar and cold plate test before neuropathic pain induction on day 5, 10 and 21 after PSNL. Early and late anxiety-like behaviour were evaluated in the elevated plus maze and in the light dark box test on

day 11 and 22 after PSNL respectively. The cognitive component was evaluated in the object recognition memory test on day 15 after PSLN. The depressive-like behaviour was assessed in the forced swimming test on day 24 after PSNL. Finally, gene expression profile in the amygdala and hippocampus was studied.

Results

Before PSNL the anxiogenic phenotype of total Penk KO mice was confirmed, whereas this effect disappeared in the conditional mutant mice. Penk deletion also produced a cognitive impairment. After PSNL, enhanced mechanical and cold allodynia were observed in total Penk KO mice, whereas conditional KO animals showed the highest nociceptive responses. However, the anxiety-like behaviour and the cognitive component associated to neuropathic pain were not enhanced in mutant mice due to a possible ceiling effect of these responses after Penk deletion. The depressive-like behaviour only remained unaltered in the conditional Penk KO mice after PSNL. The deletion of enkephalin was related with the maintenance of *Pdyn*, KOR, *Npas4* and *Nr3c1* expression in the amygdala and *Nr3c1* decreased expression in the hippocampus.

Conclusions

Enkephalins are involved in the regulation of nociceptive manifestations of neuropathic pain. Penk deletion produced a resilience effect primarily on the development of anxiety and cognitive impairment but also of depression produced by a nerve

injury. The changes in the expression patterns of several genes in amygdala and hippocampus may regulate the ceiling effect observed in the anxiety behaviour and the cognitive impairment of Penk deficient mice after PSNL. Enkephalins were identified as a key component for the development of physiological and behavioural changes induced by neuropathic pain.

Nociceptive, emotional and cognitive manifestations of neuropathic pain in pre-proenkephalin knockout mice

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Keywords: neuropathic pain, pre-proenkephalin, cognition, anxiety, depression.

Abstract

Neuropathic pain is often associated with emotional and cognitive deficits. Endogenous enkephalins modulate many physiological functions including nociceptive, emotional and cognitive responses. The aim of this study was to evaluate the involvement of pre-proenkephalin (Penk), the main precursor of enkephalins, on the nociceptive, emotional and cognitive manifestations of neuropathic pain. For this purpose, Penk was completely abolished (total Penk KO) or partially deleted in forebrain GABAergic neurons of mice (conditional Penk KO). Neuropathic pain was induced by a partial

sciatic nerve ligation (PSNL) to evaluate pain sensitivity, anxiety-like behaviour, long-term memory and depressive-like behaviour in these genetically modified mice. Gene expression profile in the amygdala and hippocampus was also studied. Nociceptive manifestations produced by PSNL were associated to cognitive impairment, anxiety- and depressive-like behaviours. An anxiogenic phenotype was confirmed in total Penk KO mice, whereas a cognitive impairment was revealed in these mutant mice. After PSLN, enhanced mechanical and cold allodynia were observed in total Penk KO mice, whereas conditional KO animals showed the highest nociceptive manifestations. However, the emotional and cognitive manifestations associated to neuropathic pain were not enhanced in mutant mice due to a possible ceiling effect of these responses after Penk deletion. A decrease of enkephalin expression was related with those of *Pdyn, Npas4* and *Nr3c1* in the amygdala and *Nr3c1* in the hippocampus. The changes in the expression patterns of these genes may regulate the ceiling effect observed in the anxiety behaviour and the cognitive impairment of Penk deficient mice after PSNL. Our results suggest a resilience effect of Penk on the development of some manifestation of neuropathic pain.

1. Introduction

Neuropathic pain is a clinical entity initiated or caused by a primary lesion or dysfunction in the nervous system, which is associated with spontaneous pain, hyperalgesia and allodynia (Merskey and Bogduk, 1994), as well as emotional and cognitive manifestations

(Apkarian, 2004; La Porta et al., 2016). Indeed, neuropathic pain has been associated to emotional disorders, such as anxiety and depression (Neugebauer et al., 2004; La Porta et al., 2016), and cognitive deficits, including memory and learning impairments. Nociceptive, emotional and cognitive behaviours could influence each other in neuropathic pain leading to an impairment of the quality of life of patients (Apkarian, 2004; Conrad et al., 2007).

The endogenous opioid system (EOS) participates in the physiological control of several responses including pain, reward, emotional behaviour, learning and stress (Arico and McNally, 2014; Bowers et al., 2012; Valentino and Van Bockstaele, 2015). The EOS comprises genes that encode three opioid receptors and three opioid peptides precursors that in turn produce several endogenous opioid peptides. The three receptor genes encode mu (MOR), delta (DOR) and kappa (KOR) opioid receptors. The proteolytic cleavage of preproenkephalin (Penk), preprodynorphin (Pdyn) and proopiomelanocortin (Pomc) produces enkephalins, dynorphins and beta-endorphin, respectively (Kieffer and Gavériaux-Ruff, 2002; Le Merrer et al., 2009b). The main opioid peptides derived from Penk are met- and leu-enkephalin, both showing high affinity to δ-opioid receptors (DORs) and μ-opioid (MORs) being their affinity for DORs about tenfold higher than for MORs (Roques et al., 2012). Penk derivatives play a crucial role in nociceptive control. Met-enkephalin and leu-enkephalin are the endogenous opioids endowed with the highest antinociceptive activity (Madden et al., 1977), and mice with Penk deletion exhibit enhanced nociceptive manifestations (König et al., 1996b). Enkephalins also play a role in

locomotion, cognitive functions and affective behaviours (Nieto et al., 2005), and mice that lack enkephalins had a reduced locomotor activity and increased levels of anxiety and aggressiveness (König et al., 1996b). Enkephalins have been involved in learning and memory modulation (Gallagher, 1982; Gallagher et al., 1983; Messing et al., 1979), as well as in synaptic plasticity (Derrick et al., 1992; Do et al., 2002; Williams and Johnston, 1996). However, its specific role on cognition is not clear enough (Canli et al., 1990; Martinez et al., 1985; Palop et al., 2005). Enkephalin expression may play a role in some animal models of depression (Dziedzicka-Wasylewska and Papp, 1996; Primeaux and Holmes, 2000). Some studies have suggested an anti-depressant effect of enkephalins (Baamonde et al., 1992; Tejedor-Real et al., 1995) but others have shown any effect on this behaviour (Bilkei-Gorzo et al., 2007). The behavioural evaluation of mice with site-directed or anatomically-restricted mutations of the EOS components may provide valuable information of the molecular players in opioid pharmacology and physiology, as well as in altered physiological conditions (Kieffer and Gavériaux-Ruff, 2002).

In this study, we evaluated the involvement of Penk on the nociceptive, emotional and cognitive manifestations of neuropathic pain. To this aim, PSNL was induced to mice with total or conditional Penk deletion to evaluate nociceptive, emotional and cognitive deficits associated to this chronic neuropathic pain. Gene expression in the amygdala and hippocampus was evaluated in Penk KO mice to elucidate possible mechanisms involved in the Penk regulation of these nociceptive manifestations.

2. Materials and methods

2.1. Animals

Three strains of mice with different mutations of the Penk gene and their respective wild-type littermates were used. The first strain was a constitutive Penk KO ($Penk^{-/-}$) previously reported on a C57BL/6J background (König et al., 1996b). Two new lines of Penk KO mice obtained using the Cre-lox system were also used in this study. First, constitutive Penk KO mice were produced under the constitutive cytomegalovirus (CMV) promoter (Penk $CMV^{-/-}$). CMV promoter is considered one of the strongest naturally occurring promoters. CMV is infectious in a wide range of tissues. CMV targeting to cells seems independent of specific virus-receptor interactions. The conditional mutants in inhibitory GABAergic neurons of forebrain (Penk $Dlx^{-/-}$) were generated using the promoter of distal-less homeobox 5/6 (DLX5/6). Dlx genes are part of a highly conserved developmental pathway that regulates forebrain development and, specifically, the DLX5/6 is identified as a marker for inhibitory neurons. These new Penk KO lines have a mix background of C57BL/6J and 129 Pas strains. Penk $CMV^{-/-}$ genetic composition is 87.5% C57BL/6J and 12.5% 129 Pas, while Penk $Dlx^{-/-}$ are 78% C57BL/6J and 22% 129 Pas.

Mice were 9-18 weeks old at the beginning of the experiment, and were housed in groups of maximum 4 per cage, with water and food *ad libitum*. 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 and 20:00 h). 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. Experiments were performed under blinded conditions.

2.2. Experimental protocol

To induce neuropathic pain, mice were exposed to a partial sciatic nerve ligation (PSNL) or sham surgery. Two experimental sequences were performed. First, $Penk^{-/-}$ mice (sham $Penk^{-/-}$ n= 19; PSNL $Penk^{-/-}$ n= 19) and their respective littermates (sham $Penk^{+/+}$ n= 17; PSNL $Penk^{+/+}$ n= 18) were used. In the second, $Penk\ CMV^{-/-}$ mice (sham $Penk\ CMV^{-/-}$ n= 20; PSNL $Penk\ CMV^{-/-}$ n= 20), $Penk\ Dlx^{-/-}$ (sham $Penk\ Dlx^{-/-}$ n=20; PSNL $Penk\ Dlx^{-/-}$ n=24) and their littermates (sham $Penk\ Dlx^{+/+}$ n=23; PSNL $Penk\ Dlx^{+/+}$ n=20) were evaluated.

Nociceptive responses were assessed before surgery and on days 5, 10 and 21 after surgical procedure. Two experimental paradigms were used to evaluate anxiety-like behaviour: the elevated plus maze on day 11 after PSNL and the light dark box on day 22 after PSNL.

The cognitive performance was evaluated in the novel object recognition test on day 15 after PSNL. The forced swimming test was used to evaluate depressive-like behaviour on day 24 after PSNL. Finally, brain samples of amygdala and hippocampus were obtained at day 28 after surgery to examine transcriptional modifications. Full experimental protocol is shown in Figure 1.

Figure 1

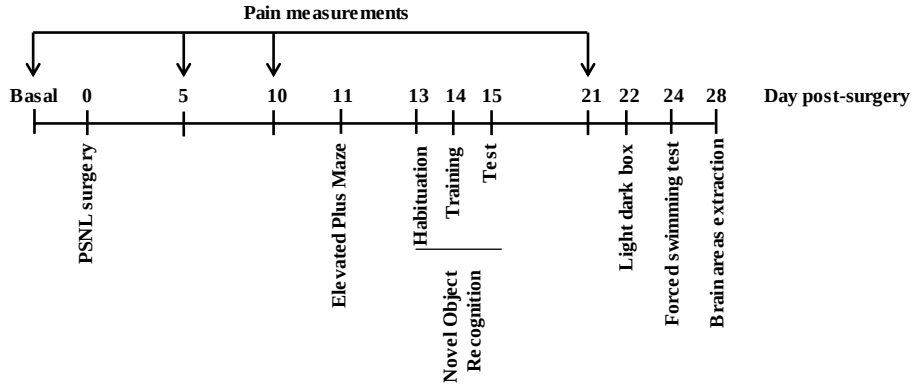

Figure 1. Experimental sequence followed to evaluate the nociceptive, emotional and cognitive components of neuropathic pain in Penk KO mice.

2.3. Neuropathic pain induction and assessment

2.3.1. Pain induction

PSLN was used to induce neuropathic pain (Malmberg and Basbaum, 1998). 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 placed around 33-50% of the sciatic nerve using an 18 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.

2.3.2. Nociceptive assessment

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 in Figure 1), using the same order of testing: mechanical allodynia, thermal hyperalgesia and cold allodynia. Before evaluations, mice were habituated during 2 h in three consecutive days to von Frey and plantar test setting.

Mechanical allodynia was evaluated by measuring the hind paw withdrawal response to von Frey filaments stimulation. Mice were placed in Plexiglas boxes (20 cm high, 9 cm diameter) with a grid surface through which the von Frey 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 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.

2.4. Behavioural tests

2.4.1. Anxiety-like behaviour

Elevated plus maze was performed 11 days after surgery 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).

Light dark box test was performed 22 days after nerve injury, as previously described (Filliol et al., 2000a). 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.

2.4.2. Cognitive behaviour

The novel object recognition test was performed as previously described (Puighermanal et al., 2009) on day 15 after nerve injury in a black Plexiglas V-maze (Panlab). 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.

2.4.3. Depressive-like behaviour

Forced swimming test was performed 24 days after surgery. 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., 1977b). 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.

2.5. Genomic analysis

2.5.1. Gene expression analysis

Tissue samples of amygdala and hippocampus from sham and PSNL Penk KO mice and wild type littermates were collected in RNAlater reagent (Qiagen Inc., USA) 4 weeks after surgery 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).

2.5.2. Quantitative PCR

Reverse transcription was performed using Omniscript Reverse Transcriptase (Qiagen Inc.) in tissue samples from amygdala and hippocampus. The qPCR reactions were performed using isoform specific TaqMan® probes (Bdnf-Mm01334042_m1, C1qa-Mm00432142_m1, Fkbp5-Mm00487401_m1, Gfap-Mm01253033_m1, Hprt1-Mm01545399_m1, Il1b-Mm00434228_m1, Il6-Mm00446190_m1, Nr3c1-Mm00433832_m1, Nr3c2-Mm01241596_m1, Pdyn-Mm00457573_m1, Tsc22d3-Mm00726417_s1, Egr1-Mm00656724_m1, Arc-Mm01204954_g1, Npas4-Mm01227866_g1, Fos-Mm00487425_m1, Oprk1-Mm01230885_m1, Oprm1-Mm01188089_m1, Oprd1-Mm01180757_m1, Mtor-Mm00444968_m1, Gadd45g-Mm01352550_g1, Gjb6-Mm00433661_s1, Aif1-Mm00479862_g1, Grm5-Mm00690332_m1, Gabbr2-Mm01352554_m1, Cd40-Mm00441891_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 was quantified to control for variation in cDNA amounts. The abundance of RNA was calculated as 2-threshold cycle.

2.6. Statistical analysis

Data obtained in the nociception models were analysed by three-way repeated measures ANOVA (genotype and surgery as between-subject factors and day as within-subject factor). *Post hoc* analysis (Fisher's LSD) was performed after ANOVA when appropriate. In the first experimental sequence, after normality test and variance analysis were performed, parametric data obtained in elevated plus maze EPM, novel object recognition test NOR and light dark box test LDB were analysed by two-way ANOVA (genotype and surgery). Subsequent *post hoc* analysis (Fisher's LSD) was performed after ANOVA when appropriate. Data obtained in forced swimming test FST were analysed by T-test. In the second experimental sequence, after normality test and variance analysis were performed, parametric data obtained in elevated plus maze EPM (entries in open arm), novel object recognition test NOR and light dark box test LDB were analysed by two-way ANOVA (genotype and surgery). Subsequent *post hoc* analysis (Fisher's LSD) was performed after ANOVA when appropriate. T-test was performed to immobility time data in the forced swimming test FST. For non-parametric data, two-group comparisons were achieved by means of the Mann–Whitney U-test (percentage of time in open arm in the elevated plus maze EPM). STATISTICA 6.0 software was used. The differences were considered significant

when the *P* value was below 0.05. Statistical analysis of gene expression results 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 between sham and PSNL groups. Prism 6 (GraphPad) was used for statistical analysis. Data are expressed as means ± standard error of the mean (SEM).

3. Results

3.1. Pain evaluation

3.1.1. Mechanical allodynia

Three-way ANOVA was first performed for the ipsilateral responses to mechanical stimulation. For the first experimental sequence (Fig. 2A), a significant effect of surgery, genotype, day and interaction between these factors was observed (Table 1). For the second experimental sequence (Fig. 3A), a significant effect of surgery, genotype, day, interaction between surgery and genotype, day and genotype, day and surgery and all the three factors was revealed (Table 1). For the contralateral responses, no significant effects were observed in any case. Subsequent *post hoc* analysis indicated that the baseline values for ipsilateral and contralateral hind paws were similar in all mouse groups before PSNL or sham surgery. Sham surgery did not modify the nociceptive responses in any genotype. However, PSNL significantly decreased the ipsilateral withdrawal thresholds in all animals, but not in the contralateral paw. The ipsilateral withdrawal threshold in PSNL $Penk^{-/-}$ mice was significantly reduced when compared with $PENK^{+/+}$ mice during all measures performed in the first

experimental sequence (Fig. 2A). During all the second experimental sequence, the ipsilateral withdrawal threshold in PSNL Penk CMV$^{-/-}$ and PSNL Penk Dlx$^{-/-}$ mice was significantly reduced when compared with Penk Dlx$^{+/+}$ (Fig. 3A). Also, significant reduction in withdrawal threshold was revealed in PSNL Penk Dlx$^{-/-}$ when compared with PSNL Penk CMV$^{-/-}$ and (Fig. 3A).

3.1.2. Heat hyperalgesia.

Three-way ANOVA was first performed for the ipsilateral responses of both experimental sequences. In the first (Fig. 2B), a significant effect of surgery and day was revealed (Table 1). In the second sequence (Fig. 3B), a significant effect of surgery, day and interaction between day and surgery was observed (Table 1). No significant effects were seen for the contralateral responses in any group.

3.1.3. Cold allodynia.

Three-way ANOVA was performed for the score values obtained in the two experimental sequences. In the first (Fig. 2C), a significant effect of surgery and day was revealed. For the second experimental sequence (Fig. 3C), a significant effect of surgery, day, interaction between genotype and surgery and interaction between day and surgery was observed (Table 1). Sham surgery did not modify nociceptive responses.

Table 1. Three way ANOVA values for nociceptive evaluation

		Experimental Sequence							
		First				Second			
	Factor	**DF**	**Error**	**F**	***P***	**DF**	**Error**	**F**	***P***
Mechanical allodynia	Surgery	1	69	703.62	< 0.001	1	80	2024.53	< 0.001
	Genotype	1	69	10.56	< 0.05	2	80	45.25	
	Day	1	69	132.62	< 0.001	3	240	202.89	
	Interactions								
	Surgery and genotype			N/S		2	80	47.62	< 0.001
	Day and genotype			N/S		6	240	4.96	
	Day and surgery			N/S		3	240	208.91	
	Surgery, genotype and day	3	207	4.37	< 0.01	6	240	4	
Heat hyperalgesia	Surgery	1	69	78.33	< 0.001	1	80	440.55	<0.0001
	Day	3	207	28.36		3	240	193.02	
	Interactions								
	Day and surgery			N/S		3	240	163.46	<0.0001
Cold allodynia	Surgery	1	69	37.99	< 0.001	1	80	103.46	< 0.001
	Day	3	207	12.68		3	240	102.21	
	Interactions								
	Genotype and surgery			N/S		2	80	5.14	< 0.05
	Day and surgery			N/S		2	240	22.04	< 0.0001

N/S= No significant, P value for ANOVA model.

Figure 2

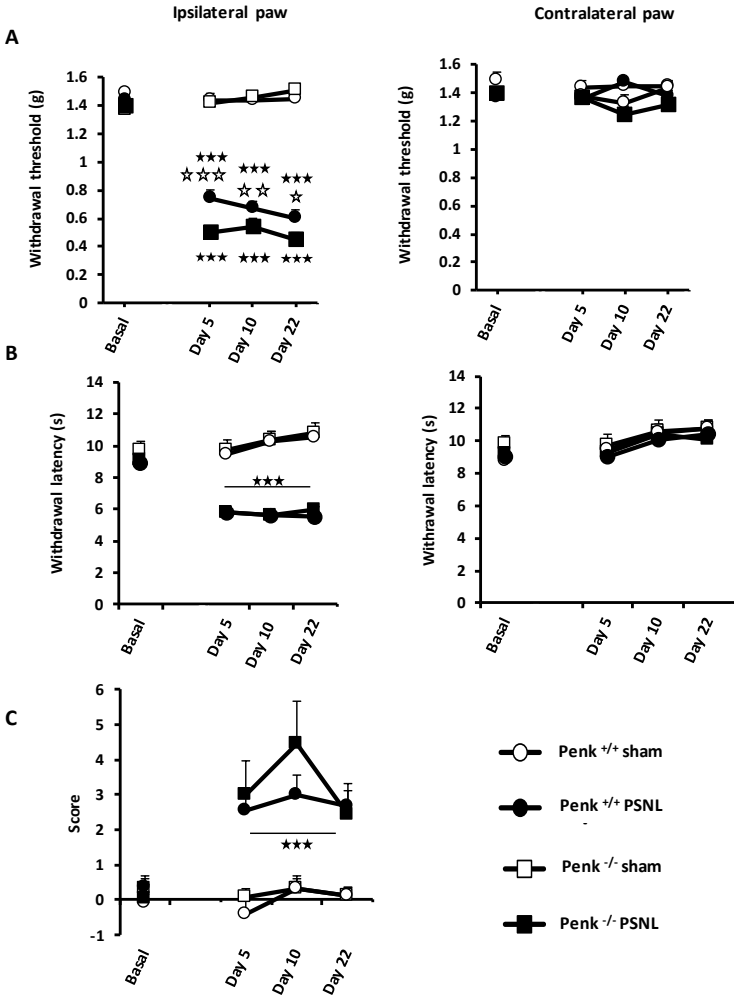

Figure 2. Evaluation of the nociceptive manifestations of neuropathic pain on Penk$^{-/-}$ and Penk$^{+/+}$ mice. (A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) heat hyperalgesia was evaluated in the plantar test and expressed as paw withdrawal 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 5, 10 and 21 after PSNL or sham surgery. Values are expressed as mean ± **standard error** of the mean (Number of animals: sham Penk$^{-/-}$ = 19, PSNL Penk$^{-/-}$ = 19; sham Penk$^{+/+}$ = 17, PSNL Penk$^{+/+}$ = 18). ★P < 0.05, ★★P < 0.01, ★★★P < 0.001 vs. sham surgery; ☆ P < 0.05, ☆☆ P < 0.01, ☆☆☆ P <0.001 001 differences between genotypes (Fisher's LSD test).

Figure 3

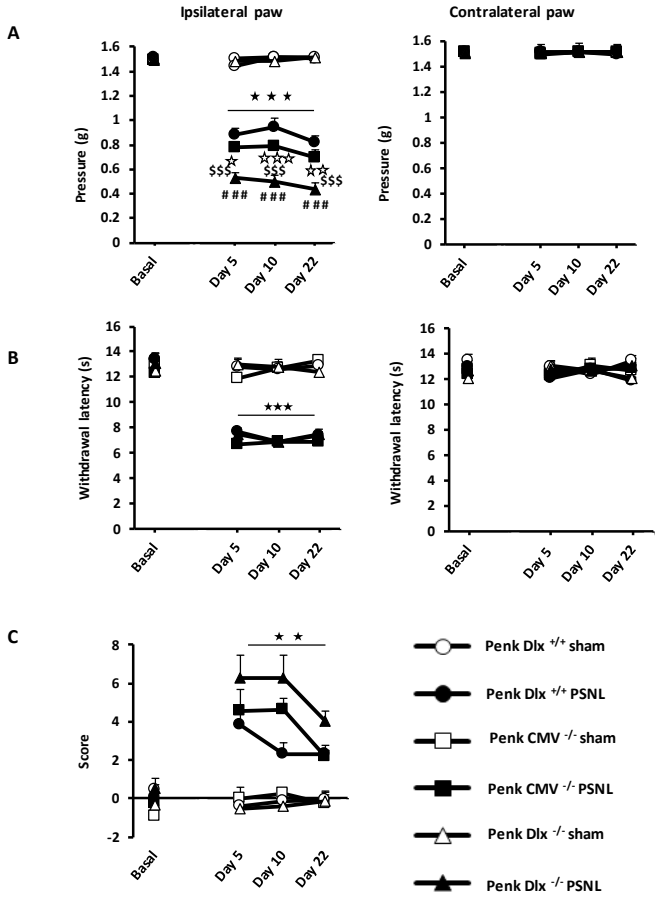

Figure 3. Evaluation of the nociceptive manifestations of neuropathic pain on Penk CMV$^{-/-}$, Penk Dlx$^{+/+}$ and Penk Dlx$^{-/-}$ mice. (A) Mechanical allodynia was evaluated by the von Frey model and expressed as withdrawal thresholds in g, (B) Heat hyperalgesia was evaluated in the plantar test 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 5, 10 and 21 after PSNL or sham surgery. Values are expressed as mean ± **standard error** of the mean (Number of animals: sham Penk CMV$^{-/-}$ = 20, PSNL Penk CMV$^{-/-}$ =20; sham Penk Dlx$^{+/+}$ = 23, PSNL Penk Dlx$^{+/+}$ = 20, sham Penk Dlx$^{-/-}$ = 20; PSNL Penk Dlx$^{-/-}$ = 24). ★★★P < 0.001 vs. sham surgery; ☆ P < 0.05, ☆☆P < 0.01, ☆☆☆P < 0.001 Dlx$^{+/+}$ vs. Penk CMV$^{-/-}$, ### P < 0.001 Penk Dlx$^{+/+}$ vs Penk Dlx$^{-/-}$; $$$ P < 0.001 Penk Dlx$^{+/+}$ vs. Penk CMV$^{-/-}$ (Fisher's LSD test).

3.2. Behavioural evaluation

3.2.1. Early anxiety-like behaviour in elevated plus maze

On day 11 after PSNL, two-way ANOVA revealed in the first experimental sequence a significant effect of genotype ($F_{(1,19)}$= 4.97; P< 0.05) for the percentage of time in the elevated plus maze open arms and a significant effect of the interaction between genotype and surgery ($F_{(1,19)}$ = 28.84; P<0.05) for the number of entries in the elevated plus maze open arms. Subsequent *post hoc* analysis for the open arms number of entries showed anxiogenic behaviour due to the deletion of enkephalins in the $Penk^{-/-}$ mice. Interestingly, the increase of anxiety after neuropathic pain only was observed in $Penk^{+/+}$ (Fig. 4A). In the second experimental sequence, on day 11 after PSNL two-way ANOVA for the number of entries in open arms revealed a significant effect of genotype ($F_{(2,77)}$=604.55; P<0.0001) and surgery ($F_{(1,77)}$=614.05; P<0.0001). Mann–Whitney U test showed a significant reduction in the percentage of time in elevated plus maze open arms of PSNL Penk $Dlx^{+/+}$ when compared with sham Penk $Dlx^{+/+}$ (U=59.00; P=0.016) and in sham Penk $CMV^{-/-}$ when compared with sham Penk $Dlx^{-/-}$ (U=27.00; P=0.006).

3.2.2. Late anxiety-like behaviour in the light dark box

On the first experimental sequence, two-way ANOVA indicated on day 22 after surgery a significant effect of surgery ($F_{(1,60)}$= 13.80; P<0.001) and interception between genotype and surgery ($F_{(1,60)}$= 7.28; P<0.01) in the number of white entries, and a significant effect of genotype ($F_{(1,60)}$= 6.46; P<0.05) in the number of distal entries.

Subsequent *post hoc* analysis confirmed the anxiogenic effect of genotype in the Penk$^{-/-}$ mice in the number of white entries that was not increased after the PSNL surgery in this genotype. Only in wild-type animals the anxiety-like behaviour was increased after PSLN (Fig. 4C). In the two-way ANOVA of the late anxiety evaluation performed on day 22 after surgery of the second experimental sequence, no significant effect of genotype, surgery or interaction between genotype and surgery was observed (Fig. 5C, D).

3.2.3. Cognitive evaluation

On the first experimental sequence, two-way ANOVA for novel object recognition discrimination index on day 15 after PSNL revealed a significant effect of surgery ($F_{(1,67)} = 4.05$; $P<0.05$) (Fig. 4E). In the second experimental sequence, two-way ANOVA revealed a significant effect of surgery ($F_{(1,108)}=9.28$; $P< 0.05$) and interaction between genotype and surgery ($F_{(2,108)}=5.05$; $P<0.05$). The subsequent *post hoc* analysis revealed a significant reduction in the discrimination index of sham Penk Dlx$^{-/-}$. Significant reduction of the cognitive function in terms of long-term memory produced by neuropathic pain was only observed in the Penk Dlx$^{-/-}$ (Fig. 5E).

Figure 4

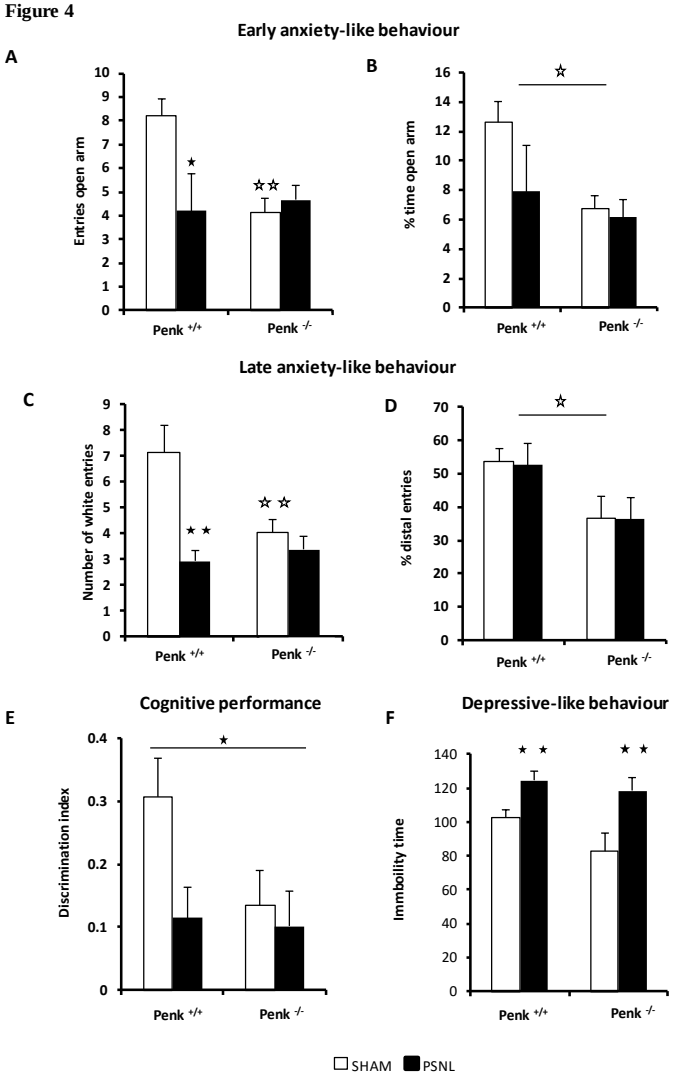

Figure 4. Emotional and cognitive behaviours in Penk$^{-/-}$ and Penk$^{+/+}$ mice exposed to PSNL. (A, B) Entries and time spent in EPM open arms, (C, D) number of white entries and distal entries in LDB test, (E) NOR discrimination index and (F) FST immobility time were evaluated on different time points after the PSNL or sham surgery. Data are expressed as mean ± **standard error** of the mean. ★P < 0.05, ★★P < 0.01, ★★★P < 0.001 vs. sham surgery; ☆ P < 0.05, ☆☆ P < 0.01, ☆☆☆ P < 0.001 differences between genotypes (Fisher's LSD test). PSNL, partial sciatic nerve ligation; EPM, elevated plus maze; LDB, light dark box; NOR, Novel object recognition test; FST, forced swimming test.

Figure 5

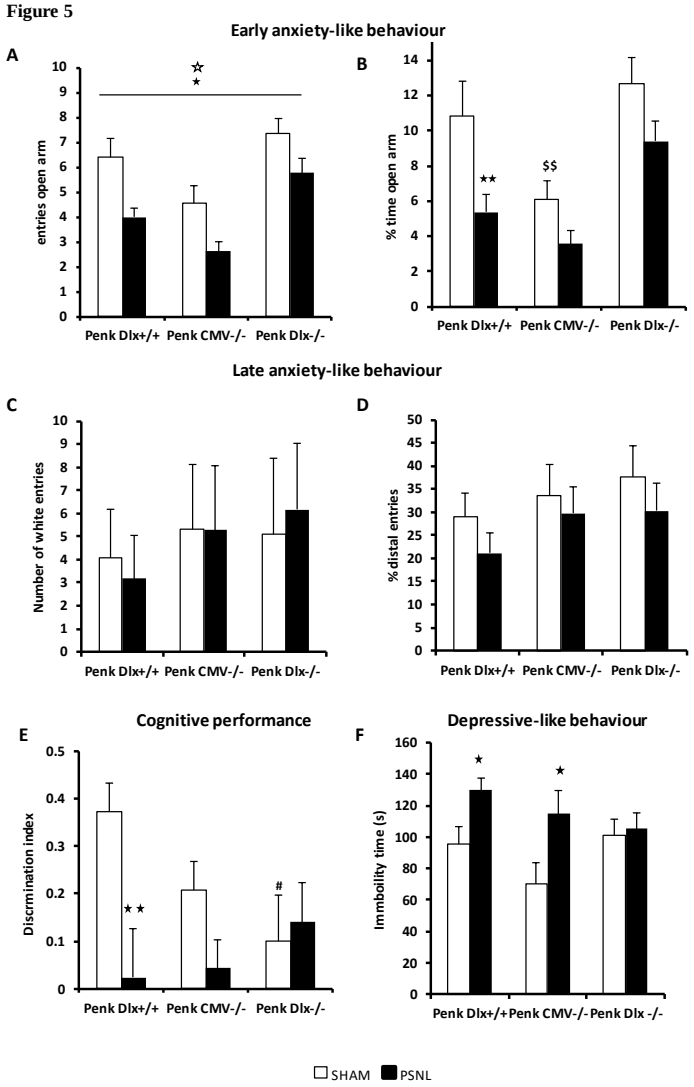

Figure 5. Emotional and cognitive behaviours on Penk CMV$^{-/-}$, Penk Dlx$^{+/+}$ and Penk Dlx$^{-/-}$ mice exposed to PSNL. (A, B) Entries and time spent in EPM open arms, (C, D) number of white entries and distal entries in LDB test, (E) NOR discrimination index and (F) FST immobility time were evaluated on different time points after the PSNL or sham surgery. Data are expressed as mean ± **standard error** of the mean. ★P < 0.05, ★★P < 0.01 vs. sham surgery; ☆ P < 0.05 Dlx$^{+/+}$ vs. Penk CMV$^{-/-}$; # P < 0.05 Penk Dlx$^{+/+}$ vs Penk Dlx$^{-/-}$; **$$** P < 0.001 Penk Dlx$^{+/+}$ vs. Penk CMV$^{-/-}$. PSNL, partial sciatic nerve ligation; EPM, elevated plus maze; LDB, light dark box; NOR, Novel object recognition test; FST, forced swimming test.

3.2.4. Depressive-like behaviour

In the first experimental sequence (Fig. 4F), T-test of the evaluation on day 24 after PSNL revealed significant increase in the immobility time of PSNL Penk$^{+/+}$ group when compared with sham Penk$^{+/+}$ (t=2.92, df=29.20) and in PSNL Penk$^{-/-}$ immobility values when compared with sham Penk$^{-/-}$ group (t=2.72, df=34.29). In the second experimental sequence, T-test revealed significant differences in the immobility time of PSNL Penk$^{-/-}$ and sham Penk$^{-/-}$ (t= 2.55, df=34.10) and in PSNL Penk CMV$^{-/-}$ when compared with sham Penk CMV$^{-/-}$ (t=2.25, df=35.62) (Fig. 5F).

3.3. Genomic analysis

PSNL enhanced the expression of genes selected for their role in the neuronal activity (*Npas4*), and the opioid signalling (*Pdyn* and the opioid receptor *Oprk1*) in the amygdala in the PSNL Penk$^{+/+}$ mice 4 weeks after surgery. *Pdyn* mRNA levels in the amygdala were increased almost two-fold ($F_{(3,33)}$= 6.8, P< 0.01). Subsequent *post hoc* analysis revealed significant *Pdyn* expression level increment in PSNL Penk$^{+/+}$ when compared with sham Penk$^{+/+}$ (Fig. 6A). An increase of *Oprk1* (Fig. 6B) receptor mRNA ($F_{(3,33)}$= 3.9, P< 0.05), as well as slight increase of *Crh* mRNA levels (Fig. 6C), were also revealed in Penk$^{+/+}$ mice (t=2.74 df=16). Furthermore, expression of opioid receptor *Oprd1* and *Oprm1* expression was not altered by PSNL (data not shown). A higher level of glucocorticoid receptor *Nr3c1* gene expression was found in the amygdala ($F_{(3,33)}$= 4.7, P< 0.01) and in the hippocampus ($F_{(3,30)}$= 8.5, P< 0.001) of PSNL mice (Fig 6E, 7A). Interestingly, enhancement of expression of activity-

dependent gene *Npas4* as well as *Pdyn* in the amygdala of Penk$^{-/-}$ mice following nerve injury was inhibited in the PSNL mice (Fig. 6A, D).

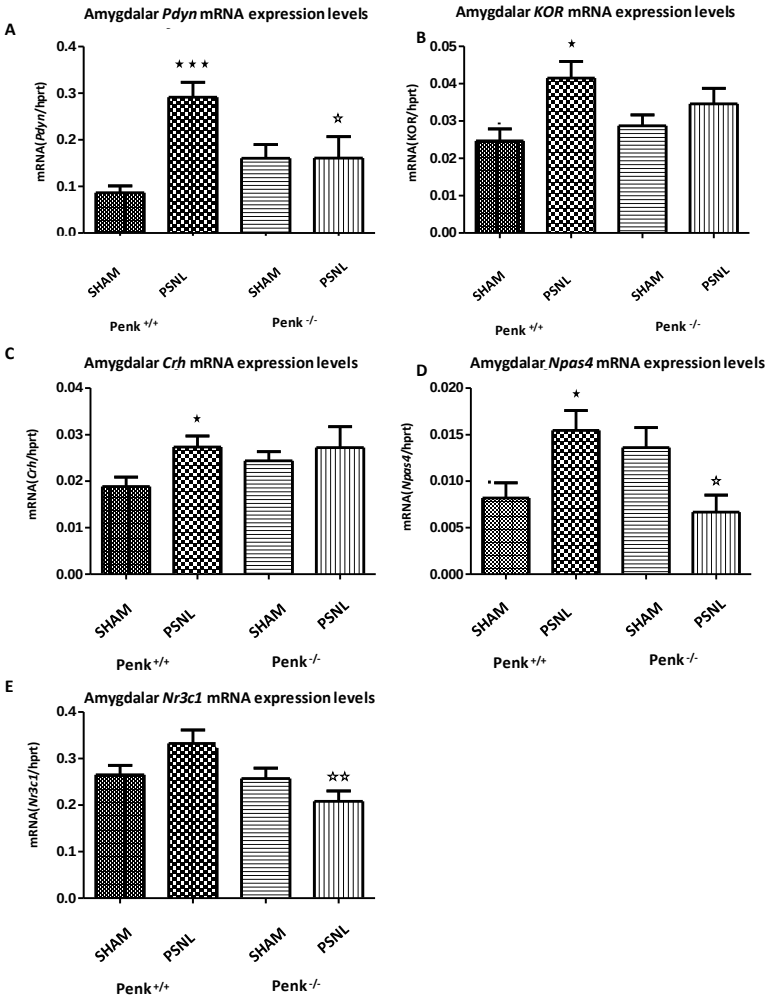

Figure 6. The influence of PSNL on selected mRNAs expression in the amygdala of Penk$^{-/-}$ and Penk$^{+/+}$. (A) *Pdyn*, (B) KOR, (C) *Crh*, (D) *NPas4*, and (E) *Nr3c1* mRNA levels were evaluated in the amygdala of Penk$^{-/-}$ and Penk$^{+/+}$ on day 28 after PSNL surgery. Changes in *Pdyn*, KOR and *Crh* levels were observed after PSNL in Penk$^{+/+}$. *Pdyn*, *NPas4* and *Nr3c1* were modified in Penk$^{-/-}$ by PSNL. ★P < 0.05, ★★★P < 0.001 vs. sham surgery; ☆ P < 0.05, ☆☆ P < 0.01 differences between genotypes (Fisher's LSD test).

Figure 7

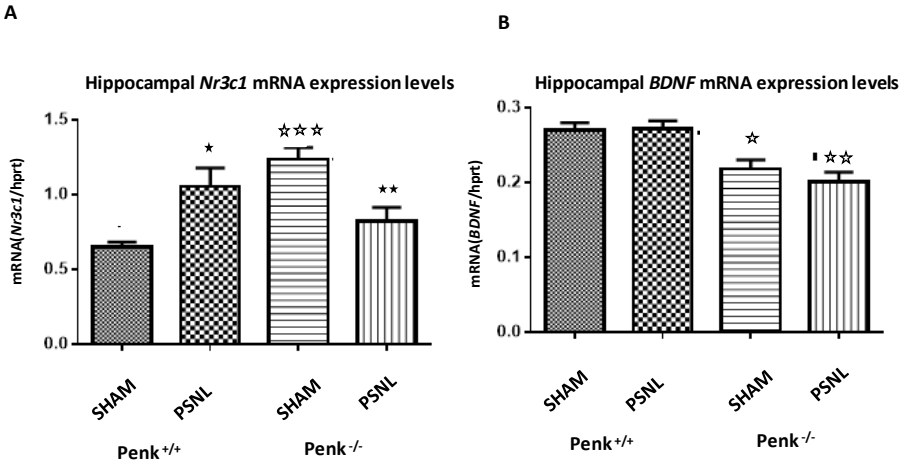

Figure 7. The influence of PSNL on selected mRNAs expression in the hippocampus of Penk$^{-/-}$ and Penk$^{+/+}$. (A) *Nr3c1* and (B) *BDNF* mRNA levels were evaluated in the hippocampus of Penk$^{-/-}$ and Penk$^{+/+}$ on day 28 after PSNL surgery. Changes in *Nr3c1* expression were affected by enkephalin deletion and PSNL; while changes in *BDNF* were just related to genotype. ★P < 0.05, ★★P < 0.01 vs. sham surgery; ☆ P < 0.05, ☆☆ P < 0.01, ☆☆☆ P < 0.001 differences between genotypes (Fisher's LSD test).

4. Discussion

Endogenous opioids are important modulators of nociceptive stimuli. We found enhanced pain sensitivity on the two strains of total Penk KO mice (Penk$^{-/-}$, Penk CMV$^{-/-}$) and in the mice with the conditional deletion in inhibitory GABAergic neurons of forebrain (Penk Dlx$^{-/-}$). The most marked effect among genotypes was observed in mechanical and cold allodynia. There were no differences among genotypes in terms of thermal hyperalgesia. The highest hypersensitivity to pain was a consequence of the conditional deletion of Penk. Enkephalins were reported as effective

in the attenuation of tactile allodynia and thermal hyperalgesia when administered spinally (Zou et al., 2011). Moreover, they are involved in the modulation of supraspinal but not spinal analgesia (König et al., 1996b).

Endogenous opioids modulated emotional behaviours throughout their actions in the limbic system. As a consequence of Penk deletion, an important increase of MOR and DOR sites (>300%) was observed in the central nucleus of the amygdala and the ventral pallidum, (Brady et al., 1999). In the early anxiety evaluation before nerve injury, Penk$^{-/-}$ mice spent less time and had fewer entries in the elevated plus maze open arm than Penk$^{+/+}$ animals, confirming the increase in anxiety state previously reported (König et al., 1996a). In the same line, even not significant, Penk CMV$^{-/-}$ mice presented a reduction in elevated plus maze parameters, suggesting increased anxiety. However, this anxiogenic response produced by enkephalins deletion was not found in Penk Dlx$^{-/-}$ mice, these animals had similar anxiety behaviour to Penk Dlx$^{+/+}$. This finding agrees with results previously reported in DOR Dlx $^{-/-}$ mice (Chung et al., 2015). Both results suggest that enkephalin activation of DORs expressed in GABAergic forebrain neurons contributes to increased anxiety. After PSNL surgery, increased anxiety-like behaviour was only observed in Penk$^{+/+}$ and Penk Dlx$^{+/+}$ mice. Enkephalins deletion in Penk$^{-/-}$, Penk CMV$^{-/-}$ and Penk Dlx$^{-/-}$ mice hinders the development of anxiety due to neuropathic pain. The increase in anxiety behaviour was maintained during the time as revealed in the late anxiety evaluation performed in the light dark box. In the first experimental sequence, the baseline anxiogenic

phenotype of Penk$^{-/-}$ mice was corroborated. It was also confirmed that neuropathic pain did not exert any influence on the already altered anxiety-like behaviour of Penk$^{-/-}$. Nevertheless, we were unable to replicate light dark box anxiety responses in the second experimental sequence. These different responses to anxiety paradigms obtained in sequence one and two are probably due to the different genetic background of mice used in each of them. In accordance with our different anxiety responses, it has been found that behavioural phenotype was strongly dependent on the anxiety experimental paradigm. Penk KO mice in DBA/2J genetic background revealed increased anxiety in the zero maze and social interaction tests, but not in the light dark box and startle response paradigms. C57BL/6J Penk KO mice showed increased anxiety in the light dark box and startle response tests, but not in the social interaction test (Bilkei-Gorzo et al., 2004).

It has been suggested a relation between enkephalin and depressive-like behaviour. Some studies have shown an anti-depressant effect of enkephalins in several paradigms. A reduction in the depressive-like behaviour was observed when wild type animals were treated with inhibitors of enkephalin metabolism (Baamonde et al., 1992) or enkephalin exogenous analogues (Tejedor-Real et al., 1995, 1998).DOR deletion significantly increased immobility time in genetically modified mice (Filliol et al., 2000a). However, when DORs were conditionally deleted in GABAergic forebrain neurons (DOR Dlx$^{-/-}$), this depressive-like behaviour disappeared (Chung et al., 2015). Additionally, it was reported that Penk deficient animals did not show depressive-like phenotype in the forced swimming or

in the tail suspension tests (Bilkei-Gorzo et al., 2007). Later, it was found that the anxiety and depressive-like behavioural in Penk KO mice remained unaltered after chronic mild stress, suggesting a resistant genotype to this situation (Melo et al., 2014). Our results agree with those reported by Bilkei-Gorzo *et al*. (Bilkei-Gorzo et al., 2007) and Melo *et al* (Melo et al., 2014), who did not find significant differences in the depressive-like behaviour in Penk KO mice. Responses in the depressive-like behaviour have been found to be specific of genetic background. Penk KO mice in C57BL/6J genetic background had a lower frequency of depressive-like behaviour in stress-induced hypoactivity and ultrasonic vocalization models (Bilkei-Gorzo et al., 2007). After PSNL, the immobility time was increased in Penk$^{+/+,}$ Penk$^{-/-}$ and Penk CMV$^{-/-}$, but not in Penk Dlx$^{-/-}$ mice where remained unaltered. Therefore, the lack of enkephalin in GABAergic neurons of forebrain conferred resistance to a depressive-like behaviour produced by neuropathic pain.

Regarding cognitive function, it has been observed that enkephalin increases learning and memory deficits in the dentate gyrus (Palop et al., 2003, 2005). However, the administration of opioid antagonists improved spatial (Decker et al., 1989) and working memories (Canli et al., 1990; Gallagher, 1982). DOR and Penk deletions are involved in deficits in hippocampal dependent learning (Le Merrer et al., 2011, 2012). The expression of some components of the EOS, such as Pdyn and Penk, were reduced in DOR$^{-/-}$ mice hippocampus and might explain the altered hippocampal activity (Le Merrer et al., 2013). We found that the lack of enkephalins impaired cognitive responses. A decrease of long-term memory was

observed in all the strains of Penk KO mice. DOR are involved in hippocampal dependent behaviours and the high DOR expression in GABAergic interneurons of hippocampus (Rezäi et al., 2012; Scherrer et al., 2006) is related with our finding that the only significant reduction in cognition was observed in Penk $Dlx^{-/-}$. An interesting ceiling effect of enkephalins on the development of cognitive impairment due to neuropathic pain was revealed in Penk KO mice.

We have investigated the expression of genes involved in the control of nociceptive, emotional and cognitive responses in the amygdala and the hippocampus to clarify the possible mechanisms underlying the behavioural changes induced by the suppression of Penk gene. The amygdala has been identified as reactive to fearful stimuli and anxiogenic situations (Phelps and LeDoux, 2005b). Hyperactive amygdalar function is found under emotional stimuli (Bruhl et al., 2011; Shah et al., 2009), and anxiogenic situations (Blair et al., 2008; Dilger et al., 2003; van den Heuvel et al., 2005). The interaction of amygdala and hippocampus is important in emotional learning, modulation of memory, and emotional contributions to social behaviour (Benarroch, 2015). Additionally, hippocampus is related with many cognitive functions such as spatial processing (Frings et al., 2006; Maguire et al., 2000), working memory (Axmacher et al., 2010), episodic memory, and the construction of mental images (Bird and Burgess, 2008; Squire et al., 2010).

Dynorphins (McLaughlin et al., 2003) and KOR (Land et al., 2008) are implicated in stress and anxiety modulation. Decrease anxiety-

like behaviour is observed in mice with KOR deletion in the basolateral amygdala (Crowley et al., 2016). We found an increased expression of *Pdyn* and KOR in the amygdala of $Penk^{+/+}$ animals after PSNL, but not in the $Penk^{-/-}$. These components of EOS have been associated with development of behavioural manifestations of chronic pain (Narita et al., 2006a; Negrete et al., 2016). The similar expression levels of *Pdyn* and KOR in sham and PSNL $Penk^{-/-}$ mice could be related with the ceiling effect on anxiety-like behaviour that was observed in our experimental setting. This finding suggests a regulatory effect of enkephalin on *Pdyn* and KOR in neuropathic pain.

Overexpression of corticotropin releasing hormone *Crh* is related with increased anxiety-like behaviour (Stenzel Poore et al., 1996). In our study, the increased expression of *Crh* was also related with anxiety development after PSNL in wild type animals. In $Penk^{-/-}$ mice PSNL did not increase *Crh* levels or behavioural anxiety. The transcription factor neuronal PAS domain containing protein 4 (*Npas4*) regulates neuronal networks homeostasis that is essential for normal behaviour and cognition (Bloodgood et al., 2013; Li et al., 2009) and alterations in its inhibitory pathways are associated with increased anxiety (Blundell et al., 2009; Hines et al., 2008). Mice lacking *Npas4* exhibit an anxious and hyperactive phenotype (Li et al., 2009). However, we found an increased expression of *Npas4* in sham $Penk^{-/-}$ and in PSNL $Penk^{+/+}$ animals with anxious behaviour, which are in accordance with the observation that *Npas4* KO animals exhibited a decreased anxiety-like behaviour in elevated plus maze (Jaehne et al., 2015). After PSNL the reduction

of *Npas4* levels in Penk$^{-/-}$ mice suggested its involvement in the regulation of the anxiety–like behaviour.

Dysregulated secretion of glucocorticoid receptors (GR) is found in several psychiatric conditions, including anxiety and depressive disorders (Guerry and Hastings, 2011; Heim and Nemeroff, 2001). It is also known, that enduring loss of GR is responsible for the maintenance of altered anxiety responsiveness over the lifetime (Arnett et al., 2015). PSNL surgery reduced *Nr3c1* expression levels in the PSNL Penk$^{-/-}$ mice when compared with PSNL Penk$^{+/+}$. In accordance with Arnett *et al* (Arnett et al., 2015), who related a GR reduction with decreased anxiety behaviour, we suggest that the loss of *Nr3c1* expression in PSNL Penk$^{-/-}$ mice might be related with the maintenance of the anxiety-like behaviour.

GR mediate the impairing effects of stress and corticosterone level on hippocampal function. Stress or elevated corticosterone levels inhibit long-term potentiation (Diamond et al., 1992), impair spatial memory (Luine et al., 1994), suppress synaptic plasticity (Joels et al., 2004) by spine loss, dendritic atrophy (Liston and Gan, 2011), and neuronal cell death (Haynes et al., 2004), and decrease neurogenesis (Schoenfeld and Gould, 2012). We consider that increased expression levels of *Nr3c1* in hippocampus may be associated with cognitive impairments. High expression levels of *Nr3c1* are related with the reduction of the discrimination index observed in the Penk$^{-/-}$ mice. As *Nr3c1* expression levels are reduced in these animals, it seems that the ceiling effect in the cognitive impairment after PSNL involves *Nr3c1* regulation.

Significant decrease in *BDNF* expression in the hippocampus was related with the cognitive impairment produced by the lack of enkephalins, but did not influence the cognitive impairment produced by neuropathic pain. It has been previously reported a relation between EOS and *BDNF* expression, and the administration of morphine and DAMGO induced up-regulation of *BDNF* mRNA expression (Zhang and Ko, 2009).

In our study, we observed that enkephalins are involved in the regulation of nociceptive manifestations of neuropathic pain. It has been previously proposed a mechanism for resilience phenotype of Penk KO mice under chronic mild stress conditions (Melo et al., 2014). Our results suggested a resilience effect (Melo et al., 2014) of Penk, primarily on the development of anxiety and cognitive impairment but also in depression produced by neuropathic pain. We identified *Pdyn*, *Npas4* and *Nr3c1* in the amygdala and also *Nr3c1* in the hippocampus to be related with enkephalin deletion. We suggest that they are probably responsible of the ceiling behavioural effect observed in anxiety and cognition after neuropathic pain induction.

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DISCUSSION

Pain is a complex condition that involves nociceptive, emotional and cognitive components. To understand the changes of behavioural components induced by neuropathic pain, we first validated the sensitivity of traditional models to evaluate changes on the depressive-like behaviour, anhedonic state, anxiety-like behaviour and cognitive function after nerve injury. Later, we explored the influence of sociability, anxiety and depressive traits on the development of neuropathic pain and its manifestations. Finally, we explored the involvement of the opioid system in the neuropathic pain and its emotional and cognitive manifestations. Specifically, we studied the role of the opioid peptide preproenkephalin.

Validation of behavioural models to assessing the emotional and cognitive manifestations of neuropathic pain by partial sciatic nerve ligation

Co-morbidities present during neuropathic pain as anxiety, depression and cognitive problems are rarely taken into account during therapeutic management, although they have a significant impact on the quality of life of patients and are associated with poor therapeutic responses (Moriarty et al., 2011; Radat et al., 2013). Currently, new treatments that target also the emotional and cognitive components of this chronic condition are needed. To achieve this goal, the validation of new reliable preclinical models should accomplish two crucial features. First, these models should allow the evaluation of nociceptive, emotional and cognitive

manifestations of neuropathic pain. Second, it should grant the assessment of effectiveness of new drugs in neuropathic pain symptoms.

In the first part of the thesis, it was found that traditional paradigms to evaluate anxiety-like, depressive-like behaviours and cognitive function in mice allowed the assessment of changes in these conditions associated to neuropathic pain. The nociceptive, emotional and cognitive manifestations were evaluated at different time points to include the beginning, development and steady state phases of neuropathic pain. Additionally, pregabalin was chosen as reference drug to allow the pharmacological validation of these paradigms, as it has proven to be effective in preclinical models and in the clinical management of neuropathic pain (Verma et al., 2014). In some animal models of neuropathic pain, such as chronic constriction injury, spared nerve injury, and spinal cord contusion, pregabalin significantly attenuated spontaneous pain behaviour, hyperalgesia and allodynia (Baastrup et al., 2011; Bender et al., 2010; Gustafsson and Sandin, 2009; Tanabe et al., 2008).

First, we evaluated nociceptive manifestations of neuropathic pain produced by PSLN. Pregabalin administered at a dose of 20 mg/kg twice a day, almost reversed the mechanical allodynia, heat hyperalgesia and cold allodynia after nerve damage. These data are in agreement with those reported showing the effectiveness of pregabalin doses of 10 mg/kg and 30 mg/kg in the relief of

neuropathic pain secondary to spinal cord injury (Tanabe et al., 2009).

We also evaluated possible emotional and cognitive alterations associated with neuropathic pain. Affective responses are highly dependent on the behavioural paradigm used (Liu and Chen, 2014). Anxiety evaluation in the elevated plus maze or in the light dark box test is based on the examination of exploratory behaviour patterns, where "anxious" mice restrict their activity. Fear and anxiety share many sign and symptoms as well as brain areas and regulatory circuits, including the bed nucleus of the stria terminalis and the amygdala. However, it has been reported that the involvement of the bed nucleus and the stria terminalis is more important in anxiety than in fear, and the amygdala has a higher implication in the modulation of fear than of anxiety (Davis et al., 2010; Davis and Whalen, 2001). Both structures have similar efferent connections to various hypothalamic and brain stem target areas, which are known to be involved in fear and anxiety. These areas receive highly processed sensory information from the basolateral nucleus of the amygdala and can react to emotional stimuli (Davis et al., 2010; Davis and Whalen, 2001). During stress or anxiety CRH is released from CRH-containing neurons in the amygdala, which project to the bed nucleus of the stria terminalis and act on CRH receptors. Thus, activation of the amygdala by some stressors could lead to long-term activation of the bed nucleus of the stria terminalis via CRH (Davis et al., 1997). In our study, anxiety-like behaviour was evaluated in the elevated plus maze. The

decrease in the percentage of time in open arms was evident since day 7 after PSNL and was maintained until day 21 after pain induction in the absence of drug treatment. Interestingly, pregabalin (20 mg/kg twice a day) reduced anxiety in PSNL animals. This is in agreement with the widely reported anxiolytic effect of pregabalin in animals and humans (Micó and Prieto, 2012; Navarrete et al., 2012).

There are some characteristics of depression that must be taken into account to develop a reliable animal model. In humans, depression is not an illness but a highly heterogeneous syndrome; key symptoms of human depression (guilt, tendency to suicide and sadness) cannot be assessed in animals (Russo and Nestler, 2014). Furthermore, the biological substrates underlying human depression remains poorly understood. However, the risk for depression is increased when the subject is exposed to stress (Kessler, 1997); consequently, most rodent depression models rely on environmental stressors to induce depressive-like symptoms that can be studied from a mechanistic point of view (Russo and Nestler, 2014). Currently, available animal models of depression can be generally classified in four groups based on the nature of their induction phase. The first is based on the application of acute or sub-chronic stressors to induce the depressive-like symptoms. The second uses long-term exposure to stressors to generate depressive-like symptoms such as anhedonia or changes in appetite. The third applies biochemical and pharmacological concepts to mimic clinical observations. The last group involves the application of genetic and

surgical techniques to change permanently phenotypes and behaviours of animals (Yin et al., 2016).

We analysed the depressive-like behaviour using paradigms from the first two groups to provide a more valid model to evaluate the effect of neuropathic pain on the development of depression. We used the forced swimming test and the sucrose preference test and evaluated the behavioural responses specific for each paradigm. The forced swimming test belongs to the despair-based models. The immobility analysed in this test is directly related to the inability or reduced motivation to maintain effort in an inescapable situation; in contrast, the sucrose preference includes different components of the reward processing (wanting, liking and learning) that are related to the appetitive, consuming and satiety phases of a pleasure cycle (Thomsen, 2015). Anhedonia is defined as the loss of the ability to experience pleasure from normally rewarding stimuli, such as food, sex and social interactions. Several brain areas are related to the hedonic impact of reward in the human brain. They include cortical regions such as orbitofrontal cortex, cingulate cortex, and insular cortex, and subcortical regions such as nucleus accumbens, ventral pallidum, amygdala, and brainstem, ventral tegmental area and periaqueductal gray matter (Kringelbach, 2005; Kringelbach and Berridge, 2009). Despite the involvement of hippocampus and frontal cortex in the depression and its treatment, it is unlikely that these regions can be accountable for all symptoms of the disorder (Nestler et al., 2002). In consequence, it is important to consider all these brain areas as functioning parts of highly overlapping and

interacting circuits. The way in which this circuitry contributes to depression remains uncertain. The best-characterized reward circuit in the brain comprises dopamine neurons in the ventral tegmental area that project to the nucleus accumbens, which is part of the ventral striatum. The main neuronal population of the nucleus accumbens are GABAergic medium spiny neurons (Koob and Le Moal, 2008). Several regions of the prefrontal cortex, central amygdala, basolateral amygdala and hippocampus are innervated by dopamine neurons of the ventral tegmental area. All these reward regions have complex interconnections. For example, the nucleus accumbens receives glutamate innervations from the prefrontal cortex, amygdala and hippocampus; and the prefrontal cortex, amygdala and hippocampus form reciprocal glutamatergic connections with each other (Hnasko et al., 2012; Tritsch et al., 2012). The functional output of these regions is modulated by GABAergic interneurons and, in the nucleus accumbens, by cholinergic interneurons as well. Moreover, each of these regions receives serotonin inputs from midbrain raphe nuclei and noradrenergic inputs from the pontine locus coeruleus, and several are innervated by hypothalamic peptide systems. Also, there is some evidence that ventral tegmental area dopamine neurons also release glutamate or GABA, which may contribute to their functional effects (Russo and Nestler, 2014).

In our study, we observed a decrease in the sucrose preference in PSNL mice since the first day after surgery, while the immobility time measured in the forced swimming test was only increased until

day 25 of neuropathic pain development. The responsiveness to pregabalin treatment was also paradigm-dependent. The anhedonic-like state induced by PSNL completely disappeared after chronic pregabalin treatment, while the effect on the depressive-like behaviour was null. These results are in agreement with those observed in a chronic inflammatory pain model in which pregabalin was chronically administered and had no effect on the depressive-like behaviour despite its analgesic effect (Maciel et al., 2013). Additionally, affective manifestations were maintained even though mechanical allodynia was abolished in another chronic pain model (Dimitrov et al., 2014). These results suggest that depressive-like symptoms, once established, do not necessarily disappear when pain is treated.

In general, we observed that anxiety and depressive-related behaviours were induced in a time-dependent manner, suggesting a critical role of time in the development of the affective consequences of neuropathic pain. The increased anxiety-like behaviour produced by the PSNL appeared by week 1 and persists at week 3 after pain induction. Some manifestations of the depressive-like behaviour, as the development of an anhedonic state, become evident in early states of pain induction, while others appear in a late state of neuropathic pain development. In accordance with our results, it has been shown that anxiety-like behaviours increase before the appearance of the depressive-like behaviour (Yalcin et al., 2011).

Many cognitive aspects, such as recognition memory (Kodama et al., 2011), attention, working and short term memory (Low et al., 2012; Ren et al., 2011), can be disrupted under neuropathic pain. Therefore, we decided to characterize the consequences of neuropathic pain on different cognitive functions using sophisticated operant (food maintained operant behaviour) and non-operant (novel object recognition) behavioural models of learning and memory. The first paradigm used was the food maintained operant behaviour to evaluate learning throughout conditioning. In this paradigm, animals learn to predict positive outcomes after repeated successful pairings of a reward with a conditioned stimulus. In the beginning of the decision-making process, mice learn to integrate the action–outcome association (in our case, lever-pressing leads to a pellet delivery as reward) with the value attributed to such outcome (high value for pellets when hungry). The striatum has been linked to several types of learning involving procedural skills, habits and reward learning (Solway and Botvinick, 2012). Dopamine is crucial for reinforcement learning in the striatum (Wise, 2004) and it is also important for learning of positive outcomes and avoidance of the aversive ones. It enhances the first through its action on D1 receptors on striatonigral neurons, whereas the decreased dopamine transmission via D2 receptors on striatopallidal neurons modulates the aversive outcomes (Frank and O'Reilly, 2006).

On the other hand, the novel object recognition test is a non-reward test based on the spontaneous exploratory behaviour of rodents that

measure recognition memory. Recognition memory is a fundamental facet related with the ability to remember. It requires an ability for both identification and judgement of the prior occurrence of what has been identified (Brown and Aggleton, 2001). Novelty recognition memory requires an intact anterior subhippocampal cortex (transentorhinal, entorhinal and perirhinal cortices) (Kang et al., 2016). Interaction between the prefrontal cortex, hippocampus and amygdala is also critical for the storage of sensory inputs into perceptual information and, as a result, memory acquisition. Sensory information is initially handled by the dorsolateral prefrontal cortex as working memory. However, when information is presented repetitively or has long-term relevance, the hippocampus and amygdala send this information to the associated cortex for long-term storage. The hippocampus is the main processor of contextual input, and stores episodic events after linking them with time and space information. Upon encountering an emotionally challenging event, activation of the amygdala facilitates the storage of information by interacting with the hippocampus and prefrontal cortex (Kang et al., 2016).

In the first approach of the cognitive model, when we evaluated memory in the novel object recognition test, the cognitive impairment produced by PSNL was evident at day 10 after nerve injury and was maintained until day 24 after surgery. The deficit in long-term memory was attenuated by the pregabalin treatment as demonstrated by the increase of the discrimination index when compared with the PSNL control group at day 24 after pain

induction. This cognitive impairment was also revealed in the food-maintained operant behaviour. In this paradigm, a reduction in the number of active responses of the PSNL without treatment during the second and third phases was observed. In these late phases, palatable food was delivered as a reward. Motivation for food seeking was affected in PSNL control groups in all the progressive ratio (PR) schedules performed at the end of each training phase. Similar results were reported in a model of selective injury of the sciatic nerve (Schwartz et al., 2014). It was reported that neuropathic pain caused a selective impairment in performance on a difficult PR task but have no detectable effects on easier tasks or the value of rewards (Schwartz et al., 2014). In our case, the highest difference in the motivation for seeking palatable food was revealed in the PSNL without treatment when the task became more difficult (FR5 schedule). Pregabalin treatment abolished the impairment induced by neuropathic pain in the food-maintained operant responses under the FR5 schedule. However, a reduction in the breaking point during the PR was observed in the sham group receiving treatment probably due to a sedative effect of the drug. The altered responses found in the most difficult operant tasks (FR5 and PR) may be partially attributed to a learning impairment, as mentioned above. Operant responding highly depends on proper cognitive functions.

Several limitations of the operant cognitive model were identified during the study. Therefore, the first food-maintained operant behaviour protocol was improved by several modifications. First,

we tried to suppress possible anhedonic component due to the palatable pellets used as reward and, therefore, standard pellets were delivered. Therefore, we could identify and evaluate the learning component of this paradigm. Second, as a complement of the cognitive component evaluation, two assessments of long-term memory in the novel object recognition test, were performed on day 14 and 20 after pain induction. Finally, as in all PRs sham animals receiving pregabalin had a reduction in the breaking point, probably due to a sedative effect of pregabalin, we decided to reduce the dose to 10 mg/kg twice a day. To induce the conditional learning by standard pellets, a slight food deprivation of 85% of the initial body weight was maintained during the drug treatment period. It was observed that the low dose of pregabalin effectively reduced mechanical allodynia and thermal hyperalgesia and partially the cold allodynia. In the food-maintained operant behaviour, animals with pain had a reduction in the number of active responses and, as showed in the previous experiment, this effect was more evident when the complexity of the task was increased. With the reduction in the dose of pregabalin, no differences in motivation when evaluated by means of comparing PR in PSNL and sham animals were seen. Therefore, the results in the operant behaviour were more related to a learning process than to a motivational effect. These findings were supported by the impairment in long-term memory evaluated in the non-operant behaviour paradigm, which was observed in animals with neuropathic pain, supports this finding. In both experiments, it was confirmed that cognitive impairment appeared in early stages of neuropathic pain

development and was maintained at least during all the time points evaluated (day 20 and 24 after nerve injury). In the second approach of the cognitive model, memory impairment was partially reversed at day 16 after surgery and completely reversed at day 24 after pain induction in animals that received pregabalin treatment. This is in accordance with the recovery observed in the first experimental sequence when the cognitive component was improved at week 3 after PSNL and 2 weeks of pregabalin treatment. These results could be controversial since some drug side effects on cognition performance, especially memory, have been attributed to pregabalin. In clinical trials performed on healthy volunteers comparing pregabalin, alprazolam and placebo, pregabalin exhibits negligible adverse effects on cognition and psychomotor behaviour (Hindmarch et al., 2005). High doses of pregabalin (≥600 mg/day) impaired several cognitive functions when administered in healthy volunteers (Salinsky et al., 2010). However, pregabalin in a relative high dose (mean dosage of 510 mg/day) improved cognitive function in female patients with long-term use of benzodiazepines (Oulis et al., 2014). Thus, the recovery observed in the novel object recognition in terms of long-term memory is probably more associated with the relief of pain and the decrease in anxiety produced by the pregabalin than with a direct improving effect on cognition.

Influence of specific behavioural traits on the nociceptive, emotional and cognitive manifestations of neuropathic pain

Recently, it has been proposed that cognitive and biological variables such as gender, age, attention, self-efficacy and personality traits may have a modulator role in pain perception and its manifestations (Tang and Gibson, 2005). The management of patients with pain would have a better outcome if each personality trait would be considered (Conrad et al., 2007). For this reason, we were interested in evaluate the influence of sociability, anxiety and depression on the inter-individual variability of nociceptive, emotional and cognitive manifestations of neuropathic pain. Behavioural, electrophysiological and genetic approaches were used in combination to elucidate mechanisms involved in personalised responses.

In our experimental conditions, sociability trait was related to spontaneous central amydgala (CeA) activity and with an increase of *Pdyn* expression levels after PSNL. However, this trait did not exert a significant influence on nociception, anhedonia, anxiety and long-term memory.

Pharmacological studies or lesions of amygdala have reported its functional role in social behaviours and social aggression in nonhuman primates (Kling, 1974; Kling and Steklis, 1976; Machado et al., 2008) and rodents (Bunnell et al., 1970; Gonzalez et al., 1996; Jonason and Enloe, 1971; Sanders and Shekhar, 1995a,

1995b). In humans, it has been observed a close relation between disturbance of social behaviour and amygdalar dysfunction (Adolphs et al., 2005; Spezio et al., 2007; Tranel and Hyman, 1990). Besides these evidences, an involvement of this brain structure in social processing or social cognition in humans has been also reported (Bickart et al., 2011; Killgore and Yurgelun-Todd, 2005; Schultz, 2005).

Electrophysiological recordings of rat basolateral amygdala (BLA) have observed changes in neuronal activity during social interaction behaviours, such as increased firing in the BLA related to the augmentation of general social behaviour (Katayama et al., 2009). These data agree with our results. Before pain induction, we observed a clear relation of the spontaneous central amydgala (CeA) activity and sociability trait. The high CeA activity was recorded in the group of animals with high sociability while the lowest activity was found in mice with low sociability.

We have shown that mice displaying low sociability phenotype tend to develop increased mechanical and thermal allodynia when compared with the intermediate and high sociability PSNL mice. Although these results did not reach statistical significance they suggest that a low sociability phenotype could be a predisposing factor to develop pain hypersensitivity. Sociability is a complex behavioural trait with direct effect on pain perception. It has been reported that interaction with conspecifics, as part of social interaction, enhances positive emotions and, in consequence, reduce

pain sensitivity. The opposite effect is observed in social isolation cases. Long lasting decrease in acute pain sensitivity is directly related to the duration of the period of isolation (D'Amato and Pavone, 2012).

Genetic analysis of amygdala samples revealed a relation between the mRNA expression of prodynorphin (*Pdyn*) and the sociability trait. The highest *Pdyn* expression was found in sham low sociability group, while the high sociability group had a decreased *Pdyn* mRNA levels. After pain induction, *Pdyn* gene expression was increased in all sociability groups maintaining the same pattern of expression found in sham animals. Components of the *Pdyn* system are found in the amygdala. High *Pdyn* levels are expressed in the CeA, whereas the BLA comprises high density of KOR (Schwarzer, 2009). The role of *Pdyn* in social behaviour was demonstrated in a study where it was found that the duration of social partner recognition was at least six times longer in dynorphin KO mice than in wild-type mice (Bilkei-Gorzo et al., 2014). These authors also found an enhanced social memory when the KOR was pharmacological blocked in wild type animals. These results and ours relate *Pdyn* with the sociability trait. Furthermore, the *Pdyn* expression pattern remains after neuropathic pain.

In our study anxiety trait was related to *Pdyn* mRNA expression before and after PSNL. A role of this trait on the development of mechanical allodynia, anxiety and cognitive impairment is

suggested. However, spontaneous activity of CeA was not associated with anxiety-like behaviour.

Electrophysiological analysis in the amygdala of the anxiety-like behaviour selected mice did not reveal a relation between this trait and the spontaneous CeA activity. The amygdala comprises several functionally distinct nuclei. The circuitry within and among amygdala subnuclei, and the various long-range projections from the amygdala, may result in different modulator functions in anxiety (Felix-Ortiz et al., 2013; Tye et al., 2011). Moreover, the circuitry that is required to detect, evaluate and process anxiogenic stimuli is complex, even more than the circuits linked to fearful stimuli (Tovote et al., 2015). Anxiety is modulated by local and long-range connections between multiple brain areas. The bed nucleus of the stria terminalis (BNST) is part of the so-called extended amygdala. It is a major target of projections from the BLA and CeA and develops an important role in mediating anxiety. In turn, the BNST projects to the lateral hypothalamus, the ventral tegmental area (VTA) and the parabrachial nucleus (Kim et al., 2013). The hippocampus role in anxiety, specifically of the ventral portion, is highly supported by several reports (Fanselow and Dong, 2010; Strange et al., 2014). Recently, an input pathway from the PFC to the CeA has been implicated in heightened anxiety (Birn et al., 2014). The septohippocampal system has long been hypothesized to play a major part in stress-induced anxiety (Gray and McNaughton, 2008). Due to all the brain areas involved in anxiety and the different functional roles of amygdala subnuclei, it is not

completely surprising the lack of relation between anxiety and the CeA activity found in our experimental conditions.

The influence that the anxiety-like behaviour has on mechanical and thermal hypersensitivity induced by neuropathic pain in our results was unclear. Anxiety can modulate nociceptive pain manifestations in different ways. Roeska *et al* (2009) evaluated the influence of trait anxiety on mechanical hypersensitivity after chronic constriction injury in rats with high and low anxiety-like behaviour. In pain measurements of day 14 and 21 after pain induction, rats with high anxiety-like behaviour display a stronger response to mechanical stimuli than those with low anxiety-like behaviour. This pain response was inverted in day 36 and 57 after the chronic constriction injury was done. This suggests that, while the mechanical hypersensitivity remained constant in the low anxiety animals, the pain withdrawal threshold was increased in the rats with high anxiety levels (Roeska et al., 2009). Even not statistical significant, we also observed an enhanced mechanical allodynia in mice with the more anxious phenotype.

Our results suggested that anxiety trait could be a predisposing factor to develop higher emotional and cognitive manifestation of neuropathic pain. After pain induction, selected mice for high anxiety showed the most severe impairment in anxiety-like behaviour and in long-term memory. Patients with anxiety disorders frequently complain about cognitive difficulties, such as excessive distractibility and poor mental concentration (Rinck et al., 2003).

On the other hand, cognition can exert an influence on anxiety, suggesting a feedback between these behaviours (Clark and Beck, 2010). Cognitive treatment reduces the activity in the amygdala and hippocampal subcortical regions that involve bottom-up emotion processing. Additionally, cognitive therapy improves activity of top-down processes areas that are involved in cognitive control of emotion, such as the medial prefrontal, orbitofrontal and dorsal anterior cingulate cortex (Kumari, 2006; Ochsner and Gross, 2005; Ressler and Mayberg, 2007). All these findings demonstrated a complex relation between cognition and anxiety. In line with literature, our results suggested that high anxiety level influences in a negative way the cognitive component of neuropathic pain.

No consistent description of *Pdyn* role in anxiety has been shown in the literature. In rats, anxiety reduction was associated with low expression of *Pdyn* (Ménard et al., 2014) but other study reported that *Pdyn* deletion increased anxiety-like behaviours and impaired the anxiolytic effect of bromazepam (Femenía et al., 2011b). Other authors agree with the results of the first group and reported that mice lacking *Pdyn* showed an anxiolytic behaviour measured in the open field, the elevated plus maze and the light-dark tests. (Kastenberger et al., 2012). Some reports have suggested that dynorphins are released in response to stress (Bruchas et al., 2009; Land et al., 2008). In our study, we observed that *Pdyn* mRNA expression has a direct relation with anxiety levels before and after pain induction. Lower *Pdyn* levels were found in the amygdala of low anxiety animals while higher expression of this gene was

observed in mice with high anxiety-like behaviour. *Pdyn* expression levels increased after pain induction maintaining the same pattern of expression found in sham animals. Additionally, we observed a general increase of corticotropin releasing hormone (*Crh*) gene expression in the amygdala of selected mice as result of PSNL. The role of *Crh* is clearly related to anxiety. It has been observed that the intraventricular injection or overexpression of *Crh* produce anxiogenic effect on mice (Stenzel Poore et al., 1996). A down-regulation of *Crh* expression in the CeA that was induced by the lack of *Pdyn* has been also reported (Kastenberger et al., 2012). We also observed an association between *Pdyn* and *Crh* expression and we may relate the over-expression of *Pdyn* after pain induction to the increased *Crh* levels.

In our experimental conditions, depression trait was related to nociceptive manifestations of neuropathic pain such as mechanical and thermal allodynia. We also found an association between depression and biomarkers expression such as *Nr3c1, Gadd45,* interleukin 6 (*il-6*) and interleukin 1 beta (*il-1 beta*). However, this trait was not related to spontaneous central amydgala (CeA) activity and did not have influence on emotional and cognitive manifestations of neuropathic pain.

We did not find association between depressive trait and the CeA spontaneous neuronal activity. However, some studies have shown amygdalar involvement in depression. In rats, the immobility time measured in the forced swimming test was direct associated with

the expression of mitogen-activated protein kinase in the amygdala (Huang and Lin, 2006; Porsolt et al., 1977a). In humans, decreased volume of amygdala was found in depressed patients that do not receive an antidepressant treatment (Hamilton et al., 2011), and the amygdalar baseline activity has been found increased and positively correlated with the severity of the depression (Drevets et al., 1992; Ketter et al., 2001). Additionally, the increased and sustained amygdala activity that accompanied acutely depressed state (Drevets et al., 2002) returns to normal after treatment with antidepressants (Fu et al., 2004).

Nociceptive manifestations of neuropathic pain such as mechanical and thermal allodynia were affected by the depressive trait. Even not statistical significant, we observed that mice with low depressive-like behaviour exhibited increased mechanical and thermal allodynia. In accordance with our results, it has been observed the presence of mechanical and cold allodynia but heat hyperalgesia, in neuropathic pain induced by spinal nerve ligation in olfactory bulbectomized rats (Burke et al., 2013). Nevertheless, in clinic, both positive and negative relationship of depression with pain sensitivity was reported. For example, an association of depression poor sleep and reduced pain thresholds was reported (Chiu et al., 2005), while an increased thermal pain threshold was reported in female patients suffering from major depressive disorder (Bär et al., 2007).

We found no influence of depressive trait on emotional and cognitive manifestations of neuropathic pain. However, depressive trait was associated with *Nr3c1*gene expression. An indirect relation between the *Nr3c1* mRNA levels and the depressive-like behaviour group was found. The highest levels of *Nr3c1* were observed in the low depressive group before PSNL. After pain induction, the *Nr3c1* mRNA expression levels of the low depressive phenotype were not increased. However, in the case of the high depressive phenotype, the levels of *Nr3c1* mRNA were increased after PSNL. In the same direction of our results, some authors reported an association between depression and the glucorticoid receptor mRNA expression (Nantharat et al., 2015). They found that higher levels of methylation at the *Nr3c1* promoter may be associated with major depressive disorders, and proposed that DNA methylation of the *Nr3c1* gene promoter may decrease *Nr3c1* mRNA expression. However, they did not find significant differences in *Nr3c1* mRNA expression levels between patients with major depressive disorders and control subjects. However, there was a trend of lower *Nr3c1* expression related to depressive patients (Nantharat et al., 2015). Our results suggest that decreased level of *Nr3c1* receptor mRNA is a predisposing factor to the development of depressive-like behaviour. Depressive-like behaviour also 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 before PSNL. Expression of *Gadd45* mRNA was increased by neuropathic pain in mice with low level of depression. Nevertheless, this gene is mainly related to synaptic plasticity and

memory formation (Sultan and Sweatt, 2013). Synaptic plasticity is also involved in depression. There are some reports suggesting that antidepressants increase the activity of signalling pathways mediating positive changes of synaptic plasticity in the amygdala (Abelaira et al., 2011; Gourley et al., 2009; Réus et al., 2011; Thome et al., 2000). The increase observed in amygdala volumes of patients with mayor depressive disorders may be due to antidepressant action (Hamilton et al., 2011). However, there is little information about the activity of signal transduction pathways in the amygdala during depression. Increased MEK-ERK signalling and CREB activity in the amygdala have been related to depressive-like behaviour (Huang and Lin, 2006; Wallace et al., 2004). Abnormalities in signalling pathways have been involved in mechanisms of antidepressant effects and a decreased ERK, Akt and mTOR signalling and GluR1 phosphorylation in the amygdala have been reported (Chandran et al., 2013). In our experimental conditions, we propose the existence of a link between *Gadd45* mRNA in the amygdala and the regulation of depressive-like behaviour produced by neuropathic pain.

Neuroinflammatory processes have important roles in the pathophysiology of depression and chronic pain (Burke et al., 2013). Regarding inflammatory genes, in mice showing a depressive-like behaviour we observed changes in the expression of il-6 and il-1beta. After PSNL a decrease in il-6 mRNA expression and increased levels of il-1 beta were reported. In accordance with the il-6 over-expression after PSNL found in our study, a decrease

in il-6 has been reported in olfactory bulbectomizated rats with neuropathic pain and a lack of effect of SNL on il-1beta expression (Burke et al., 2013). It is known that the il-6 in the amygdala increases immobility in the forced swimming test (Wu and Lin, 2008) and that chronic stress exposure increases il-1beta production specifically in the amygdala (Porterfield et al., 2012). Additionally, increased glial activation and il-1beta within the amygdala may be responsible for induced mechanical and cold allodynia in bulbectomized rats (Burke et al., 2013). It has been shown that intracerebroventricular administration of non-pyrogenic doses of il-1 beta results in thermal (Oka et al., 1993) and mechanical hyperalgesia (Yabuuchi et al., 1996). In agreement with these two studies, our results related the increase in il-1 beta expression with the hypersensitivity induced by neuropathic pain. They also indicated that neuroimmune processes in the amygdala might partially underlie the nociceptive behavioural changes observed. We can suggest a regulation of pain sensitivity in depressed animals throughout the down-regulation of il-6 and the over-expression of il-1 beta.

The relationship between neuropathic pain and anxiety, cognition, depression is complex because it is bidirectional. Therefore, the effects of “high” traits such as sociability, anxiety and depression on neuropathic pain can be masked at least by the neuropathic pain symptoms *per se*. Specifically, chronic pain induces anxiety, impaired memory, and depressive-like behaviour and therefore we can talk about a ceiling effect (basic anxiety-trait plus anxiety

produced by neuropathic pain). In addition, the criteria used to select the extreme phenotype is not very strict leading to a weak phenotype. As an example, an effect on anxiety on chronic pain nociceptive outcomes has been actually reported in mice selectively bred for high or low anxiety-like behaviour across several generation based on EPM (Roeska et al., 2009).

The role of the preproenkephalins on the nociceptive, emotional and cognitive manifestations of neuropathic pain.

The endogenous opioid system (EOS) participates in the regulation of several physiological responses such as pain, reward, emotional behaviour, learning and stress regulation (Arico and McNally, 2014; Bowers et al., 2012; Valentino and Van Bockstaele, 2015). Within this system, preproenkephalin (Penk) is the opioid peptide precursor of met-enkephalin and leu-enkephalin. Penk derivatives have the highest antinociceptive activity (Madden et al., 1977). Enkephalins also play a role in locomotion, cognitive functions and affective behaviours such as anxiety, aggressiveness and depression (König et al., 1996a; Nieto et al., 2005).

In the first part of this study, the nociceptive manifestations on Penk KO mice were evaluated. No differences in the basal pain sensitivity were detected between the wild type and the Penk KO animals, although the endogenous opiates are known as important modulators of painful stimuli (Stein, 2016). However, the enkephalin modulation was evident after PSNL. Enhanced pain

sensitivity was found in two strains of total Penk KO mice ($Penk^{-/-}$, Penk $CMV^{-/-}$) and also in the mice with the conditional deletion in inhibitory GABAergic neurons of forebrain (Penk $Dlx^{-/-}$). Pain sensitivity differences between genotypes were observed in the mechanical and cold allodynia evaluations. The highest hypersensitivity to pain was a consequence of the conditional deletion of Penk and there were no differences among genotypes in thermal hyperalgesia. It has been reported that enkephalins are effective in the attenuation of tactile allodynia and thermal hyperalgesia when administered spinally (Zou et al., 2011). Moreover, they are involved in the modulation of supraspinal but not spinal analgesia (König et al., 1996a).

Endogenous opioids modulate emotional behaviours throughout their actions in the limbic system. As a consequence of Penk deletion, MOR and DOR sites increase in more than 300% in the central nucleus of the amygdala and in the ventral pallidum (Brady et al., 1999). In the early anxiety evaluation, $Penk^{-/-}$ mice spent less time and had fewer entries in the elevated plus maze open arm than $Penk^{+/+}$ animals before PSNL, confirming the anxiogenic phenotype previously reported (König et al., 1996a). In the Penk $CMV^{-/-}$, it was also observed a not statistically significant increase in anxiety as a reduction in the number of entries and in the percentage of time in elevated plus maze open arm. The anxiogenic phenotype produced by Penk deletion also corresponded with a high anxiety-related behaviour reported in constitutive DOR KO animals (Kieffer et al., 2000; Roberts et al., 2001) and with the systemic

administration of DOR antagonists (Perrine et al., 2006; Saitoh et al., 2005). In contrast, this anxiogenic response produced by enkephalins deletion lacked in Penk $Dlx^{-/-}$ mice. These animals had similar anxiety behaviour to Penk $Dlx^{+/+}$. Unexpected, lower anxiety was previously reported in DOR $Dlx^{-/-}$ mice. (Chung et al., 2015). Our results also suggest that enkephalin activation of DOR expressed in GABAergic forebrain neurons contributes to increased anxiety. It has been proposed a modulatory effect of DOR in anxiety (Randall-Thompson et al., 2010). DOR agonist injection into the rat central nucleus of the amygdala reduced anxiety and DOR antagonist did not have any effect on this behaviour. Therefore, it was concluded that the activation of DOR in the central amygdala reduces anxiety-like behaviour and the presence of DOR in this area is important for regulating anxious states (Randall-Thompson et al., 2010). We can also propose that enkephalin modulation trough DOR exerts anxiolytic and anxiogenic activities, possibly involving midbrain (amygdala) and forebrain structures, respectively. After PSNL, increased anxiety-like behaviour was only observed in $Penk^{+/+}$ and in Penk $Dlx^{+/+}$ mice. Enkephalins deletion in $Penk^{-/-}$, Penk $CMV^{-/-}$ and Penk $Dlx^{-/-}$ mice hinders the development of anxiety due to neuropathic pain. The increased anxiety behaviour of Penk KO mice was maintained as revealed in the late anxiety evaluation performed in the light dark box. In the first experimental sequence, the baseline anxiogenic phenotype of $Penk^{-/-}$ mice was corroborated. These animals had less number of entries in the white space and less number of distal entries in the light dark box test. It was also confirmed that

neuropathic pain did not exert any influence on the already altered anxiety-like behaviour of Penk$^{-/-}$. Nevertheless, we were unable to replicate light dark box anxiety responses in the second experimental sequence. These different responses to anxiety paradigms obtained in sequence one and two are probably due to the different genetic background of mice used in each of them. In accordance with our different anxiety responses, it has been found that behavioural phenotype was strongly dependent on the anxiety experimental paradigm. Penk KO mice in DBA/2J genetic background revealed increased anxiety in the zero maze and social interaction tests, but not in the light dark box and startle response paradigms. C57BL/6J Penk KO mice showed increased anxiety in the light dark box and startle response tests, but not in the social interaction test (Bilkei-Gorzo et al., 2004).

We have investigated the expression of genes involved in the control of emotional responses in the amygdala, to clarify the possible mechanisms underlying the changes in the anxiety-like behaviour induced by PSNL in Penk KO mice. The amygdala has been identified as reactive to fearful stimuli and anxiogenic situations (Phelps and LeDoux, 2005b). Hyperactive amygdalar function is found under emotional stimuli and anxiogenic situations (Bruhl et al., 2011; Shah et al., 2009). Additionally, DOR has been proposed as a regulator receptor of anxious states in the central amygdala (Randall-Thompson et al., 2010). *Pdyn, KOR, Npas4* and *Nr3c1* expression levels were maintained in the amygdala of Penk$^{-/-}$ after PSNL. These genes are probably responsible of the ceiling

behavioural effect observed in anxiety after neuropathic pain. More detail of each gene function and it expression under chronic pain condition is described below.

Other components of the EOS such as the dynorphins (McLaughlin et al., 2003) and KOR (Land et al., 2008) are implicated in stress and anxiety modulation. Decrease anxiety-like behaviour is observed in mice with KOR deletion in the basolateral amygdala (Crowley et al., 2016). We found an increased expression of *Pdyn* and KOR in the amygdala of $Penk^{+/+}$ animals after PSNL, but not in the $Penk^{-/-}$. These components of EOS have been associated with development of behavioural manifestations of chronic pain (Narita et al., 2006a; Negrete et al., 2016). The similar expression levels of *Pdyn* and KOR in sham and PSNL $Penk^{-/-}$ mice could be related with the ceiling effect on anxiety-like behaviour that was observed in our experimental setting. This finding suggests a regulatory effect of enkephalin on *Pdyn* and KOR in neuropathic pain.

Overexpression of corticotropin releasing hormone (*Crh)* has been related with increased anxiety-like behaviour (Stenzel Poore et al., 1996). In our study, the increased expression of *Crh* was also related with anxiety development after PSNL in wild type animals. The increased *Crh* levels found in the PSNL $Penk^{+/-}$ mice are similar to those expressed in sham $PENK^{-/-}$ animals. Interestingly, in $Penk^{-/-}$ mice PSNL did not increase *Crh* levels or anxiety-like behaviour.

The transcription factor neuronal PAS domain containing protein 4 (*Npas4*) regulates neuronal network homeostasis that is essential for normal behaviour and cognition (Bloodgood et al., 2013; Li et al., 2009) and alterations in its inhibitory pathways are associated with increased anxiety (Blundell et al., 2009; Hines et al., 2008). Mice lacking *Npas4* exhibit an anxious and hyperactive phenotype (Li et al., 2009). However, we found an increased expression of *Npas4* in sham $Penk^{-/-}$ and in PSNL $Penk^{+/+}$ animals with anxiety-like behaviour, which are in accordance with the observation that *Npas4* KO animals exhibited a decreased anxiety-like behaviour in elevated plus maze (Jaehne et al., 2015). After PSNL the reduction of *Npas4* levels in $Penk^{-/-}$ mice suggested its involvement in the regulation of the anxiety–like behaviour.

Dysregulated secretion of glucocorticoid receptors (GR) is found in several psychiatric conditions, including anxiety and depressive disorders (Guerry and Hastings, 2011; Heim and Nemeroff, 2001). It is also known, that enduring loss of GR is responsible for the maintenance of altered anxiety responsiveness over the lifetime (Arnett et al., 2015). PSNL surgery reduced *Nr3c1* expression levels in the PSNL $Penk^{-/-}$ mice when compared with PSNL $Penk^{+/+}$. In accordance with Arnett *et al* (Arnett et al., 2015), who related a GR reduction with decreased anxiety behaviour, we suggest that the loss of *Nr3c1* expression in PSNL $Penk^{-/-}$ mice might be related with the maintenance of the anxiety-like behaviour.

Regarding cognitive function, it has been observed that enkephalin increases learning and memory deficits in the dentate gyrus (Palop et al., 2003, 2005). However, the administration of opioid antagonists improved spatial (Decker et al., 1989) and working memories (Canli et al., 1990; Gallagher, 1982). DOR and Penk deletions are involved in deficits in hippocampal dependent learning (Le Merrer et al., 2011, 2012). The expression of some components of the EOS, such as *Pdyn* and Penk, was reduced in $DOR^{-/-}$ mice hippocampus and might explain the altered hippocampal activity (Le Merrer et al., 2013). We found that the lack of enkephalins impaired cognitive responses. A decrease of long-term memory was observed in all the strains of Penk KO mice. DOR are involved in hippocampal dependent behaviours and the high DOR expression in GABAergic interneurons of hippocampus (Rezäi et al., 2012; Scherrer et al., 2006) is related with our finding that the only significant reduction in cognition was observed in Penk $Dlx^{-/-}$. An interesting ceiling effect of enkephalins on the development of cognitive impairment due to neuropathic pain was revealed in Penk KO mice.

In order to suggest a mechanism underlying the changes in the cognitive function induced by neuropathic pain in the absence of Penk gene, we investigated the expression of *Nr3c1* and BDNF in the hippocampus. The interaction of amygdala and hippocampus is important in emotional learning, modulation of memory, and emotional contributions to social behaviour (Benarroch, 2015). Additionally, hippocampus by itself is related with many cognitive

functions such as spatial processing (Frings et al., 2006; Maguire et al., 2000), working memory (Axmacher et al., 2010), episodic memory, and the construction of mental images (Bird and Burgess, 2008; Squire et al., 2010). In our study, *Nr3c1* was found to be overexpressed in the hippocampus of Penk$^{-/}$. We propose that this gene is partially contributing to the regulation of the ceiling behavioural effect observed in the cognitive impairment after PSNL. BDNF was related with the long-term memory impairment in Penk$^{-/-}$ but not with the cognitive component of neuropathic pain. Below we described in detail *Nr3c1* and BDNF function and it expression under chronic pain condition.

GR mediate the impairing effects of stress and corticosterone level on hippocampal function. Stress or elevated corticosterone levels inhibit long-term potentiation (Diamond et al., 1992), impair spatial memory (Luine et al., 1994), suppress synaptic plasticity (Joels et al., 2004) by spine loss, dendritic atrophy (Liston and Gan, 2011), and neuronal cell death (Haynes et al., 2004), and decrease neurogenesis (Schoenfeld and Gould, 2012). We consider that increased expression levels of *Nr3c1* in hippocampus may be associated with cognitive impairments. Over expression of *Nr3c1* is related with the reduction of the discrimination index observed in Penk$^{-/-}$ mice before PSNL and in Penk$^{+/+}$ mice after PSNL. As *Nr3c1* expression levels are reduced in Penk$^{-/-}$ animals after PSNL, it seems that the ceiling effect in the cognitive impairment after PSNL involves *Nr3c1* regulation.

Significant decrease in *BDNF* expression in the hippocampus was related with the cognitive impairment produced by the lack of enkephalins, but did not influence the cognitive impairment produced by neuropathic pain. It has been previously reported a relation between EOS and *BDNF* expression, and the administration of morphine and DAMGO induced up-regulation of *BDNF* mRNA expression (Zhang and Ko, 2009).

Finally, it has been suggested a relation between enkephalin and depressive-like behaviour. Some studies have shown an anti-depressant effect of enkephalins in several paradigms. A reduction in the depressive-like behaviour was observed when wild type animals were treated with inhibitors of enkephalin metabolism (Baamonde et al., 1992) or enkephalin exogenous analogues (Tejedor-Real et al., 1995, 1998). Additionally, it was reported that Penk deficient animals did not show depressive-like phenotype in the forced swimming or in the tail suspension tests (Bilkei-Gorzo et al., 2007). Later was found that the anxiety and depressive-like behavioural in Penk KO mice remained unaltered after chronic mild stress, suggesting a resistant genotype to this situation (Melo et al., 2014). Our results agree with those reported by Bilkei-Gorzo *et al*. (Bilkei-Gorzo et al., 2007) and Melo *et al* (Melo et al., 2014), who did not find significant differences in the depressive-like behaviour in Penk KO mice. Responses in the depressive-like behaviour have been found to be specific of genetic background. Penk KO mice in C57BL/6J genetic background had a lower frequency of depressive-like behaviour in stress-induced hypoactivity and ultrasonic

vocalization models (Bilkei-Gorzo et al., 2007). DOR deletion significantly increased immobility time in genetically modified mice (Filliol et al., 2000a). However, when DOR were conditionally deleted in GABAergic forebrain neurons (DOR $Dlx^{-/-}$), this depressive-like behaviour disappeared (Chung et al., 2015). In accordance, in our study, after PSNL, the immobility time was increased in $Penk^{+/+,}$ $Penk^{-/-}$ and Penk $CMV^{-/-}$, but not in Penk $Dlx^{-/-}$ mice where remained unaltered. Therefore, the lack of enkephalin in GABAergic neurons of forebrain conferred resistance to a depressive-like behaviour produced by neuropathic pain.

As interesting final remark, it was reported that Penk KO mice exhibited resistant to develop anxiety, anhedonic state and depression as results of chronic mild stress (Melo et al., 2014). In our case, we suggested a resilience effect on Penk KO mice, primarily to the development of anxiety and cognitive impairment but also of depression produced by neuropathic pain. This finding identifies enkephalins as a key component for the development of physiological and behavioural changes induced by neuropathic pain.

CONCLUSIONS

1. Traditional paradigms to evaluate anxiety and depressive-like behaviours, and cognitive function allowed the assessment of consequences of neuropathic pain in mice.
2. Changes on affective and cognitive behaviours induced by neuropathic pain were time-dependent.
3. Anxiety-like behaviour was more evident after pain induction and was maintained during the development and maintenance of neuropathic pain.
4. Depressive-like behaviour, specifically the development of an anhedonic-like state, became evident in early pain induction while the despair state appears later.
5. Neuropathic pain caused cognitive impairment that could be detected by operant and non-operant paradigms. Learning and long-term memory were disrupted at early stages of neuropathic pain.
6. Pregabalin treatment was able to diminish the mechanical allodynia, heat hyperalgesia and cold allodynia after nerve injury.
7. Pregabalin reduced anxiety, completely suppressed the anhedonic-like state and did not have any effect on the depressive-like behaviour induced by neuropathic pain.
8. Chronic pregabalin treatments attenuated cognitive manifestations of neuropathic pain.
9. Sociability trait was directly related with spontaneous activity of central amygdala. This trait was indirectly associated with amygdalar *Pdyn* expression levels before and after nerve injury.
10. Sociability trait had little influence on nociception, anhedonia, anxiety and long-term memory.

11. A lack of association between spontaneous central amygdala activity and anxiety-like behaviour was seen.

12. Anxiety trait was found as an inter-individual vulnerability factor that aggravates mechanical allodynia, anxiety and cognitive impairment in neuropathic pain, and *Pdyn* mRNA was a concomitant biomarker in the amygdala.

13. Depression trait was unrelated with spontaneous central amygdala activity and it did not have any influence on emotional and cognitive manifestations of neuropathic pain.

14. Depression was related with mechanical and thermal allodynia. This trait conferred resistance to the development of neuropathic pain that might be attributable to the expression of the *Gadd45* gene in the amygdala.

15. Penk deletion enhanced mechanical and cold allodynia after nerve injury.

16. Enkephalin modulation of nociceptive responses was associated to inhibitory GABAergic neurons of forebrain.

17. The conditional Penk deletion in inhibitory GABAergic neurons of forebrain produced a resilient phenotype to the development of depression produced by neuropathic pain.

18. The maintained expression of *Pdyn, KOR, Npas4* and *Nr3c1* in the amygdala was involved in the ceiling behavioural effect in anxiety associated to Penk deletion after neuropathic pain.

19. Decreased expression of *Nr3c1* in the hippocampus was related with Penk deletion and the ceiling cognitive impairment observed after neuropathic pain.

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