ROLE OF THE SIGMA-1 RECEPTOR IN THE PATHOPHYSIOLOGY OF OSTEOARTHRITIS PAIN

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“The noblest pleasure is the joy of understanding”

(Leonardo da Vinci)

“…y que toda tu risa le gane ese pulso al dolor…”

(El canto del loco)

Abstract

Osteoarthritis is the most common musculoskeletal disease worldwide, characterized by degradation of the articular cartilage, chronic joint pain and disability. Currently available treatments for osteoarthritis have limited efficacy and significant side effects. Understanding the neurobiological mechanisms involved in the development and maintenance of this chronic pain condition and the pain-related comorbidities is crucial to develop novel therapeutic alternatives. The present thesis is focused on the role of the sigma-1 receptor (σ1R), a chaperone expressed in key areas for pain control, modulating chronic osteoarthritis pain and opioid analgesic tolerance. Using a mouse model of osteoarthritis pain, we demonstrated that the pharmacological blockade of the σ1R produces acute and long-lasting effects inhibiting osteoarthritis pain and its cognitive and emotional manifestations. Moreover, the σ1R antagonist restored morphine-induced antinociception in opioid-tolerant individuals, constituting a potential therapeutic strategy for the multimodal management of chronic pain. We found that the σ1R antagonist modulates a neurobiological pathway common to osteoarthritis pain and opioid tolerance, involving μ-opioid receptor activity, neuroinflammation and glutamatergic signalling. The relevance of this pathway is highlighted through the identification of a promising treatment, based on simultaneous blockade of σ1R and stimulation of the μ-opioid receptor, which relieves osteoarthritis pain without inducing tolerance. Overall we combined behavioural, biochemical and electrophysiological approaches to advance in the understanding of the role of σ1R on osteoarthritis pain manifestations, and identified

σ1R antagonists as efficient therapeutic agents to inhibit chronic osteoarthritis pain and the deleterious side effects of opioid prescription drugs.

Resum

La osteoartritis es la malaltia musculoesquelètica més comú arreu del món, caracteritzada per la degradació del cartílag articular, dolor crònic a les articulacions i discapacitat física. Els tractaments disponibles actualment per la osteoartritis tenen una eficàcia limitada i presenten efectes secundaris significatius. Comprendre els mecanismes neurobiologics implicats en el desenvolupament i el manteniment d'aquest tipus de dolor crònic i les comorbiditats associades és crucial per desenvolupar noves alternatives terapèutiques. La present tesis està centrada en el paper del receptor sigma-1 (σ1R), una xaperona expressada en àrees clau pel control del dolor, modulant el dolor osteoartrític i la tolerància a l'analgèsia opioide. Utilitzant un model de dolor osteoartrític en ratolí, hem demostrat que el bloqueig farmacològic del σ1R produeix efectes aguts i persistents inhibint el dolor osteoartrític i les seves manifestacions cognitives i emocionals. A més, l'antagonista del σ1R restaura l'antinocicepció induïda per la morfina en individus tolerants als opioides, essent llavors una estratègia terapèutica apropiada per el control multimodal del dolor crònic. Hem observat que l'antagonista del σ1R modula una via neurobiològica comú a la osteoartritis i a la tolerància opioide, la qual implica l'activitat del receptor opioide μ, mediadors neuroinflamatoris i senyalització glutamatèrgica. La rellevància d'aquesta via queda emfatitzada per la

identificació d'un prometedor tractament, basat en el bloqueig del σ1R i la simultània estimulació del receptor opioide μ, que alleugereix el dolor osteoartrític sense induir tolerància. En general, hem combinat tècniques comportamentals, bioquímiques i electrofisiológiques per avançar en la comprensió del paper del σ1R en diferents manifestacions de la osteoartritis, i hem identificat els antagonistes σ1R com agents terapèutics eficients per inhibir el dolor osteoartrític crònic i els efectes secundaris perjudicials dels medicaments opioides.

Abbreviations

ACC: anterior cingulate cortex

AMPA: α-amino-3-hydroxy 5-methyl-4-isoxazeloproprionic acid

ATF-3: activating transcription factor

cAMP: cyclic adenosine monophosphate

CFA: Complete Freund's adjuvant

CGRP: calcitonin gene-related peptide

CNS: central nervous system

CREB: cAMP response element binding protein

DHEA: dehydroepiandrosterone

DMT: N,N-Dimethyltryptamine

DNIC: diffuse noxious inhibitory control

DRG: dorsal root ganglia

ER: endoplasmic reticulum

ERK: extracellular-signal regulated kinases

HINT1: histidine triad nucleotide binding protein 1

IASP: International association for the study of pain

IP_3: inositol-1,4,5-triphosphate

KO: Knockout

mGluR: Metabotropic glutamate receptor

MMP: matrix metalloproteinases

MOR: μ-opioid receptor

NMDA: N-methyl-D-aspartate

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NPY: neuropeptide Y

NSAIDs: non-steroidal anti-inflammatory drugs

OARSI: Osteoarthritis research society international

PAG: periaqueductal grey

PFC: prefrontal cortex

PKA: protein kinase A

PKC: protein kinase C

RVM: rostral ventromedial medulla

SSRI: selective serotonin reuptake inhibitors

WDR: wide dynamic range

WT: Wild-type

σ1R: sigma-1 receptor

σ2R: sigma-2 receptor

σR: sigma receptors

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INTRODUCTION

1 What is pain?

"*Pain is whatever the experiencing person says it is, existing whenever the experiencing person says it does*" (McCaffery and Beebe, 1989). Although this definition highlights that pain is always a subjective experience, it ignores that the inability to verbally communicate a feeling does not negate the possibility that an individual is experiencing pain being in need of relief. The most widely accepted definition of pain was developed by the International Association for the Study of Pain (IASP): "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*" (Merskey and Bogduk, 1994). A painful experience is more than just a nociceptive response to tissue damage and integrates different behaviours, sensations and thoughts that finally construct the symptom of pain. Therefore, pain can be constituted by two principal components (Baños *et al.*, 2006). First, the **nociceptive** or **sensorial** component, which is the consequence of painful stimuli transmission from nerves to the brain cortex and it provides information about the location, duration, modality and intensity of the stimuli. Second the **emotional** component, which comprises the unpleasant character of pain perception that can seriously differ depending on the cause, the moment and the memory of previous experiences of the patient.

1.1 Classification of pain

Pain has been classified in many ways considering, among others, the intensity (mild, moderate, severe), the localization (cervical, spinal, visceral), the association to disease (rheumatic, cancer, diabetic

neuropathy), the duration (acute or chronic) and the categorization based in pathophysiological mechanisms (nociceptive, inflammatory and neuropathic) (Figure 1) (Cervero and Laird, 1991; Woolf, 2010).

1.1.1 From acute to chronic pain

Acute pain is an immediate, short-lasting response to an identifiable event such as a noxious stimulus or tissue trauma. It has a biological function and it resolves with the healing of the underlying injury. By contrast, **chronic pain** persists beyond the course of an acute disease or after tissue healing is complete, it serves no biological purpose and it is not considered a symptom but rather a disease of its own (Woolf, 2010). The main distinction between these types of pain results from its specific time course. However chronic pain is not simply a temporal extension of acute pain but involves distinct pathophysiological mechanisms (Aliaga, 2002; Kuner and Flor, 2017). Current theories propose that prolonged exposure to acute pain may progress into chronic by involving functional plasticity and structural reorganization at different anatomical levels of the nociceptive pathway (Voscopoulos and Lema, 2010; Feizerfan and Sheh, 2014; Kuner and Flor, 2017).

1.1.2 Pathophysiological mechanisms

1.1.2.1 Nociceptive pain

Nociceptive pain is described as pain occurring with a normally functioning somatosensory nervous system in which the perception of pain is proportional to the intensity of the stimulus (Figure 1). It arises when a brief noxious stimulus that induces minimal or no tissue damage activates specialized high-threshold sensory neurons,

warning the organism of potentially harmful events. Nociceptive pain is well localized, transient, and plays a vital role in the normal defence mechanisms by initiating protective reflexes. The essential need of nociceptive pain for survival and wellbeing is illustrated in individuals who suffer congenital insensitivity to pain (Indo, 2001; Cox *et al.*, 2006). As a result of the absence of nociception, they do not engage appropriate protective behaviours, leading to repeated injury and unintentional self-mutilation (Costigan *et al.*, 2009).

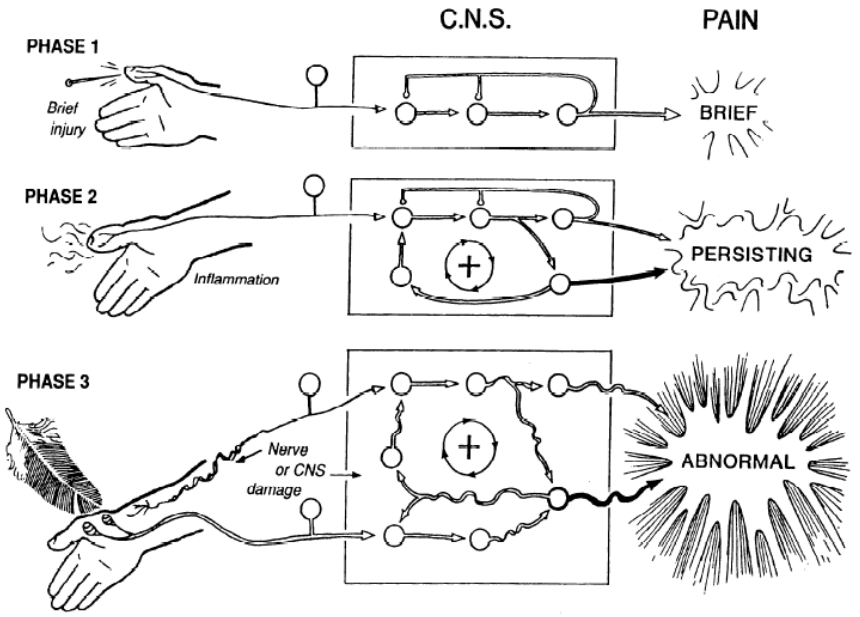


Figure 1. Models of pain processing. The nociceptive system can respond to three different conditions: 1) the processing of brief noxious stimuli, 2) the consequences of prolonged noxious stimulation leading to tissue damage and inflammation, and 3) the consequences of neurological damage, including peripheral neuropathies and central pain states. CNS, central nervous system. (Cervero and Laird, 1991).

1.1.2.2 Inflammatory pain

Inflammatory pain is associated with inflammatory processes and may arise in conditions such as trauma events, infections, or chronic inflammatory diseases (Figure 1). This type of pain is adaptive and protective and assists the healing of the injured body part by creating a situation that discourages physical contact or movement. The inflammatory response induces the release of several mediators that directly activate nociceptive fibres. The sensory nervous system is sensitized and undergoes a profound change in its responsiveness, being activated by low-threshold inputs. As a consequence of peripheral or central sensitization, hyperalgesia and allodynia are present. **Allodynia** is the pain induced by normally innocuous stimuli, whereas **hyperalgesia** is the exaggerated responses to noxious stimuli. Once the process of healing has finished, pain usually disappears, although in some cases it may persist leading to chronic pain losing its physiological purpose.

1.1.2.3 Neuropathic pain

Neuropathic pain is described as pain caused by a lesion or disease of the somatosensory nervous system, either peripheral or central (Figure 1). This type of pain is mostly chronic and can be extremely severe and disabling for the individual, who suffers a persistent, diffuse sensation with no specific location. It is not protective, but maladaptive, resulting from abnormal functioning of the nervous system. It is characterized by the existence of spontaneous and abnormal stimulus-evoked pain (allodynia and hyperalgesia), and the

relationship between the intensity of the stimulus and the painful response is almost completely lost.

1.2 Pain transmission, from the periphery to the brain

The journey between the initial exposure to a noxious stimulus and the conscious appreciation of pain is a complex series of mechanisms whereby the noxious stimulus is encoded as a nociceptive message in the periphery and is progressively transmitted to higher nervous centres, where it is processed (Millan, 1999).

1.2.1 Peripheral mechanisms

Thermal, mechanical, or chemical noxious stimuli are detected by a subpopulation of peripheral nerve fibres called **nociceptors**. As all first-order afferent neurons, the cell bodies of nociceptors are located in the dorsal root ganglia (DRG) (stimulus from the body) or the trigeminal ganglion (stimulus from the face). They have a peripheral axonal branch innervating the target organ, and a central axon making synapses with second-order neurons in the dorsal horn of the spinal cord or the trigeminal nucleus caudalis (Basbaum *et al.*, 2009; Dubin and Patapoutian, 2010).

Taking into account the myelinization, the diameter and the conduction speed, the primary sensory fibres are classified in four main groups:

- **Aα fibres:** large-diameter (13 – 20 µm), fast (80 – 120 m/sec) and myelinated fibres which conduct proprioception (sense of the relative position).

- **Aβ fibres:** Large, myelinated (6 – 12 μm) and fast driving (35 – 75 m/sec) fibres that mainly respond to innocuous stimuli such as vibration and light touch.
- **Aδ fibres:** Myelinated, medium (1 – 5 μm) and fast driving (5 – 30 m/sec) fibres. They are responsible for the acute, well-localized "first" or fast pain, upon the first adaptive response to pain (withdrawal) (Basbaum *et al.*, 2009). Electrophysiological studies have further subdivided these fibres into two categories: type I and type II fibres, which mediate the acute first pain to mechanical stimuli or to noxious heat, respectively (Giordano, 2005).
- **C fibres:** Unmyelinated, small diameter (0.2 – 1.5 μm) fibres with slow conduction (0.5 – 2 m/sec) that are typically associated with the transmission of poorly localized, diffuse, slow pain. Most C fibres are polymodal, thus responding to thermal, mechanical and chemical stimuli (Dubin and Patapoutian, 2010). A large group of C fibres are so-called "silent nociceptors", which are heat-responsive but mechanical-insensitive, and only become sensitized in the course of pathophysiological processes. In terms of neurochemistry, C nociceptors can be subdivided into peptidergic or non-peptidergic, regarding the neuroactive substances they synthesize and release (Snider and McMahon, 1998).

Under **physiological conditions**, Aδ and C fibres are responsible for nociceptive transmission, whereas Aβ fibres conduct low-threshold mechanosensitivity without eliciting pain sensation (Figure 2). After a peripheral noxious stimulation, Aδ fibres are activated and transmit the immediate acute pain, which is followed by a diffuse pain conducted by activated C fibres. However, in **sensitizing conditions**, Aβ fibres can also evoke nociceptive responses (Figure 3). Tissue damage is often accompanied by the accumulation of endogenous factors from activated nociceptors or non-neural cells within the injured area (McMahon and Bevan, 2005). These factors are referred to as the "inflammatory soup", which contains a wide array of signalling molecules, including neurotransmitters, peptides, cytokines, and chemokines, among others. These inflammatory mediators are involved in the development of **peripheral sensitization** (Schaible, 2007), provoking enormous changes in the excitability of nociceptors and amplifying the signal transduction transmitted to the spinal cord (Scholz and Woolf, 2002; Gold and Gebhart, 2010).

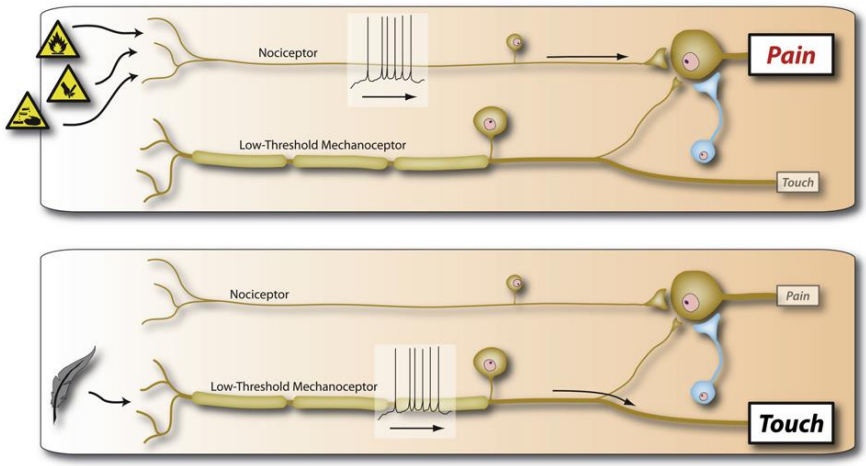


Figure 2. Normal sensation. Under physiological conditions, the highly specialized primary sensory neurons that encode low-intensity stimuli only activate the central pathways that lead to innocuous sensations, while high-intensity stimuli activating nociceptors lead to pain (Woolf, 2011).

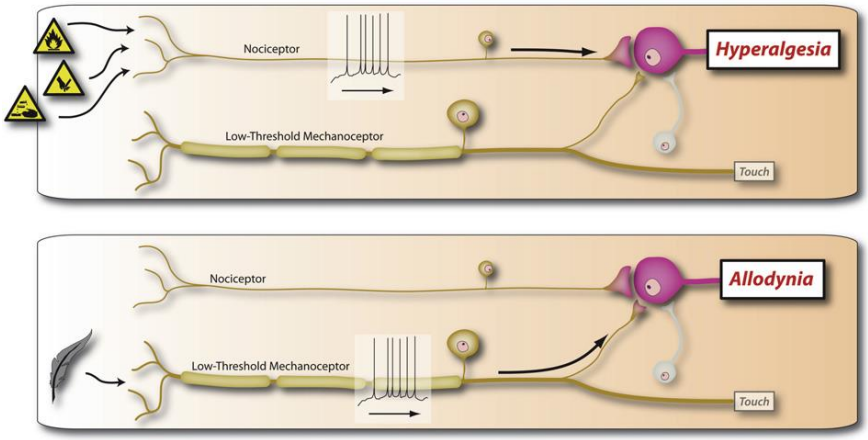


Figure 3. Sensitization of the pain pathways. Abnormal functioning of the nervous system leads to central amplification, thus enhancing the pain response to noxious stimuli in amplitude, duration and spatial extent, while the strengthening of normally ineffective synapses recruits subliminal inputs such that low threshold stimuli can now activate the pain circuit (Woolf, 2011).

1.2.2 Central mechanisms

1.2.2.1 Sensory transmission in the spinal cord

The central terminals of primary afferent fibres end in the dorsal horn of the spinal cord, which is organized into different **laminae**. Most nociceptive Aδ and C fibres project superficially to laminae I and II, with a smaller number reaching deeper layers (laminae V). By contrast, low-threshold Aβ fibres predominantly innervate laminae III, IV and V (Figure 4) (D'Mello and Dickenson, 2008; Basbaum *et al.*, 2009).

The incoming stimuli to the spinal cord activate **second order neurons**, which can be distinguished in three types depending on their specific synaptic inputs (Coghill *et al.*, 1993; Schaible and Grubb, 1993; Calvino and Grilo, 2006):

- **Nociceptive specific neurons:** They are mostly found in the superficial dorsal horn (laminae I and II) and synapse with Aδ and C fibres. They respond exclusively to noxious stimuli and are involved in the encoding of pain location, as they have restricted receptive fields.
- **Wide dynamic range (WDR) neurons:** These neurons are predominantly located in the deep dorsal horn (laminae V and VI), though they are also found in superficial layers. They receive a convergent non-noxious and noxious input via direct Aδ and Aβ fibres and indirect C fibre inputs, thus responding to a broad range of stimulation, from light touch to noxious pinch, chemicals and heat. WDRs fire action potentials in a graded manner depending on stimulus intensity, and exhibit "wind-up", a short-

lasting form of synaptic plasticity (Dubner *et al.*, 1989; Simone *et al.*, 1991).

- **Non-nociceptive neurons:** They respond to proprioceptive and low intensity innocuous stimuli, and are mainly located in laminae I, II, III and IV.

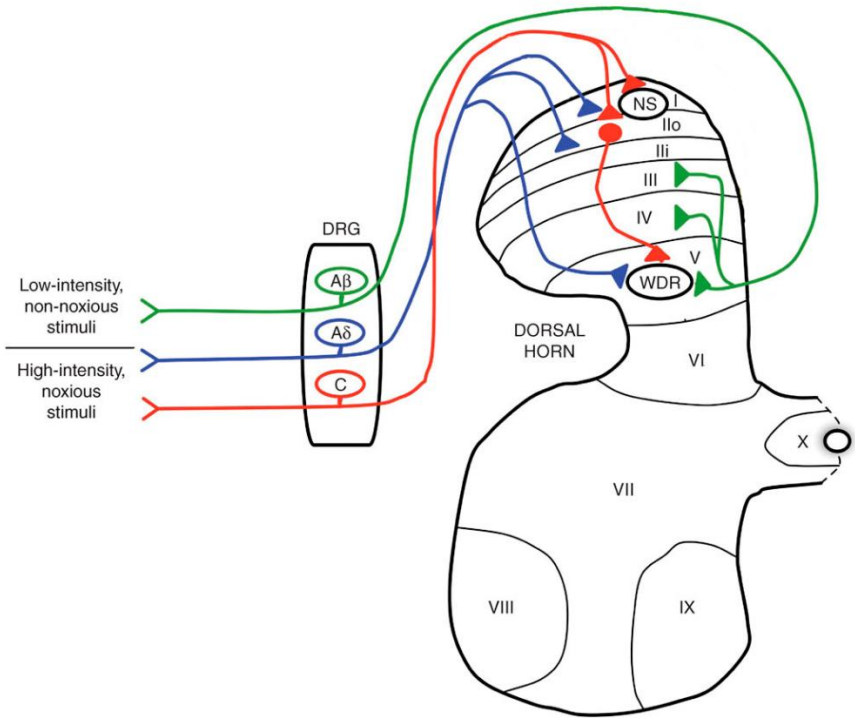


Figure 4. Pain pathways from periphery to the spinal cord. Primary afferent fibres (Aβ, Aδ and C) transmit impulses from the periphery, through the dorsal root ganglia (DRG) and into the dorsal horn of the spinal cord. Nociceptive specific (NS) cells are mainly found in the superficial dorsal horn (laminas I and II) and receive inputs from Aδ and C fibres, whereas most wide dynamic range (WDR) neurons are located deeper (lamina V) and make synapse with all primary afferent fibres. (Adapted from D'Mello and Dickenson, 2008).

The second order neurons, which respond to the peripherally generated signals, are under ongoing control by peripheral inputs, excitatory and inhibitory interneurons, and descending modulation.

Altogether, the responses of NS and WDR cells can increase or decrease, thus influencing the output from the dorsal horn. Noxious stimulation induces the release of the neurotransmitter glutamate and neuromodulators such as substance P, calcitonin gene-related peptide (CGRP) and brain-derived neurotrophic factor (BDNF) from primary afferents. **Glutamate** exerts an excitatory effect post-synaptically, leading to membrane depolarization via α-amino-3-hydroxy 5-methyl-4-isoxazeloproprionic acid (AMPA), kainate, N-methyl-D-aspartate (NMDA), and G-protein coupled metabotropic (mGluR) receptors. Acute pain is signalled by the activation of **AMPA** and kainate receptors, responsible for the initial response of spinal cord neurons. Summation of subthreshold excitatory postsynaptic currents will result in action potential firing and transmission of pain messages to higher-order neurons. Under these conditions, the activation of the **NMDA** receptor (NMDAR) is not possible, since magnesium (Mg^{2+}) ions are blocking the ion channel of the receptor. However, in the context of an injury, when there is a repetitive and high-frequency stimulation of C-fibres, there is enough depolarization to remove the Mg^{2+} and to activate NMDARs. The consequence is an amplification and prolongation of the response of dorsal horn neurons, hence exacerbating responses to noxious stimuli. Besides, excessive glutamate induces the activation of mGluRs (Wang *et al.*, 2012), which has been reported to play a key role in sustaining heightened central excitability in chronic pain, particularly mGluR1 and **mGluR5**, with minimal involvement in acute nociception (Walker *et al.*, 2001; Hudson *et al.*, 2002; Urban *et al.*, 2003). Altogether, the intracellular calcium levels are increased, thus activating downstream

signalling pathways and second messenger systems, notably kinases, which further enhance neuronal excitability and facilitate pain transmission. **Substance P**, **CGRP** and **BDNF** contribute to this activation of intracellular kinases by binding to neurokinin-1, CGRP and tyrosine receptor kinase B receptors, respectively. In **pathological pain**, primary afferent neurons exhibit transcriptional changes in response to inflammatory signals or nerve injury, leading to an over-expression of the listed neuromodulators, which are crucially involved in the generation and maintenance of **central sensitization** (Latremoliere and Woolf, 2009) (Figure 5). Consequently, neurons in the dorsal horn spinal cord may exhibit an increase of spontaneous activity, reduction in the activation thresholds by peripheral stimuli, increased responses to suprathreshold stimulation and/or enlargement of their receptive fields (Latremoliere and Woolf, 2009).

Besides the participation of neuronal modulation, several evidences have shown that non-neuronal cell types, namely **astrocytes** and **microglia**, are also able to influence pain transmission through the dorsal horn of the spinal cord, particularly under pathological neuropathic conditions (Coyle, 1998; Salter and Beggs, 2014). It has been reported a dramatic activation of spinal microglia in several models of chronic pain (Tsuda *et al.*, 2005; Negrete *et al.*, 2017). Activated microglial cells release signalling molecules such as cytokines (**interleukin-1β** (**IL1β**) and **tumor necrosis factor α** (**TNFα**)), **nitrous oxide** and **BDNF**. These neuroinflammatory mediators contribute in turn to the central sensitization by enhancing excitatory and reducing inhibitory currents in the second order neurons, leading

to an enhanced processing of nociceptive information (DeLeo and Yezierski, 2001; Chacur *et al.*, 2009) (Figure 5). Astrocytes also become activated after tissue damage (Garrison *et al.*, 1994; Raghavendra *et al.*, 2004; Hald *et al.*, 2009), with a slower onset and more prolonged time course than microglia, suggesting that they may play a role in the maintenance of chronic pain hypersensitivity (Ji *et al.*, 2006; Zhang and Koninck, 2006).

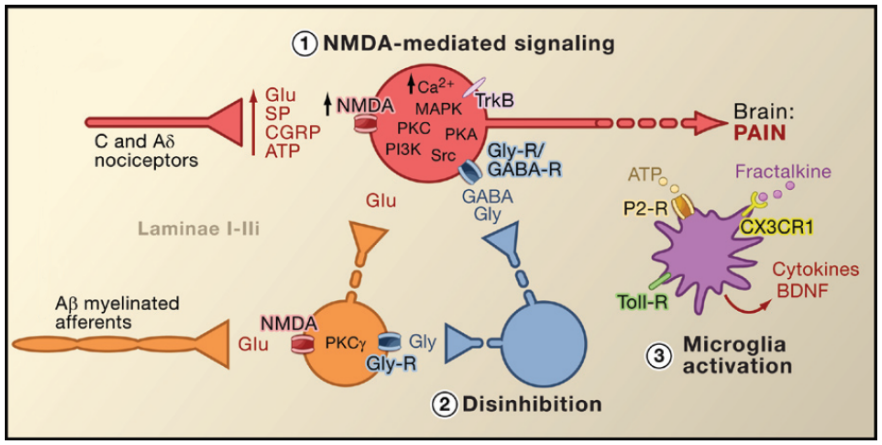


Figure 5. Central sensitization. (1) After intense stimulation or persistent injury, activated C and Aδ nociceptors release a variety of neurotransmitters, including glutamate (Glu), substance P (SP), calcitonin-gene related peptide (CGRP) and ATP, onto output neurons in the dorsal horn (red). As a consequence, NMDA receptors are activated, increasing intracellular calcium and activating several calcium-dependent signalling pathways and second messengers. This cascade of events increases excitability and facilitate the transmission of pain messages to the brain. (2) Under normal circumstances, inhibitory interneurons (blue) continuously release inhibitory neurotransmitters to decrease the excitability of spinal neurons and modulate pain transmission. However, under pathological conditions, this inhibition can be lost, resulting in hyperalgesia. (3) Peripheral nerve injury promotes the activation of microglial cells, resulting in a release of brain-derived neurotrophic factor (BDNF) and pro-inflammatory cytokines. These factors promote increased excitability and enhanced pain in response to both noxious and innocuous stimulation (Basbaum *et al.*, 2009).

1.2.2.2 Ascending pathways

The output from the dorsal horn to higher centres in the brain is carried by spinal projection of second order neurons along ascending pathways. Most neurons from deep laminae (III to VI) and a portion of lamina I neurons contralaterally project to the ventroposterior and ventrobasal thalamus (**spinothalamic tract**), and from there nociceptive information is transmitted to cortical regions forming the "pain matrix" (primary and secondary somatosensory cortices, insular cortex, anterior cingulate cortex (ACC), and prefrontal cortex (PFC)) (Tracey and Mantyh, 2007). On the other hand, neurons from laminae I and II project to medial thalamus, periaqueductal grey (PAG) and mainly to the parabrachial area (**spinoparabrachial tract**). These regions in turn project to brain areas such the hypothalamus and amygdala, which are crucial for the cognitive, emotional and neurovegetative components of chronic pain (Bester *et al.*, 2000; La Porta *et al.*, 2016) (Figure 7). Therefore, the spinothalamic pathway is crucial for the sensory discriminatory aspects of pain, whereas the spinoparabrachial tract plays a central role in the emotional component of the pain experience (Wall *et al.*, 1988; Suzuki and Dickenson, 2005).

1.2.2.3 Central processing

At the supraspinal level, several brain regions are activated by nociceptive inputs and participate in **pain perception**. Brain imaging studies in normal subjects consistently show that the regions most commonly activated by **acute noxious stimulation** are the thalamus,

the somatosensory cortices, the ACC, the insular cortex, the PFC and the amygdala (Apkarian, 2004; Apkarian *et al.*, 2005).

The **thalamus** is crucial for the first conscious perception of pain and is a key relay station for the transmission of nociceptive information from subcortical areas to the cerebral cortex. On the other hand, the **hypothalamus** has a central role in the integration of autonomic and endocrine responses necessary for the homeostasis and adaptation to painful stimuli (De Menezes *et al.*, 2009; Cortelli *et al.*, 2013). **Primary and secondary somatosensory cortices** are important for the perception of sensory characteristics of pain, which include quality, location and duration of the stimulus (Coghill *et al.*, 1999). Impairment of somatosensory cortex function reduces the ability to localize or describe the nature and intensity of painful stimuli, without affecting the perception of an unpleasant feeling (Ploner *et al.*, 1999; Uhelski *et al.*, 2012). The **insular cortex** is related to both the sensory and the cognitive aspects of pain perception (Mesulam and Mufson, 1982; Apkarian *et al.*, 2005), whereas the **ACC** is linked to the cognitive-evaluative processing and the aversiveness of ongoing pain (Sellmeijer *et al.*, 2018). Several studies report that a distraction, a negative emotional state, an alteration of pain expectations or the suggestion of a change in the pain unpleasantness can selectively modulate ACC activity (Rainville *et al.*, 1997; Apkarian *et al.*, 2005; Bushnell *et al.*, 2013). Therefore, activation of ACC correlates with the modified final perception of pain rather than with its actual intensity. The activation of the **PFC** does not correlate with stimulus intensity but with the identification of a stimulus as painful (Coghill *et al.*,

1999). In particular, the **medial PFC** (**mPFC**) is associated with the voluntary control of emotional suffering (Apkarian *et al.*, 2011), and is active in subjects anticipating or expecting pain (Porro *et al.*, 2002) and in patients complaining of pain in the absence of peripheral stimulation (Ohira *et al.*, 2006). Finally, the **amygdala** is critically involved in the emotional-affective dimensions of pain (Veinante *et al.*, 2013). When the amygdala activity is disrupted, noxious stimuli are still detected and discriminated but are devoid of unpleasantness perception and thus do not motivate avoidance (Hebben *et al.*, 1985; Corder *et al.*, 2019).

Although the pattern of brain regions involved in **chronic pain** overlaps with those activated by acute stimulation, there are some significant differences in their activity and their involvement in pain perception (Apkarian, 2011). For instance, the activation of the **somatosensory cortices** is less consistent in patients with chronic pain (Hsieh *et al.*, 1995; Apkarian *et al.*, 2005), pointing to a devaluation of the discrimination of the stimuli in subjects with ongoing pain. Additionally, chronic pain conditions are often associated with decreased baseline activity or stimulus-related activity in the **thalamus** (Iadarola *et al.*, 1995; Gustin *et al.*, 2011, 2014), suggesting that this area may undergo adaptive changes. The correlation between **ACC** activity with perceived pain intensity observed in normal subjects is lost in patients suffering from chronic pain (Silverman *et al.*, 1997; Mertz *et al.*, 2000; Lorenz *et al.*, 2002). On the other hand, the **mPFC** and **amygdala** were found to exhibit a consistent and increased activation in chronic pain conditions

(Apkarian *et al.*, 2005), implying that persistent pain alters the cognitive and emotional perception of everyday experiences. However, caution should be taken when comparing these data, since different clinical pain conditions might have distinct brain activity patterns (Apkarian *et al.*, 2011; Baliki *et al.*, 2011).

Apart from differential brain activity, there is rising evidence for **functional and structural plasticity** in supraspinal structures during chronic pain (Figure 6). Morphological alterations of grey matter volume and density in the brain, variations in cortical representations, changes in dendritic spines, remodelling of structural and functional connectivity between brain areas and reactivation of glial cells are the main changes reported (Boadas-Vaello *et al.*, 2017; Kuner and Flor, 2017).

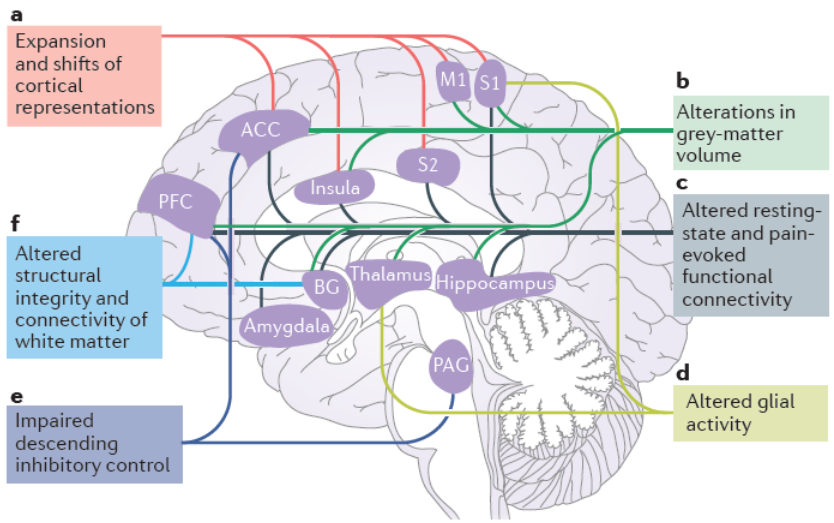


Figure 6. Structural and functional changes in the human brain in chronic pain conditions. ACC, anterior cingulate cortex; BG, basal ganglia; M1, primary motor cortex; PAG, periaqueductal grey; PFC, prefrontal cortex; S1, primary somatosensory cortex; S2, secondary somatosensory cortex (Kuner and Flor, 2017).

1.2.2.4 Descending pathways

The brain has long been known to importantly influence pain sensation by modulating processing of somatosensory information at the spinal level. This descending control of pain underlies changes in pain thresholds as a response to attention, emotions and mood, context and expectations and internal states (Millan, 2002; Chen and Heinricher, 2019). As a result, nociceptive transmission can be exacerbated or attenuated, and the balance between inhibition or facilitation greatly influences the final behavioural outcome, allowing a rapid adaptation to the environmental circumstances. Thus, acute stress and expected pain relief can produce analgesia (stress-induced and placebo analgesia) (Butler and Finn, 2009; Wager and Atlas, 2015), while chronic stress and anxiety can facilitate pain (post-traumatic stress disorders or pain catastrophizing) (Palyo and Beck, 2005; Quartana *et al.*, 2009; Jennings *et al.*, 2014).

Descending control arises from several supraspinal sites, but the best-characterized pathway originates within PAG, which is pivotal in modulating **descending facilitation** or **inhibition** of nociceptive input (Figure 7). Impulses from supraspinal centres are integrated into the midbrain **PAG**, that receive projections from the thalamus, hypothalamus, amygdala and cortical areas such as the PFC, as well as from collaterals of ascending pathways. PAG neurons project downstream to the **rostral ventromedial medulla** (**RVM**), which also receives inputs from the thalamus, the parabrachial area and the locus coeruleus, and is considered the final common relay in descending modulation of pain. OFF- and ON-cells from the RVM

inhibit or facilitate pain perception, respectively, sending outputs to the spinal cord or the trigeminal nucleus caudalis (Fields *et al.*, 2005; Ossipov *et al.*, 2014). However, during **chronic pain**, this adaptative system can be overrun, and instead, there is a marked enhancement in excitability that can result from dysregulation of descending inhibition, increased facilitation or a combination of both (Bingel and Tracey, 2008; Denk *et al.*, 2014). Studies both in human patients (Zambreanu *et al.*, 2005; Mainero *et al.*, 2007) and animal models (Gebhart, 2004; De Felice *et al.*, 2011; Wang *et al.*, 2013) have demonstrated that altered activity in the PAG and the RVM play a key role in the generation and maintenance of central sensitization and hyperalgesia.

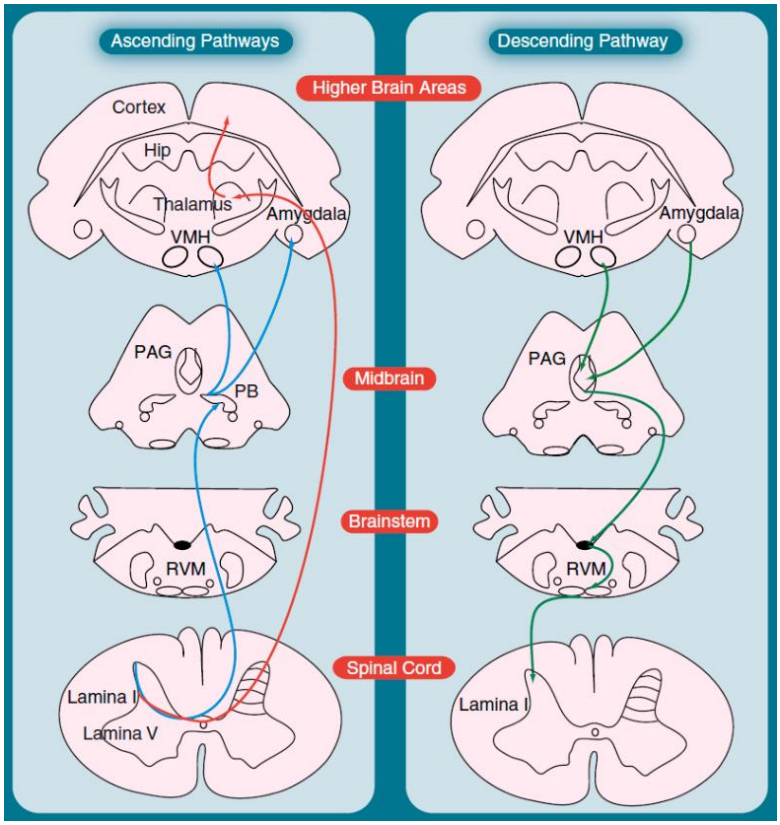


Figure 7. Main ascending and descending pain pathways. The spinoparabrachial tract (blue line) originates from the superficial dorsal horn and feeds areas of the brain concerned with affect. The spinothalamic pathway (red line) distributes nociceptive information to areas of the cortex concerned with both discrimination and affect. The descending pathway highlighted (green line) originates from the amygdala and hypothalamus and terminates in the periaqueductal grey (PAG). Neurons project from here to the lower brainstem and control many of the antinociceptive and autonomic responses that follow noxious stimulation. Hip: Hippocampus; PB: Parabrachial area; RVM: Rostroventral medial medulla; VMH: Ventral medial nucleus of the hypothalamus (Bee and Dickenson, 2007).

2 Osteoarthritis

2.1 Epidemiology

Osteoarthritis is the most common **musculoskeletal disease** and one of the most prevalent chronic diseases worldwide (Gabriel and Michaud, 2009; Neogi and Zhang, 2013; Puig-Junoy and Ruiz Zamora, 2015). Initially, osteoarthritis was regarded as a degenerative condition involving articular cartilage and subchondral bone, but nowadays it is considered a syndrome of the whole joint with many complex aetiologies rather than a single disease entity (Kidd, 2006). Osteoarthritis develops progressively over decades, and it is characterized by cartilage loss, structural and functional deterioration of the synovium, bones and joint tissue, loss of range of motion and pain. All joints of the body can be affected, but the most common are the large weight-bearing joints, such as knees and hip, and small peripheral joints, including the hands (Sofat *et al.*, 2011) (Figure 8). Symptoms can vary from mild to severe joint pain and stiffness, that often lead to the loss of joint function and partial or permanent disability. Importantly, osteoarthritis represents a vast socio-economic cost for the health system burdens, not only limited to the direct costs of healthcare use but also in terms of productivity losses and associated care for patients with osteoarthritis (Puig-Junoy and Ruiz Zamora, 2015).

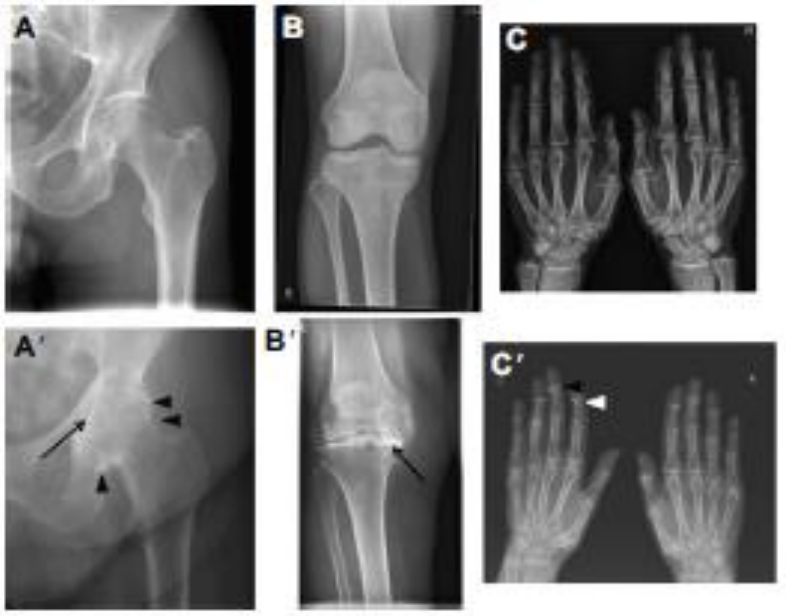


Figure 8. X-ray radiographic images showing structural alterations of the joints most commonly affected by osteoarthritis. Normal (A, B, C) and severely affected joints (A', B', C') of the hip, knee and hand, respectively. Arrows indicate joint space narrowing, and arrowheads indicate the presence of bone outgrowths (Thysen *et al.*, 2015).

Several factors can contribute to osteoarthritis development, such as trauma, ageing, obesity, reduced physical activity, diet and genetic predisposition (Iannone and Lapadula, 2010; Adatia *et al.*, 2012). However, age is the strongest predictor for osteoarthritis, which most commonly affects the middle-aged and elderly, even though younger people may be affected mainly as a result of injury or overuse. Therefore, the **prevalence** of osteoarthritis increases with age, reaching up to 18% of women and 10% of men over the age of 60 in the world population (Maiese, 2016). Importantly, prevalence not only differs depending on age and sex, but also depending on the disease definition used (radiological or clinical), the joint affected, and geographical area (Pereira *et al.*, 2011; Litwic *et al.*, 2013). According

to the Global Burden of Disease study, osteoarthritis could be placed as the 9th cause of disability-adjusted life years in developed countries by the year 2020 (Murray and Lopez, 1997). This rise on the number of cases is probably due to the extended life expectancy and the increased prevalence of risk factors, such as obesity and reduced physical activity (Hunter, 2011; Puig-Junoy and Ruiz Zamora, 2015).

2.2 Physiopathology of osteoarthritis

The joints are specialized structures organized around a cavity that connect the different bones of the skeleton and that allow movement within well-defined ranges and axes (Thysen *et al.*, 2015). Different tissues functionally cooperate within the joint to achieve the required balance between connection and articulation of the skeletal elements. The **articular cartilage** caps the ends of the bones providing a smooth and deformable environment that supports movement. This tissue is composed of articular chondrocytes embedded in a specific extracellular matrix containing type II collagen and proteoglycans, which are responsible for the resistance against tension and the capacity to deform and adapt upon loading. In physiological conditions, chondrocytes have low metabolic activity and a limited regeneration potential, but they maintain the synthesis of proteoglycans. In the deepest zone of the cartilage, a thin layer of chondrocytes calcifies their extracellular matrix to form the interface with the **subchondral bone**, which plays a critical role in stress and load distribution. The joint cavity is surrounded by the **synovium**, a thin connective tissue composed by synovial fibroblasts and tissue-resident macrophages that produces lubricating synovial fluid. The

outer layer of the synovial membrane is well vascularized and represents the source of nourishment for the articular cartilage, which is avascular and aneural. Finally, **ligaments** and the **capsule** provide strength and limit the degree of movement of the whole joint (Firestein *et al.*, 2012; Lories and Luyten, 2012) (Figure 9).

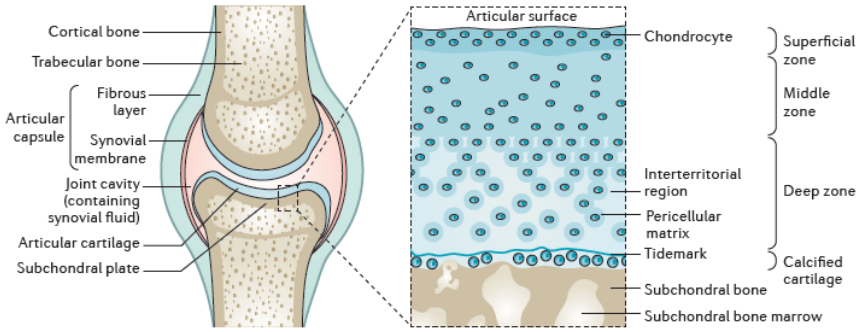


Figure 9. Joint structure. (a) Joint connecting two adjacent bones that are covered by a layer of specialized articular cartilage and are encased in a connective tissue capsule lined by a synovial membrane, consisting of a thin cell layer of macrophages and fibroblasts. (b) Cross-section of the articular surface of a joint illustrating the main structural elements, including the articular cartilage (with chondrocytes), the calcified cartilage, and the subchondral bone (Martel-Pelletier *et al.*, 2016).

Osteoarthritis occurs when there is an imbalance between the breakdown and the repair of joint tissue, thus leading to the disruption of the normal homeostasis of the joint (Lories and Luyten, 2012). Despite the identification of several risk factors, numerous causes may lead to the initiation and progression of osteoarthritis. The first sign of osteoarthritis at the cellular and molecular level appears to be a shift in the quiescent state of the articular chondrocytes. In the early stages of osteoarthritis, chondrocytes exhibit increased synthetic activity and produce additional extracellular matrix molecules, showing attempts to repair (Sofat *et*

al., 2011). At the same time, chondrocytes also produce **pro-inflammatory cytokines** such as interleukin-1 and tissue-destructive enzymes, such as **matrix metalloproteinases** (**MMPs**). In the short term, endogenous protective mechanisms are able to compete with the destructive cascades, but in the long term, they fail to stop the degeneration. Thus, these molecules produce a progressive loss of cartilage with cell death and depletion of the extracellular matrix (Lories and Luyten, 2012). During advanced stages of osteoarthritis, many of the chondrocytes, particularly in the deeper layers of the cartilage, express markers of chondrocyte hypertrophy like collagen type X, and angiogenic factors, contributing to the expansion of cartilage calcification and vascular invasion (Eyre, 2004; Heinegård and Saxne, 2011). Fissures in the superficial layer gradually extend into deeper layers and finally lead to severe destruction and disintegration of cartilage structure and volume. This pathophysiological process will then result in secondary changes to the subchondral bone and other tissues of the joint including synovium, menisci, capsule, tendons and ligaments (Thysen *et al.*, 2015; Martel-Pelletier *et al.*, 2016). Hence, progressive loss of cartilage, remodelling of the subchondral bone, formation of bone outgrowths (osteophytes) at the joint margins, synovial inflammation or damage of the menisci, capsules and tendons are among the processes that characterize the pathophysiology of osteoarthritis and potentially contribute to joint pain and functional impairment (Thysen *et al.*, 2015) (Figure 10).

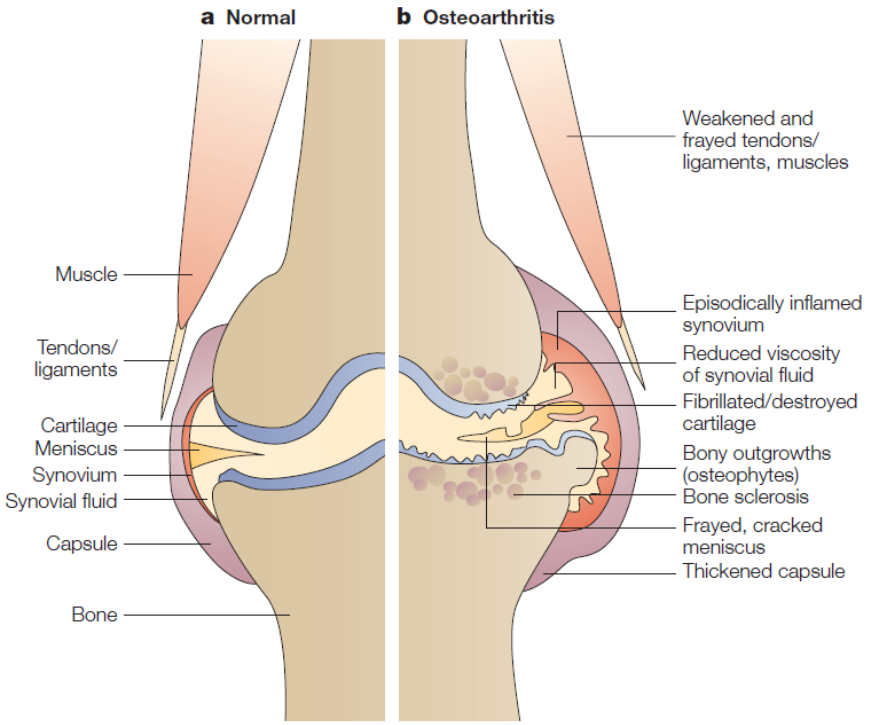


Figure 10. Structural affectations during osteoarthritis. (a) Healthy tissue is shown, with normal cartilage and bone structure, high levels of joint fluid and synovial membrane. (b) Osteoarthritis knee presents remodelling bone and cartilage, outgrowth of osteophytes and altered joint synovial fluid (Wieland *et al.*, 2005).

Currently, there are no sensitive techniques available for osteoarthritis diagnosis beyond classical radiography. Although structural molecules and fragments derived from bone, cartilage and synovium have been suggested as potential **biomarkers** for osteoarthritis (Lotz *et al.*, 2013; Ishijima *et al.*, 2014), the progression of the disease cannot be predicted and, consequently, cannot be prevented or stopped.

2.3 What makes osteoarthritis painful?

Pain is the most predominant symptom of osteoarthritis and is what usually leads those affected to seek medical care. In the early stages of the disease, pain occurs episodically during movement and loading of the joint, but as the disease progresses, it becomes more severe and constant pain at rest may arise (Felson, 2009; Lluch *et al.*, 2014). Several studies have shown the presence of spontaneous pain, allodynia and hyperalgesia in osteoarthritis patients, as well as impaired joint proprioception, loss of cutaneous vibration sensitivity, and hypoaesthesia to punctate mechanical and thermal stimuli (Wylde *et al.*, 2012). However, it is widely recognised that the presence and severity of joint pain correlate poorly with structural evidences of joint damage based on plain x-ray (Lawrence *et al.*, 1966; Dieppe, 2004). Most patients present pain and disability after a significant loss of cartilage has occurred, but it is estimated that up to 40% of individuals showing radiological damage have no pain (Kidd, 2006). Such discordance may rely on the fact that pain perception arises in response to a complex series of neurophysiologic events involving transduction of stimuli, transmission of encoded information, and subsequent modulation of this activity at both peripheral and central levels .

2.3.1 Peripheral mechanisms

Within the joint, sensory nerve fibres have been identified in many anatomic tissues including the periosteum and the subchondral bone, and in soft tissues like ligaments, menisci, and the synovium (Hukkanen *et al.*, 1992; Hirasawa *et al.*, 2000). Joint sensory

innervation is predominantly proprioceptive and nociceptive, indicating that the perception of potentially harmful movements is fundamental for a proper joint function. It has been reported that the 80% of all afferent neurons in the knee joint of rats and cats are nociceptors (McDougall, 2006), whereas in humans a 70-80% of the articular branches of the tibial nerve that innervate the posterior knee capsule are C-fibres and sympathetic nerves (Hines *et al.*, 1996). Although cartilage loss is an important structural alteration associated with osteoarthritis, cartilage is not innervated and thus it cannot be directly linked with the occurrence of pain. Studies in humans showed that the application of noxious mechanical, thermal or chemical stimuli to the fibrous structures such as ligaments and capsule elicited pain (Dye *et al.*, 1998), whereas stimulation of normal synovial tissue rarely evoked pain, and no pain was produced by stimulation of cartilage (Kellgren and Samuel, 1950; Schaible *et al.*, 2009). However, under disease conditions, there is plasticity of the innervation territories, and patients with osteoarthritis have shown innervation of normally aneural tissues with substance P and CGRP-positive nerves (Suri *et al.*, 2007).

Excitation of nociceptors occurs as a result of morphological and/or biochemical alterations related to the local pathophysiology of osteoarthritis. Inflammatory mediators such as bradykinin, prostaglandins, neuropeptides, cytokines and chemokines are released into the joint (Malfait and Schnitzer, 2013). These mediators cause localized tissue damage as well as reduction of the firing threshold of joint nociceptors, making them more likely to respond to

both non-noxious and noxious stimuli (Figure 11). In patients with osteoarthritis, levels of the pro-inflammatory cytokines **IL1β** and **TNFα** are elevated in the synovial fluid, synovial membrane, subchondral bone and cartilage (Kapoor *et al.*, 2011). It is well known that these mediators injected into the joint cavity can directly sensitize afferent neurons and trigger hyperalgesia (Richter *et al.*, 2010; Schaible, 2014). In osteoarthritis, considerable evidence shows cyclooxygenase over-expression in the synovium, bone and surrounding joint tissues (Adatia *et al.*, 2012), as well as in spinal cord neurons (Vardeh *et al.*, 2009). Furthermore, joint inflammation enhances local levels of nerve growth factor, a major contributor to **peripheral sensitization** (Woolf *et al.*, 1994).

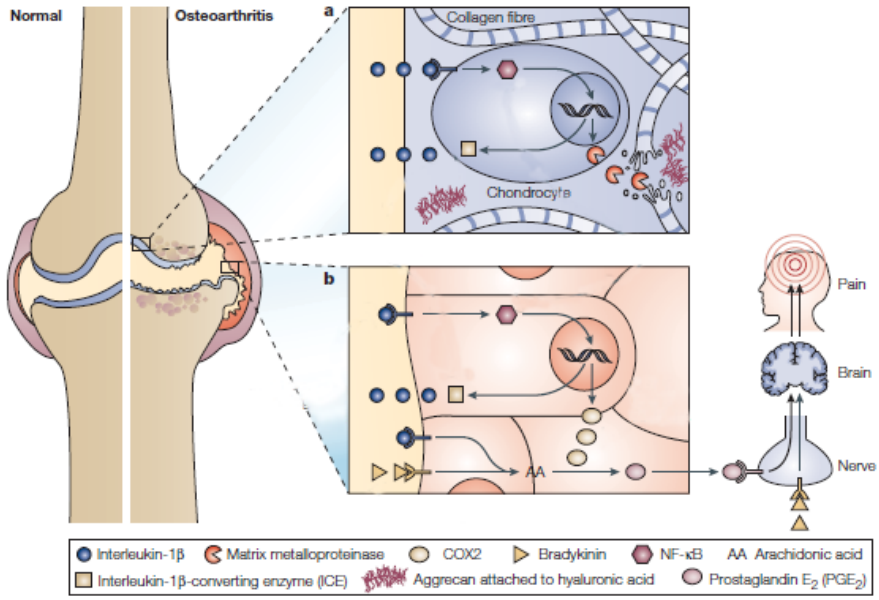


Figure 11. Pro-inflammatory mediators in the joint during osteoarthritis. Increased levels of pro-inflammatory mediators, such as cytokines, prostaglandins or chemokines, among others, contribute to cartilage degradation and inflammation, driving to pain generation. (Adapted from Wieland *et al.*, 2005).

2.3.2 Spinal mechanisms

Preclinical studies in rodent models of osteoarthritis showed increased spinal levels of glutamate, SP and CGRP (Puttfarcken *et al.*, 2010; Ferland *et al.*, 2011), and over-activation of microglial cells (Orita *et al.*, 2011), which facilitate the generation of spinal hyperexcitability. These changes underlie **central sensitization** processes, which are considered to be essential for osteoarthritis pain, as it might contribute to the apparent discordance between pain and structural joint damage. Therefore, nociceptive spinal cord neurons receiving inputs from the joint develop, during osteoarthritis, a state of prolonged hyperexcitability, which leads to enhanced responses to peripheral stimulation and decreased firing thresholds. Furthermore, second order neurons show enlargement of the receptive field and increased responses to stimuli applied to regions distant from the joint (Eva Kosek and Ordeberg, 2000; Bajaj *et al.*, 2001; Suokas *et al.*, 2012), reflecting the convergent inputs to spinal cord neurons from primary afferents of the affected joint and from remote tissues (Thakur *et al.*, 2014). Additionally, it has been reported a higher temporal pain summation score upon repetitive stimulation of the osteoarthritis joint (Arendt-Nielsen *et al.*, 2010; Neogi *et al.*, 2015).

2.3.3 Supraspinal mechanisms

The plastic changes occurring during central sensitization are not restricted to the spinal cord and also involve supraspinal structures (Lluch *et al.*, 2014). Neuroimaging studies of osteoarthritis patients show increased activity in the thalamus, somatosensory cortex,

insular cortex, and ACC after noxious mechanical knee stimulation. Such brain activity was reduced after local treatment with lidocaine (Baliki *et al.*, 2008), suggesting that supraspinal activation mediates pain during osteoarthritis. Moreover, stimulus-evoked brain activity differs from the activation associated with spontaneous pain (Parksl *et al.*, 2011). Patients with osteoarthritis showed enhanced activation of the **PFC** (Apkarian *et al.*, 2009), a decrease in grey matter volume of the **thalamus** (Gwilym *et al.*, 2010), and specific morphological changes in the cortical grey matter (Baliki *et al.*, 2011). Interestingly, brain re-organization in osteoarthritis patients was unique to this condition, reflecting the exclusive maladaptive physiology of different types of chronic pain (Baliki *et al.*, 2011).

2.3.4 Descending modulation

In addition to the ascending modulation, descending pathways also play an important role in the central pathophysiological mechanisms involved in osteoarthritis. **Diffuse noxious inhibitory control** (**DNIC**) is a descending pain modulatory pathway often described as “pain inhibits pain”. It occurs in healthy individuals when the initial pain is inhibited by a noxious stimulus applied to another remote location in the body. In patients with osteoarthritis, as in many individuals suffering chronic pain, DNIC is defective (E Kosek and Ordeberg, 2000; Arendt-Nielsen *et al.*, 2010). Interestingly, this mechanism seems to be restored after successful joint replacement (E Kosek and Ordeberg, 2000; Arendt-Nielsen *et al.*, 2010), providing evidence that chronic peripheral inputs are essential for the maintenance of the central nervous system (CNS) alterations associated with chronic pain.

Furthermore, functional imaging in patients with hip osteoarthritis showed increased activation of the **PAG** during cutaneous stimulation in referred pain areas, suggesting an involvement of this brain region in supraspinal sensitization (Gwilym *et al.*, 2009). Additionally, preclinical studies in rodent models of osteoarthritis also demonstrate the dysregulation of DNIC in late, but not early, phases of the disease (Lockwood *et al.*, 2019), as well as the presence of a continuous serotonergic facilitation from the RVM that modulates low threshold evoked neuronal responses (Rahman *et al.*, 2009).

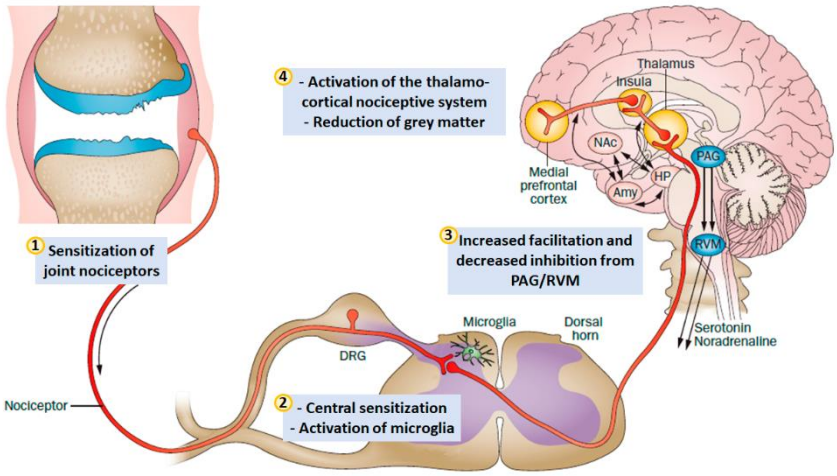


Figure 12. Representation of the pain mechanisms involved in osteoarthritis. (1) Hypersensitive afferent terminals in the dorsal root ganglia (DRG) synapse to second order neurons in the dorsal horn of the spinal cord. (2) Activated microglial cells release inflammatory mediators that contribute to central sensitization and pain processing. (3) PAG-RVM system modulates descending pathways through decreased inhibitory and increased excitatory control. (4) Altered activation of different supraspinal regions involved in both the nociceptive and the emotional components of osteoarthritis. RVM: rostro ventral medulla; PAG: periaqueductal gray; HP: hypothalamus; Amy: amygdala; NAc: nucleus accumbens. (Adapted from Malfait and Schnitzer, 2013).

2.4 Osteoarthritis pain, inflammatory or neuropathic?

In the past years, an increasing number of studies have described sensory abnormalities that accompany osteoarthritis pain (Thakur *et al.*, 2014). It has been estimated a 5 to 50% prevalence of **neuropathic pain** during osteoarthritis (Ohtori *et al.*, 2012; Hochman *et al.*, 2013; Soni *et al.*, 2013), reflecting a great heterogeneity in patients with this chronic pain condition. These patients reported higher pain intensity, more referred pain, pain at more sites and longer osteoarthritis duration than patients with no neuropathic signs.

Structural magnetic resonance imaging of knee is not yet sensitive enough to identify nerve fibre lesions, but samples of synovium from patients undergoing total knee replacement allow direct observation of articular somatosensory alterations. Compared with healthy individuals, osteoarthritis joints showed infiltration of immune cells, increased angiogenesis and increased growth factor expression (Suri *et al.*, 2007). It was also observed a simultaneous loss of innervation in synovial lining together with increased innervation of cartilage and the osteochondral junction (Suri *et al.*, 2007; Walsh *et al.*, 2010; Eitner *et al.*, 2013), demonstrating the occurrence of plasticity in intra-articular somatosensory structures during the course of the disease and further supporting the presence of a neuropathic component in osteoarthritis pain.

These clinical evidences are additionally reinforced by preclinical findings in models of osteoarthritis pain. The monosodium iodoacetate (MIA) rat model has features that are consistent with neuropathy, including upregulation of the neuronal damage marker

activating transcription factor (ATF-3) in peripheral nerves that innervate intra-articular structures, and morphological and proliferative changes of glial cells in the ipsilateral spinal cord (Ivanavicius *et al.*, 2007; Orita *et al.*, 2011; Thakur *et al.*, 2012). Such overexpression of ATF-3 was dependent on the MIA dose used, suggesting a link between nerve damage and the progression and severity of osteoarthritis. Indeed, greater pain behaviours were observed when markers of nerve damage were upregulated, compared with the less severe variant, where nerve damage was absent (Thakur *et al.*, 2012).

2.5 Comorbidities associated with chronic osteoarthritis pain

2.5.1 Emotional manifestations

As joint degeneration progresses, pain in osteoarthritis patients is accompanied by a gradual decrease in functional movements and difficulty in everyday simple tasks, such as walking, climbing stairs and housekeeping. This leads to the loss of functional and autonomous capability, exerting a major negative effect on the quality of life and increasing the risk of developing other medical comorbidities (Smith *et al.*, 2014). Thus, osteoarthritis patients may often suffer sleep disturbances, anxiety, feelings of helplessness and depression (Sharma *et al.*, 2016). Indeed, it has been observed that over 40% of a cohort of patients with lower limb osteoarthritis suffered from clinically significant anxiety and depression (Axford *et al.*, 2010). This percentage is at least 2.5 times greater than the one expected in the general population (Kirmayer *et al.*, 1993; Hirschfeld, 2001). Importantly, the severity of pain reported by patients correlates with

the levels of anxiety and depression. In agreement, other clinical studies show increased prevalence of depression among patients with osteoarthritis (Rosemann *et al.*, 2007; Sale *et al.*, 2008). Interestingly, measures of self-perceived quality of life in osteoarthritis patients correlate better with pain and depression than radiological signs (Goldenberg, 2010). Anxiety and depressive-like behaviours have also been observed in rodent models of osteoarthritis pain (La Porta *et al.*, 2015; Negrete *et al.*, 2017).

2.5.2 Cognitive manifestations

Cognitive processing has also been widely investigated in different chronic pain conditions, which are commonly associated with the impairment of cognitive functions (Moriarty *et al.*, 2011). A broad range of cognitive outputs can be negatively affected during chronic pain, including attention, concentration, psychomotor activity, executive function, decision-making or memory (Liu and Chen, 2014). Cognitive deficits have been found in osteoarthritis patients (Tassain *et al.*, 2003; Karp *et al.*, 2006; La Porta *et al.*, 2015) and in rodent models of osteoarthritis (La Porta *et al.*, 2015; Negrete *et al.*, 2017).

2.5.3 Reciprocity between pain and emotional and cognitive impairments

The causal relationship between pain and emotional and cognitive alterations is difficult to establish because experiencing pain contributes to a negative affective and cognitive state and, in turn, this negative state magnifies and worsens pain perception (Bushnell *et al.*, 2013) (Figure 13).

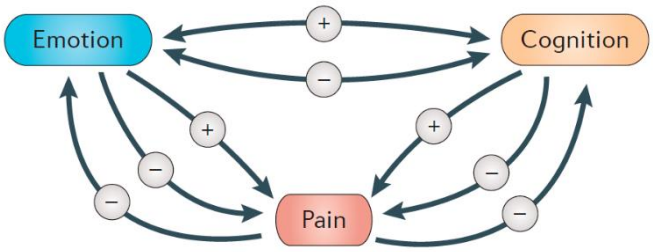


Figure 13. Feedback loop between pain, emotions and cognition. Pain can have negative effects on emotions and on cognitive function. Conversely, a negative emotional state can lead to increased pain, whereas positive state can reduce it. Similarly, cognitive states such as attention and memory can either increase or decrease pain. The minus sign refers to a negative effect and the plus sign refers to a positive effect (Bushnell *et al.*, 2013).

It has been well documented that emotional and cognitive traits are important factors to modulate pain perception (Rhudy *et al.*, 2008; Villemure and Bushnell, 2009; Wiech, 2016). Clinical studies revealed that patients with high anxiety sensitivity or anxiety disorders displayed amplified pain intensity (Keogh and Mansoor, 2001; Defrin *et al.*, 2008). Conversely, social support has been associated with lower pain intensity in response to experimental stimuli and in chronic pain conditions (Montoya *et al.*, 2004). The influence of depression modulating pain perception is still not conclusive, since both pain-attenuating and enhancing effects of depressive disorders have been reported (Chiu *et al.*, 2005; Bär *et al.*, 2006; Schwier *et al.*, 2010).

There are now substantial evidences suggesting that patients with chronic pain may have anatomical alterations within regions involved in cognitive and emotional modulation of pain, such as the dorsolateral and medial PFC, the ACC and the insular cortex. There is less grey matter in these brain areas of patients with several chronic

pain disorders such as back pain, fibromyalgia, complex regional pain syndrome and osteoarthritis (Davis and Moayedi, 2013). It has been suggested that excessive nociceptive input impairs the function and structure of grey matter, including neuronal loss related to excitotoxicity (Bushnell *et al.*, 2013). Studies supporting this idea show increased levels of glutamate and decreased neuronal marker N-acetyl aspartate in frontal cortices of patients with chronic back pain and fibromyalgia (Grachev *et al.*, 2000, 2002; Harris *et al.*, 2009). Furthermore, patients with joint pain have shown a significant correlation between depression and mPFC activation (Schweinhardt *et al.*, 2008). Additionally, studies in rodents also suggest that chronic pain can cause supraspinal neuroinflammatory responses (Apkarian *et al.*, 2006; Norman *et al.*, 2010) with alterations of glial cells and pro- and anti-inflammatory cytokines, in addition to an imbalance of inhibitory and excitatory neurotransmission (Figure 14) (Humo *et al.*, 2019). Changes in dendritic and synaptic structure and function in regions involved in pain processing (Xu *et al.*, 2008; Metz *et al.*, 2009) have also been reported. Such anatomical alterations are temporally coincident with emotional impairments, as shown in a rodent model of neuropathic pain. Indeed, months after the injury and the onset of hypersensitivity, rats exhibited anxiety-like behaviour and attentional deficits together with a reduced volume of PFC (Seminowicz *et al.*, 2009; Low *et al.*, 2012).

In summary, it has been widely demonstrated that chronic pain is detrimental to the brain, and long-term pain itself can decrease the endogenous ability to control pain and lead to many of the

comorbidities that affect individuals with persistent pain. Importantly, patients with chronic pain and emotional comorbidities exhibit worse prognosis and poorer treatment responses than those with chronic pain alone (Holmes *et al.*, 2013; Sheng *et al.*, 2017). Therefore, further studies are vastly needed to better characterize these pain manifestations and its influence on pain perception and to investigate novel therapeutic approaches that simultaneously control the nociceptive, affective and cognitive manifestations of osteoarthritis pain.

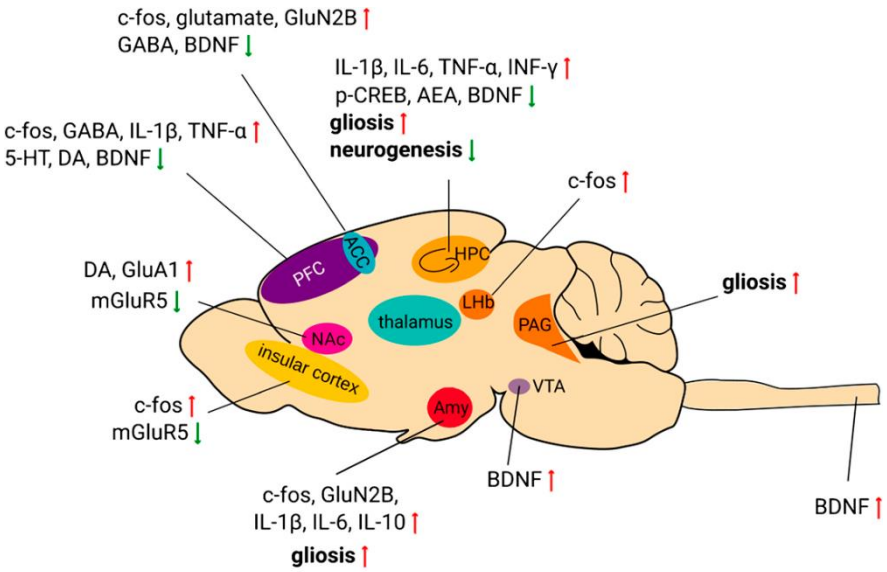


Figure 14. Summary of molecular alterations involved in preclinical rodent models of comorbid chronic pain and anxiodepressive-like behaviours. Red up-arrows, increased levels; green down-arrows, decreased levels; 5-HT, serotonin; ACC, anterior cingulate cortex; AEA, anandamide; AMY, amygdala; BDNF, brain-derived neurotrophic factor; DA, dopamine; GABA, gamma-aminobutyric acid; GluA1, AMPA receptor subunit; GluN2B, NMDAR 2B; HPC, hippocampus; IL-6, interleukin 6; IL-10, interleukin 10; IL-1β, interleukin 1 beta; INF-γ, interferon-gamma; LHb, lateral habenula; mGluR5, metabotropic glutamate receptor 5; NAc, nucleus accumbens; PAG, periaqueductal gray; p-CREB, phospho c-AMP-response element-binding; PFC, prefrontal cortex; TNF-α, tumor necrosis factor-alpha; VTA, ventral tegmental area. (Adapted from Humo *et al.*, 2019).

2.6 Current treatment strategies for osteoarthritis

Clinical management of osteoarthritis has been described in guidelines from musculoskeletal organizations based on results from existing clinical trials and expert opinions. According to the **Osteoarthritis Research Society International** (**OARSI**), treatments are directed towards reducing joint pain and stiffness, maintaining and improving joint mobility, limiting the progression of joint damage, reducing physical disability, improving health-related quality of life and educating patients about the nature of the disorder and its management (Zhang *et al.*, 2007; Mcalindon *et al.*, 2014). Clinical management of osteoarthritis is mainly symptomatic and includes a limited combination of pharmacological and non-pharmacological approaches to reduce pain (Figure 15).

2.6.1 Non-pharmacological management of osteoarthritis

Non-pharmacological interventions are greatly recommended for individuals with osteoarthritis and are usually used as a complementary strategy to pharmacological treatments (Fernandes *et al.*, 2013). It has been widely proposed a multidisciplinary combination of education, self-management, exercise and physical therapy, weight loss, walking aids and regular reassessment (Sarzi-Puttini *et al.*, 2005). Muscle weakness plays a major part in the development of disability, thus muscle strengthening through **exercise** is especially effective at reducing pain (Ruhdorfer *et al.*, 2016). Furthermore, since **weight loss** is positively correlated with improvement in knee osteoarthritis symptoms (Atukorala *et al.*, 2016), weight reduction is crucial to diminish pain and recover

function and might also be associated with reduced progression of structural damage (Felson *et al.*, 1992; Messier *et al.*, 2013).

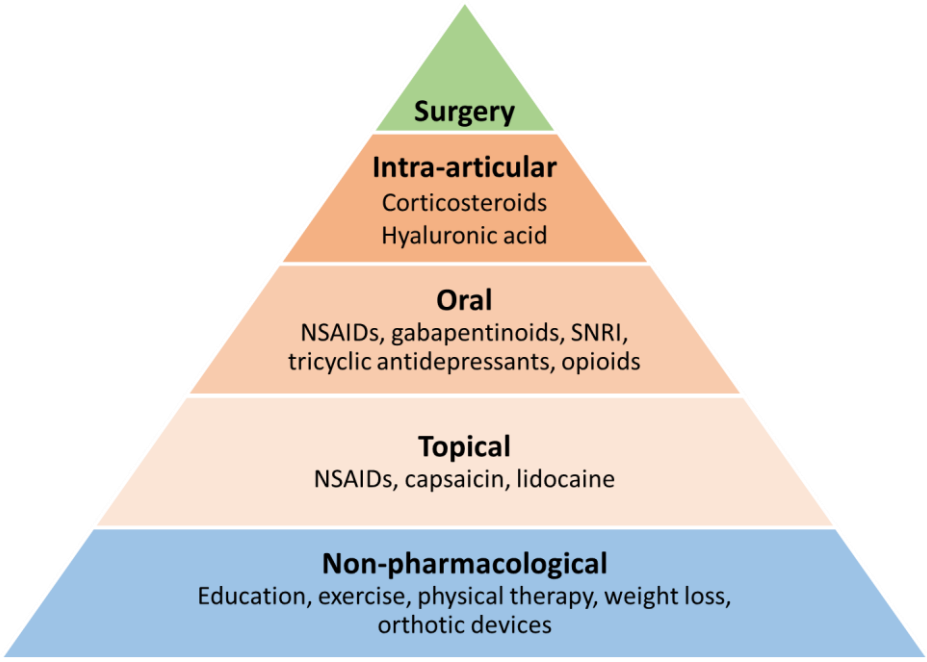


Figure 15. Recommended stepped-care approach for the treatment of osteoarthritis. In addition to non-pharmacological measures such as weight loss and physical exercise (blue), international guidelines include symptomatic treatments with topical, oral or intra-articular analgesics (orange). Because no approved drugs exist that prevent or halt osteoarthritic joint destruction, the ultimate measure is joint replacement. NSAID: non-steroidal anti-inflammatory drugs; SNRI: serotonin-noradrenalin reuptake inhibitors.

2.6.2 Topical treatments for osteoarthritis

First-line **pharmacological** therapies include **topical drugs**, which have better safety profile than orally-administered drugs but are limited by joint penetration and multiple daily applications. Topical **non-steroidal anti-inflammatory drugs** (**NSAIDs**) were found to be effective relieving osteoarthritis-related pain, but no evidence supports their long-term use (Lin *et al.*, 2004). Topical **lidocaine** also

resulted in significant improvements in pain intensity, physical function and stiffness (Burch *et al.*, 2004). On the other hand, the usefulness of topical **capsaicin** in osteoarthritis is controversial. This chilli pepper extract was effective treating mild-to-moderate osteoarthritis pain, although a large number of patients reported local adverse effects (Kosuwon *et al.*, 2010). Topical application of these compounds is generally used for hand and knee osteoarthritis in combination with systemic agents to obtain a localized pain relief (Zhang *et al.*, 2010).

2.6.3 Oral medication for osteoarthritis

Oral NSAIDs have effects relieving pain and increasing mobility for approximately 60% of patients with osteoarthritis (Lee *et al.*, 2004). However, NSAIDs present a short-term efficacy and are associated with gastrointestinal, kidney and cardiovascular complications, especially in patients of advanced age and comorbidities (Tonge *et al.*, 2014). Patients who cannot tolerate or should not be exposed to NSAIDs and continue to have severe pain may be considered candidates for other therapeutic options like opioids (see sub-heading 2.6.3.1) or **non-standard analgesics**. Among these, **gabapentinoids**, such as pregabalin, have demonstrated efficacy in animal models of osteoarthritis (Rahman *et al.*, 2009; Vonsy *et al.*, 2009) and in a recent clinical trial of patients with hand osteoarthritis (Sofat *et al.*, 2017). However, the pregabalin-treated group showed adverse events, the most common of which were mental disturbance, headaches, weight gain, sleepiness and dizziness, and the treatment did not improve the depression or anxiety associated to chronic pain (Sofat *et al.*, 2017).

Moreover, the **serotonin-noradrenalin reuptake inhibitor** duloxetine also showed a moderate analgesic effect in osteoarthritic rats (Chandran *et al.*, 2009), and it reduced pain in individuals with osteoarthritis (Chappell *et al.*, 2009, 2011; Micca *et al.*, 2013). This drug has been approved in the USA, but not in Europe, for the treatment of musculoskeletal pain. Importantly, osteoarthritis patients presenting neuropathic pain characteristics are more likely to respond to non-standard analgesics than to NSAIDs (Thakur *et al.*, 2014). In agreement, tricyclic antidepressants and gabapentin remained efficacious relieving pain in rat models of osteoarthritis, whereas NSAIDs only maintained its analgesic effect during the first two weeks after pain induction, but it showed a vast reduction of effectiveness beyond this time point (Fernihough *et al.*, 2004; Ivanavicius *et al.*, 2007; Rashid *et al.*, 2013). For osteoarthritis patients with neuropathic features, combination therapies might be a promising option. Indeed, the combination of an NSAID and pregabalin reported significantly greater analgesia than the single administration of the NSAID or pregabalin in patients with knee osteoarthritis (Ohtori *et al.*, 2013). All these non-conventional analgesics may have a beneficial effect on pain, but the adverse effects linked with many of these centrally acting drugs, such as nausea, headaches, somnolence or dry mouth, would limit their clinical use.

2.6.3.1 Opioid treatments for chronic osteoarthritis pain: analgesia and tolerance

Opioid drugs usually show affinity for more than one opioid receptor subtype (μ, δ, and κ) and the activation of all subtypes leads to **analgesia**. However, the great majority of opioid analgesic drugs used in the clinical practice predominantly induce analgesia by activating the **μ-opioid receptor** (**MOR**), such as morphine, oxycodone or fentanyl (Kieffer, 1999; Pasternak and Pan, 2011, 2013). MOR is a member of the G protein-coupled receptor superfamily, with 7 transmembrane helices and the N- and C-terminus facing at the extracellular and the intracellular sides, respectively (Connor and Christie, 1999; Serohijos *et al.*, 2011). Once the opioid agonist binds MOR, the inhibitory α subunit of the G protein (Giα) dissociates from the Gβγ subunit. Thus, the activated Giα inhibits the activity of the adenylate cyclase with the consequent decrease in cyclic adenosine monophosphate (cAMP) production and thereby protein kinase A (PKA) functioning. At the same time, the Gβγ subunit, which is still anchored to the membrane, inhibits the activity of voltage-dependent calcium channels while opening inwardly rectifying potassium channels. Altogether, these mechanisms contribute to the hyperpolarization of the cells stimulated by MOR agonists, therefore decreasing the neuronal activity (Al-Hasani and Bruchas, 2011; Raehal *et al.*, 2011) (Figure 16).

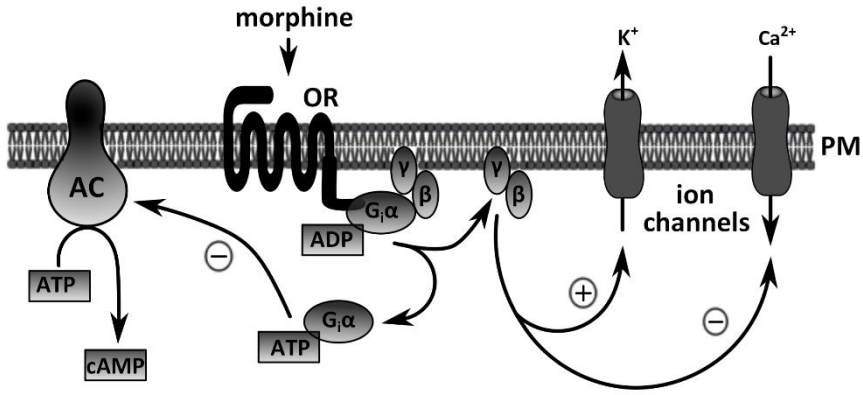


Figure 16. Schematic representation of mu-opioid signalling. Activation of the opioid receptor (OR) separates Giα and Gβγ subunits. Giα subunit inhibits the adenylate cyclase (AC), which results in reduced formation of cyclic adenosine monophosphate (cAMP). Concurrently, Gβγ subunits activate and inhibit de potassium (K^+) and calcium (Ca^{2+}) channels, respectively, leading to cell hyperpolarization. Altogether, the activation of the mu-opioid receptor leads to the reduction of neuron excitability. ADP, adenosine diphosphate; ATP, adenosine triphosphate; PM, plasma membrane (Skrabalova *et al.*, 2013).

Along with their analgesic effects, opioids have the potential to produce important **side effects**. Opioid-induced adverse events occur frequently and reduce the quality of life of patients, and they are often the cause of treatment discontinuation (Cherny *et al.*, 2001; Al-Hasani and Bruchas, 2011). Therefore, its management is still nowadays a major clinical challenge. Side effects caused by opioid action at central level include nausea and vomiting, sedation, decrease of respiration rate, increase of urinary retention or miosis (reduction of pupil size). On the other hand, decreased gastric motility and intestinal secretion, increased gastroesophageal reflux and reduced blood pressure and heart rate are some the opioid adverse effects primarily induced in the periphery (Benyamin *et al.*, 2008; Khademi *et al.*, 2016).

After repeated opioid treatment, **tolerance** to its analgesic effect can be developed, leading to the need of increased doses to maintain the same level of analgesia (Ballantyne and Mao, 2003; Raehal *et al.*, 2011). Importantly, tolerance to other non-analgesic effects of opioids, such as constipation or nausea, is minimally developed (Dumas and Pollack, 2008). An extensive variety of different and unrelated mechanisms influence on tolerance, indicating that tolerance involves the convergence of many pathways to a common behavioural response (Pasternak and Pan, 2013). The phosphorylation-dependent desensitization by different kinases is considered the predominant mechanism mediating the attenuation of opioid receptor signalling (Marie *et al.*, 2006). When MORs are phosphorylated, they are unable to associate to G proteins and, consequently, to respond to further agonist stimulation. Afterwards, they can be internalized for its subsequent recycling and resensitization. Interestingly, this is not equally applicable to all MOR agonists, since morphine provokes little or no internalization (Connor *et al.*, 2004). It has been proposed that the regulation of opioid receptors by endocytosis and recycling serves a protective role in reducing the development of tolerance. Indeed, MOR agonists like DAMGO, which promotes efficient endocytosis of the receptors, produce only weak tolerance, whereas morphine and other MOR agonists that very weakly induce endocytosis produce a high degree of tolerance (Finn and Whistler, 2001). In addition to changes in the opioid receptor, prolonged treatment with opioids also promotes cellular adaptations that oppose the effects of MOR activation. Following chronic morphine treatment, enhanced levels of cAMP have

been reported, as well as increased release of excitatory modulators including glutamate, CGRP or substance P from nociceptive primary afferents within the spinal cord, thus contributing to analgesic tolerance (Waldhoer *et al.*, 2004). In addition, it has been proposed that tolerance may also be the result of the activation of anti-opioid systems, including neuropeptides, such as nociceptine, and the NMDAR signalling (Ueda, 2004; Garzón *et al.*, 2008).

During osteoarthritis, **opioids** might be the only option for patients in whom NSAIDs are contraindicated, ineffective and/or poorly tolerated (Jordan *et al.*, 2003). The American College of Rheumatology strongly recommends opioid analgesics in patients who are not willing or had contraindications to undergo a total joint replacement after having failed medical therapy (Hochberg *et al.*, 2012). However, concerns about potential opioid misuse or abuse and harm persist, and its use for osteoarthritis-related pain remains controversial (Spitz *et al.*, 2011). In a meta-analysis of 40 studies examining opioids in the treatment of chronic non-cancer pain in older adults, where 70% of enrolled patients were suffering from osteoarthritis, opioids showed efficacy reducing pain and physical disability, but not improving quality of life (Papaleontiou *et al.*, 2010). Common adverse events comprised constipation, nausea and dizziness, and provoked opioid discontinuation in 25% of cases. This is a significant percentage, especially considering that the mean duration of treatment studies was 4 weeks, a markedly shorter duration than the one required to treat chronic pain conditions. Furthermore, a 2009 study, updated in 2014, concluded that the small to moderate beneficial effects of

opioids in hip or knee osteoarthritis pain are outweighed by large increases in the risk of adverse events, thus suggesting that opioids should not be used routinely, even if osteoarthritis pain is severe (Nüesch *et al.*, 2009; da Costa *et al.*, 2014). Clearly, the debate on the use of opioids in osteoarthritis also depends on the duration of their use. However, despite chronic opioid therapy has been defined by daily use for at least 90 days, in the practice they are often used indefinitely (Von Korff *et al.*, 2011). A long-term study (median duration of 8.4 years) evaluating safety and efficacy of opioids in patients with intractable chronic non-cancer pain (20% patients with osteoarthritis) demonstrated that a minority of patients will experience a sustained (>1 year) response with no or tolerable side effects (Watson *et al.*, 2010).

2.6.3.1.1 The opioid crisis

Knowledge of the powerful analgesic effect of opioids is millenary, and it has been used for thousands of years for both recreational and medical purposes (Schiff, 2002). In the **19th century**, tension raised between the wish to take advantage of the medical benefits of opioids and the recognition of the development of abuse and addiction, which may lead to devastating consequences (Booth, 1999). During most of the **20th century**, long-term use of opioids therapy to treat chronic pain was contraindicated by the risk of addiction, increased disability and lack of efficacy over time (Rosenblum *et al.*, 2008). It was not until the late **1980's** that the world health organization addressed the under-treatment of postoperative and cancer pain and proposed an approach for the use

of opioids in palliative care (World Health Organization, 1986), leading to a rise of opioid medical prescriptions among patients suffering from cancer pain. This soon prompted to question whether opioids were exclusively reserved for cancer pain and were totally avoided in chronic pain states, and the consideration of pain relief as a fundamental human right was extensively argued (Melzack, 1990). Thus, in the **1990's**, pain advocacy groups and pharmaceutical companies supported the use of potent analgesics to treat severe pain of whatever cause, and a number of randomized controlled trials demonstrated efficacy in both nociceptive and neuropathic pain conditions (Moulin *et al.*, 1996; Dellemijn *et al.*, 1998; Gimbel *et al.*, 2003). This led to a substantial year-to-year rise of opioid usage that continues today. However, more recently the attention has focused on the balance between the benefits and harms of opioid prescribing. Importantly, the limitations of the early opioid trials have been noticed, in particular the fact that in clinical practice opioids for non-cancer pain were prescribed for much longer and in larger doses than the regimens used in clinical trials (Ballantyne and Mao, 2003). Furthermore, opioid misuse, diversion, high addiction potential and related morbidity and mortality highly emerged (Zacny *et al.*, 2003; Von Korff and Deyo, 2004; Okie, 2010). In just the past 15 years, there has been an **epidemic** of prescription opioid misuse, mostly in the United States and Canada, with a quadruplication of prescription opioid sales and mortality (Compton and Volkow, 2006; Helmerhorst *et al.*, 2017). Alarmingly, opioids were highly prescribed after minor ambulatory surgeries such as dental interventions, introducing opioids into society and leading to diversion and abuse (Mazer-

Amirshahi *et al.*, 2014; Steinmetz *et al.*, 2017). Some pharmaceutical companies contributed significantly to the rise of the opioid epidemic, receiving considerable reprimands as a consequence (Helmerhorst *et al.*, 2017). Surprisingly, in addition to the increasing mortality, there are no studies to this date which established appropriate evidence for the long-term safety and efficacy of opioid therapy in reducing chronic pain.

2.6.4 Intra-articular therapy in osteoarthritis

Intra-articular drug delivery has advantages over systemic delivery, including increased local bioavailability, reduced systemic exposure, fewer adverse effects and reduced cost (Evans *et al.*, 2014; Emami *et al.*, 2018). However, the efficacy of intra-articular therapies remains controversial and clinical guidelines are often inconsistent (Jones *et al.*, 2019). Due to their potent anti-inflammatory effects (Wilder, 1997), **corticosteroids** have shown analgesic efficacy during osteoarthritis (Bellamy *et al.*, 2006; da Costa *et al.*, 2016), and its intra-articular administration might be recommended especially to treat acute pain episodes in patients not responding to oral analgesics (Ravaud *et al.*, 1999). However, they provide short-term analgesic benefits and frequent injections can further damage the joint. **Hyaluronic acid** is a physiological component of synovial fluid and cartilage implicated in lubrication and inhibition of prostaglandin synthesis (Lohmander *et al.*, 1996; Brandt *et al.*, 2000). Its levels are diminished in osteoarthritis joints, thus intra-articular injections of exogenous hyaluronic acid aimed to compensate for this deficiency (Brandt *et al.*, 2000). However, it showed minimal analgesia in knee

osteoarthritis (Lo *et al.*, 2003; Rutjes *et al.*, 2012), and its effectivity has been recently reported as inconclusive (Jones *et al.*, 2019).

2.6.5 Other treatment options

A new strategy for the treatment of osteoarthritis now under investigation consists of **disease-modifying osteoarthritis drugs** (Tonge *et al.*, 2014), focused on modifying the structural progression of the disease. This approach could potentially confer a delay, complete cessation or reversion of structural deterioration or even prevent the disease development (Hunter, 2011). However, despite all efforts and the promising results in some preclinical and clinical trials, none of these pharmacological agents has been approved by regulatory authorities.

The use of **nutraceuticals** for the management of osteoarthritis has also been studied in some clinical trials, although the results are controversial. It has been reported that patients deficient in **vitamin D** have an increased risk of knee damage progression (Zhang *et al.*, 2014), but a 2-years supplementary vitamin D did not reduce pain nor cartilage loss in patients with knee osteoarthritis (McAlindon *et al.*, 2013). Clinical trials with **chrondrotin sulphate** and **glucosamine** have reported beneficial effects on pain and function during osteoarthritis (Mantovani *et al.*, 2016), but its possible effectiveness is widely inconclusive and needs further elucidation (Akhtar and Haqqi, 2012; Davies *et al.*, 2013).

Hence, osteoarthritis treatment remains an enormous challenge, and there is an urgent need to better understand the aetiology and physiopathology of this disease in order to develop more effective

drugs. It is also critical that the affective and cognitive alterations associated with chronic pain start being considered when developing complete therapeutic approaches.

2.7 Experimental study of osteoarthritis pain

Reproducing features of osteoarthritis in **animal models** is crucial to gain a better understanding of disease mechanisms and to assess response to potential therapies. There have been reported over 20 different animal models of osteoarthritis in 10 different species of varying strain, age and gender, each of them with its own advantages and disadvantages (Thysen *et al.*, 2015).

2.7.1 Existing animal models; benefits and limitations

Animal models of osteoarthritis can be broadly classified into spontaneous, including naturally occurring and genetic models of the disease, and induced models by surgical manipulation or intra-articular chemical injection (Table 1).

The **spontaneous models** exhibit a slow progression of the disease, and they closely mimic the course of primary osteoarthritis in humans without the need for intervention. These models allow following the development of the disease from the early to the late stages, but they are relatively costly and time-consuming and tend to be more variable in their phenotype (Lampropoulou-Adamidou *et al.*, 2014). Osteoarthritis can **naturally occur** in certain animals like mice, rabbits, guinea pigs, dogs, sheep, and horses as they age (Kyostio-Moore *et al.*, 2011; Vandeweerd *et al.*, 2013; Yan *et al.*, 2014). Among these, the Dunkin Hartley guinea pig has been the most widely used animal to study naturally-occurring osteoarthritis (Jimenez *et al.*, 1997; Yan *et*

al., 2014). Osteoarthritis-like lesions also occur in the knee joint of C57/BL6 mice at approximately 17 months of age (Wilhelm and Faust, 1976), whereas the STR/ort mouse strain develops knee osteoarthritis between 12 and 20 weeks of age, probably partly due to their 50% higher body weight than other mouse strains (Walton, 1977; Poulet *et al.*, 2013). In addition, **genetically modified** strains of mice have been designed as models of osteoarthritis, with mutating genes that either protect the animal from the disease or worsen a structural change (Little and Hunter, 2013). In particular, mice with mutations in extracellular matrix genes often develop spontaneous osteoarthritis. Examples include *Del1*$^{\pm}$ and *Col9a1*$^{-/-}$ mice, which have a mutation on the collagen II and IX genes, respectively (Säämänen *et al.*, 2000; Hu *et al.*, 2006), mice over-expressing cathepsin K, an enzyme involved in bone remodelling and resorption (Morko *et al.*, 2005), or mice over-expressing the human MMP13, which results in an articular cartilage pathology similar to human osteoarthritis (Neuhold *et al.*, 2001). These approaches have played a crucial role in understanding specific genetic contributions to the pathogenesis of osteoarthritis (Little and Zaki, 2012). However, as naturally occurring osteoarthritis almost certainly relies on the effect of many genes, the genetic models might oversimplify the process of the disease. Thus, results of therapeutic interventions may not be easily translatable to other animal models nor to humans, and care must be taken in study design and interpretation of results.

Surgically-induced models of osteoarthritis are commonly used in mice, rats, sheep, dogs and rabbits and aim to mimic post-traumatic

osteoarthritis. The disease is induced by a joint destabilization, altered articular surface contact forces, and intra-articular inflammation. The surgical models involve different structures of the joint and can induce diverse severities of cartilage degeneration, which are proportional to the degree of the instability induced in the joint (Kamekura *et al.*, 2005; Tochigi *et al.*, 2011). Some of the most commonly used surgical models include **anterior cruciate ligament transection**, **total or partial meniscectomy**, and **destabilization of medial meniscus** (Fang and Beier, 2014; Lampropoulou-Adamidou *et al.*, 2014). In these models, the first signs of hypersensitivity may appear only after a few weeks. Animals usually develop asymmetries in weight bearing and mechanical allodynia upon paw stimulation with von Frey filaments, whereas development of thermal hyperalgesia has shown inconsistent results (Bove *et al.*, 2006; Ferland *et al.*, 2011). The advantages of surgically-induced models are a fast and reproducible time course of disease progression and a clear relationship between the development of the pathology and the event which initiates it. However, this invasive rapid induction may be too fast to study the early development of osteoarthritis as well as to measure early drug treatments (Kuyinu *et al.*, 2016). Furthermore, knee joints are the preferred articulation used in all models, but it should be considered that load distribution and gait mechanics for knee joint highly vary between species (Teeple *et al.*, 2013). Interestingly, **ovariectomy** has become a surgical model to study a potential risk factor for osteoarthritis development, since an oestrogen deficiency in postmenopausal women seems to increase the risk for this disease (Sowers *et al.*, 2006).

Chemically-induced models mostly consist in the injection of a toxic or inflammatory compound directly into the knee joint with deleterious effects on joint homeostasis and consequent destruction of joint structures. Chemical agents can produce inhibition of chondrocyte metabolism, such as **papain** (Miyauchi *et al.*, 1993) or **MIA** (Guingamp *et al.*, 1997), damage of ligaments and tendons, as the case of **collagenase** (van der Kraan *et al.*, 1989) or **quinolone** antibiotics (Sendzik *et al.*, 2009), or selective joint denervation, such as **immunotoxins** (Salo *et al.*, 1997). Among these chemical compounds, MIA is the most commonly used. It is an inhibitor of the glyceraldehyde-3-phosphate dehydrogenase activity, which produces a rapid and widespread chondrocyte death, extensive neovascularization, cartilage degeneration and subsequent subchondral bone loss, as well as a profound and prolonged inflammation (Guzman *et al.*, 2003). The pain-related behaviour developed after a single injection of MIA has been widely described in rats (Bove *et al.*, 2003; Fernihough *et al.*, 2004) and mice (Harvey and Dickenson, 2009; La Porta *et al.*, 2013) demonstrating a functional impairment similar to that observed in the human disease. The rapid development of the MIA model is clearly different from the slow progress of human osteoarthritis, and the severe structural histopathological alterations of the joint do not mimic all of the physical features associated with the human disease (Little and Zaki, 2012). Thus, this model is not suitable for the study of disease pathogenesis, but it is considered useful to investigate the mechanisms of pain and possible analgesic therapies because it generates long-lasting mechanical hyperalgesia (Lampropoulou-

Adamidou *et al.*, 2014). Furthermore, all chemical models are less invasive than surgical models, easy to perform and to reproduce and facilitate timely and cost-effective experiments.

Table 1. Animal models of osteoarthritis; benefits, limitations and outcomes of pain assessment.

Osteoarthritis animal models	Species	Benefits	Limitations	Outcomes of pain assessment
Spontaneous models				
Naturally occurring	Mouse (STR/ort, DBA/1, C57BL/6), guinea pig (Duncan Hartley), rabbit, dog, horse, sheep	- Closely mimic human osteoarthritis - Different stages of osteoarthritis present - No need for intervention	- High cost - Time-consuming - High variability of disease onset and progression - Behavioural manifestations poorly studied	- Spontaneously active knee joint afferent fibres (McDougall *et al.*, 2009)
Genetically modified	Mouse (*Del1*$^{\pm}$, *Col9a1*$^{-/-}$, overexpression of cathepsin K, overexpression of MMP13	- Critical to understand the role of specific genes in the development of osteoarthritis - Consistent phenotype - No need for intervention	- Osteoarthritis due to a single gene mutation does not correlate with human disease - High cost - Time-consuming	- Changes in gait parameters (Allen et al., 2009) - Secondary mechanical allodynia (Allen et al., 2009) - Altered motor coordination (Allen et al., 2009)

Surgically-induced models				
Anterior cruciate ligament transection (ACLT)	Mouse, rat, dog, rabbit, goat, sheep	- Mimic human post-traumatic osteoarthritis - High reproducibility of the time course - Rapid disease progression	- Invasive procedure - Strong surgical skills required - Speed of onset and severity higher than humans	- Changes in gait parameters (Moreau et al., 2011; Fu et al., 2012) - Progressive secondary mechanical allodynia (Yang et al., 2014; Silva et al., 2018) - Alterations on weight distribution (Yang et al., 2014; Tawonsawatruk et al., 2018) - Changes in skin conductance response (Moreau et al., 2011)
Partial/complete meniscectomy	Mouse, rat, dog, guinea pig	- Mimic human post-traumatic osteoarthritis - High reproducibility of the time course - Rapid disease progression - Modulation of disease severity (partial/total, medial/lateral excision, unilateral/bilateral)	- Invasive procedure - Strong surgical skills required - Speed of onset and severity higher than humans	- Secondary mechanical allodynia (Bove et al., 2006) - Alteration on weight distribution (Bove et al., 2006) - Vocalization upon knee compression (Knights et al., 2012) - Cold hypersensitivity (Knights et al., 2012) - Sensitization of knee joint afferent fibres (Bullock et al., 2014)
Destabilization of medial meniscus (DMM)	Mouse, rat	- Mimic human post-traumatic osteoarthritis - High reproducibility of the time course - Slower disease progression and mild cartilage damage more similar to humans	- Invasive procedure - Strong surgical skills required	- Secondary mechanical allodynia (Miller et al., 2017) - Primary knee hyperalgesia (Miller et al., 2017) - Alteration on weight distribution (late-onset) (Inglis et al., 2008) - Altered motor coordination (Wang et al., 2016) - Spontaneous pain behaviours (Inglis et al., 2008; Miller et al., 2012)

Ovariectomy	Mouse, rat, guinea pig, rabbit, sheep	- Mimic human post-menopausal osteoarthritis	- Maybe secondary to weight gain and/or bone changes - Invasive procedure - Strong surgical skills required	- Modest mechanical allodynia (Yang et al., 2014)
Chemically-induced models				
Monosodium iodoacetate (MIA)	Mouse, rat	- Very rapid induction of severe joint degeneration - Low cost - High reproducibility of the time course - Less invasive procedures - Easy to implement - Control of severity according to the dosage	- Less clinically relevant than other models - Speed of onset and severity much higher than humans - Lesions induced are poorly documented - Inappropriate to study disease pathogenesis	- Stable and long-lasting secondary mechanical allodynia (Thakur et al., 2012; La Porta et al., 2013) - Alterations on weight distribution (Bove *et al.*, 2003; Haywood *et al.*, 2018) - Changes in gait parameters (Ferreira-Gomes et al., 2008) - Altered motor coordination (Harvey and Dickenson, 2009) - Vocalization in response to knee bend (Im et al., 2010; Ferreira-Gomes et al., 2012) - Diminished hind limb grip force (Chandran et al., 2009; Lee et al., 2011; Marker and Pomonis, 2012) - Primary knee hyperalgesia (Marker and Pomonis, 2012) - Conditioned place preference (P. Liu et al., 2011) - Sensitization of knee joint afferent fibres (Schuelert and McDougall, 2009) - Wind-up of dorsal horn neurons (central sensitization) (Harvey and Dickenson, 2009; Thakur et al., 2012)

Animal subjects used as osteoarthritis preclinical models range in size from mice to horses (Table 1). For reasons of cost, ethics, ease of handling, greater availability of facilities for housing and opportunity for genetic manipulation, **small animals** (mice, rats, rabbits and guinea pigs) are most often used to investigate specific disease mechanisms and for initial drug screenings. The primary disadvantage of these models is related to dissimilarities in tissue structure and joint mechanics between these species and humans. **Large animal** models (dogs, sheep, goats, pigs and horses) show more similarity to human in terms of cartilage morphology, joint anatomy and joint biomechanical function. However, they have a much higher cost, handling challenges and present important ethical concerns, particularly related to public perception. Nevertheless, they are generally required for the validation of potential therapeutic strategies (Little and Smith, 2008; McCoy, 2015; Thysen *et al.*, 2015).

2.7.2 Measures of disease outcome

2.7.2.1 Histopathology

Histologic scoring of the level of joint damage has been the gold standard for outcome assessment in animal models of osteoarthritis. Several scoring systems have been used in literature (Collins and McElligott, 1960; Mankin *et al.*, 1971; Pritzker *et al.*, 2006), making difficult the comparison across studies. In mice, the modified Mankin score has been widely applied (Glasson *et al.*, 1996; Neuhold *et al.*, 2001), but it has been questioned for the differences in the architecture of the cartilage between humans and mice (McCoy, 2015). In 2010, OARSI developed a new grading system (Glasson *et al.*,

2010), which has become one of the most appropriate system of histologic scoring in osteoarthritic mice. It is a semiquantitative method that involves a scoring range from 0 to 6 for structural cartilage damage in each of the 4 quadrants of the joint (medial/lateral tibial plateau and medial/lateral femoral condyle). Multiple sections for each joint should be assessed, and a summed score of the complete joint and/or the maximal score can be reported (Glasson *et al.*, 2010). This system shows a good intra-observer reproducibility and it is sensitive enough to be an effective screening tool.

2.7.2.2 Evaluation of pain in animal models

Chronic pain and discomfort are the hallmarks of osteoarthritis and the main reason for presentation of patients to medical services. Thus, the evaluation of pain in preclinical studies is an integral part of understanding the pathogenesis of the disease and developing successful treatments. However, characterizing pain and disability in animal subjects is an enormous challenge. Proper **pain measurement** requires identification of animal behaviour alterations that reliably indicate the sensation of pain. For the purpose of this thesis, we will focus on pain assessments on rodent animals. Behavioural tests to measure knee joint pain in osteoarthritis models include **evoked pain behaviours**, static and dynamic **weight bearing** and **motor coordination**, and **spontaneous** behaviours (Neugebauer *et al.*, 2007; O'Brien *et al.*, 2017). Furthermore, spontaneous or evoked joint afferent nerve activity can also be used as an outcome of nociception (Malfait *et al.*, 2013).

Evoked mechanical sensitivity of the hind paw in animals with knee osteoarthritis is commonly measured with von Frey filaments. Rats and mice with knee osteoarthritis show decreased paw withdrawal thresholds in response to mechanical stimuli for several weeks on the affected limb (Thakur *et al.*, 2012; La Porta *et al.*, 2013; Yang *et al.*, 2014; Miller *et al.*, 2017). **Thermal sensitivity** of the injured limb has been observed in rodent models of inflammatory mono-arthritis (Zhang *et al.*, 2001; Hong *et al.*, 2006), but surgically and chemically-induced osteoarthritis models showed no differences in the paw withdrawal latency to noxious heat (Bove *et al.*, 2006; La Porta *et al.*, 2013). MIA-injected rats also display reduced **hind limb grip force** for at least 1 month after injury (Chandran *et al.*, 2009; Lee *et al.*, 2011; Marker and Pomonis, 2012). Furthermore, a **pressure application device** has been recently developed, allowing the application of the stimulus directly to the knee joint and measuring the mechanosensitivity at this level. This device has shown **primary knee hyperalgesia** in the MIA and the destabilization of medial meniscus models of osteoarthritis (Malek *et al.*, 2015; Miller *et al.*, 2017). The monitorization of **vocalization** in response to compression or bend of the affected knee has also been found effective to assess primary hyperalgesia (Im *et al.*, 2010; Ferreira-Gomes *et al.*, 2012; Knights *et al.*, 2012).

Weight bearing have also been used in osteoarthritis models induced by MIA or surgery, where the **static** weight distribution across the hind paws of osteoarthritic animals showed a significant shift from the injured limb to the contralateral site (Bove *et al.*, 2003, 2006;

Fernihough *et al.*, 2004; Yang *et al.*, 2014). The **Catwalk** apparatus allows the assessment of numerous **gait parameters** in freely moving animals (Vrinten and Hamers, 2003). Although there is currently no standard method of gait analysis for animal models of osteoarthritis, several studies have reported alterations in different gait parameters in surgical and chemical models of the disease (Ferreira-Gomes *et al.*, 2008; Ferland *et al.*, 2011; Fu *et al.*, 2012). Gait analysis techniques enable detailed, objective and functional assessments of pain-induced behavioural adaptations, but it should be taken into consideration that rodents are pray animals who tend to mask their pain to avoid becoming a target for predators. Furthermore, the rotarod and the beam walking test can quantify **motor coordination**, which has been shown to be affected in osteoarthritis pain models (Allen *et al.*, 2009; Harvey and Dickenson, 2009).

A vast amount of information can also be acquired from simple observation of the **spontaneous pain behaviours**, which are thought to be more clinically relevant than evoked pain responses but are laborious to obtain and open to subjective interpretation. Automated systems have been developed to detect and quantify altered behaviours related to osteoarthritis pain, including hind limb licking, scratching, grooming, climbing, immobility or feeding. The technique has been successfully used in rodent models which generally showed reduced locomotion, rearing and climbing behaviours (Inglis *et al.*, 2008; Miller *et al.*, 2012).

A powerful yet technically demanding tool to measure joint nociception comprises the measure of neuronal activity in the pain

pathways by ***in vivo* electrophysiology**. Recording the responsiveness of peripheral fibres innervating osteoarthritic joints and second order neurons in the dorsal horn provide crucial information of the neurophysiological properties modifications of the nervous system during osteoarthritis. It has been reported that graded doses of intra-articular MIA produce a graded sensitization of joint afferent fibres (Schuelert and McDougall, 2009), a phenomenon that was also observed in a surgically-induced model of osteoarthritis (Bullock *et al.*, 2014). *In vivo* recordings from lamina V-VI of L4-L5 dorsal horn have been carried out in MIA-injected rats (Thakur *et al.*, 2012) and mice (Harvey and Dickenson, 2009), which revealed enhanced responsiveness after stimulation of their peripheral receptive field demonstrating the presence of central sensitization during osteoarthritis. In these experiments, WDR neurons with receptive fields on the hind paw were recorded. However, no studies of the activity of spinal neurons with knee joint afferent input have been described.

As previously explained, chronic pain is often accompanied by affective and cognitive alterations which could, in turn, worsen pain perception. Therefore, pain measurements in animal models of osteoarthritis should also be accompanied by behavioural testing of learning and memory, anxiety and depression. Several well-characterized behavioural tasks have been described to assess **cognitive function** such as the novel object recognition test, the Morris water maze, the radial arm maze, the social recognition test or the fear conditioning, which are effective in evaluating different

aspects of memory (Quillfeldt, 2016; Wolf et al., 2016). **Affective behaviour** is a highly complex response and several preclinical models are well-accepted for the evaluation of the anxiety and depressive-like behaviour in rodents (Belovicova et al., 2017). In the case of depression, the forced swimming test, the tail suspension test or the sucrose preference test, among others, seem to be good experimental approaches, being the forced swimming test the most widely used paradigm to screen new antidepressants (Micale et al., 2013). The majority of studies using animal models of anxiety employ behavioural paradigms based on approach-avoidance conflict, among which the elevated plus maze, its brother the elevated zero maze, the open field and the light/dark box have become the most popular (Cryan and Sweeney, 2011). Descriptions of the different paradigms to assess learning and memory, depressive-like and anxiety-like behaviours are found in Table 2.

Table 2. Description of the main models used to study learning and memory, depressive-like and anxiety-like behaviours in rodents. Adapted from (Lee and Silva, 2009; Belovicova *et al.*, 2017).

Test/model	Description/measurements
Learning and memory	
Novel object recognition	During the training session, animals are allowed to freely explore two objects in a maze, one of which will be replaced for a novel object in the test session. It is a non-aversive and non-spatial task to study recognition memory based on the innate animal tendency to explore the novelty.
Morris water maze	Animals swim in a pool of water to find the location of a submerged platform just beneath the surface of the water. Animals are trained during several days and the time/path length they take to find the platform is the learning index. It is used to study spatial learning and memory.
Radial arm maze	The apparatus has several arms (most commonly eight) that can be baited with food pellets at the end. Food deprived animals are allowed to enter the arms and search for hidden food. Different variants of this task are done blocking or giving access to the different arms with or without food. It is used to study spatial learning.
Social recognition	Similar to the object recognition test, but in this case the objects are replaces with animals (juveniles, from different cages, different strains). It assessed social memory.
Fear conditioning	It is an aversive learning task in which animals associate a non-aversive conditioned stimuli, such as a tone or context, with an aversive unconditioned stimulus (e.g. foot shock). Conditioned responses that can be active (rearing, diving, locomotion) or passive (freezing) can be used as measures of memory.
Depressive-like behaviour	
Forced swimming (FST)	When forced to swim without possibility to escape, rodents stop moving completely after the initial period of intense activity. The immobility state is considered a situation of despair and indicate depressive-like behaviour.
Tail suspension	It induces similar behaviour as the FST. The passive immobility during inescapable upside-down suspension by the tail is measure, indicating more "depression" when longer immobility phases.
Sucrose preference	Animals have access to water without and with different concentrations of sucrose, and the preference rate is analysed. The reduced interest in the reward is a manifestation of depressive behaviour.
Learned helplessness	Animals are exposed to unpredictable and inescapable shock, and subsequently develop coping deficits for aversive but escapable situations.
Olfactory bulbectomy	Behavioural and neurochemical alterations developed after the removal of the olfactory bulbs, which results in a disruption of the limbic hypothalamic axis.
Anxiety-like behaviour	
Elevated plus maze (EPM)	Rodents tend to avoid potentially dangerous places, but at the same time they tend to explore novel environments. The avoidance of the open and elevated arms of the maze indicate higher degree of anxiety-like behaviour.
Elevated zero maze	It is based on the same conflict between two opposing innate motivations of rodent as in the EPM. Here, high "anxiety" is indicated by reduced time spent in the open quadrants.
Open field	The animal is placed in the central zone of an open field. The avoidance of the novel, brightly illuminated central area is measured as an outcome of anxiety-like behaviour.
Light/dark box	It is based on the natural aversion of rodents toward brightly illuminated spaces. The anxiety-like behaviour is analysed by the animal's preferences for the dark over the light part of the box.

3 Sigma-1 receptor

3.1 Historical overview

The **sigma receptor** (**σR**) was discovered more than 40 years ago (Martin *et al.*, 1976), and it was first misclassified as a subclass of the opioid receptor family based on the psychomimetic actions exhibited by SKF-10,047 (N-allyl-normetazocine). Such effects could not be explained by the actions on the known opioid receptors, and the existence of a σ-opioid receptor was proposed. The differences in the enantiomeric selectivity of SKF-10,047 for the opioid receptors (Su, 1982), and the fact that the effects of sigma ligands were not blocked, neither *in vivo* nor *in vitro*, by classical opioid antagonists (Iwamoto, 1981; Vaupel, 1983; Young and Khazan, 1984) led to the identification of the σR. Based on the selectivity profile of ligands and the molecular mass, two subtypes of σR were described: the **sigma-1 receptor** (**σ1R**) and the **sigma-2 receptor** (**σ2R**) (Hellewell and Bowen, 1990; Quirion *et al.*, 1992). It has been shown that both receptors colocalize in several areas of the rat brain, but they are present in different ratios (Leitner *et al.*, 1994; McCann *et al.*, 1994; Bouchard and Quirion, 1997). The σ2R subtype has been intensively studied over the past years (Monassier *et al.*, 2007; Abate *et al.*, 2018; Blass and Rogers, 2018; Vázquez-Rosa *et al.*, 2018), although the σ1R is better characterized and will be the focus of this thesis.

3.2 Gene and structure of the sigma-1 receptor

The σ1R was first cloned in 1996 from guinea pig liver (Hanner *et al.*, 1996), and later from human cell lines (Kekuda *et al.*, 1996), human brain (Prasad *et al.*, 1998), rat brain (Seth *et al.*, 1998; Mei and

Pasternak, 2001) and mouse kidney and brain (Seth *et al.*, 1997; Pan *et al.*, 1998). The human gene for the σ1R is located on chromosome 9 (chromosome 4 in mice, and 5 in rats) and encodes a 24 kDa molecular mass protein of 223 amino acids. The σ1R sequence is highly homologous among species (above 90%) and shares no homology with any known mammalian protein (Hanner *et al.*, 1996; Kekuda *et al.*, 1996; Seth *et al.*, 1997, 1998). However, it shares approximately 30% identity with the yeast gene that encodes the C7-C8 sterol isomerase, an enzyme necessary for cholesterol synthesis (Moebius *et al.*, 1997). This finding led to think about a possible role of the σ1R in sterol biosynthesis, which would be in agreement with its affinity for steroids (Su *et al.*, 1988). However, it has been revealed that σ1R does not possess the sterol isomerase activity (Hanner *et al.*, 1996; Seth *et al.*, 2001).

The σ1R was firstly proposed as a single transmembrane protein (Hanner *et al.*, 1996; Dussossoy *et al.*, 1999), but more recent evidences indicate that it has two alpha-helical transmembrane segments with the NH_2 and COOH termini on the cytoplasmic side of the plasma membrane or in the lumen of the endoplasmic reticulum (Aydar *et al.*, 2002; Hayashi and Su, 2007) (Figure 17). Apart from the transmembrane domains, there are two other hydrophobic regions that form the “steroid-binding domain like”, which is involved in the formation of the ligand-binding site (Pal *et al.*, 2007, 2008). Therefore, the binding site of the σ1R is located in the inner surface of the membrane, thus enabling hydrophobic molecules to associate with the receptor.

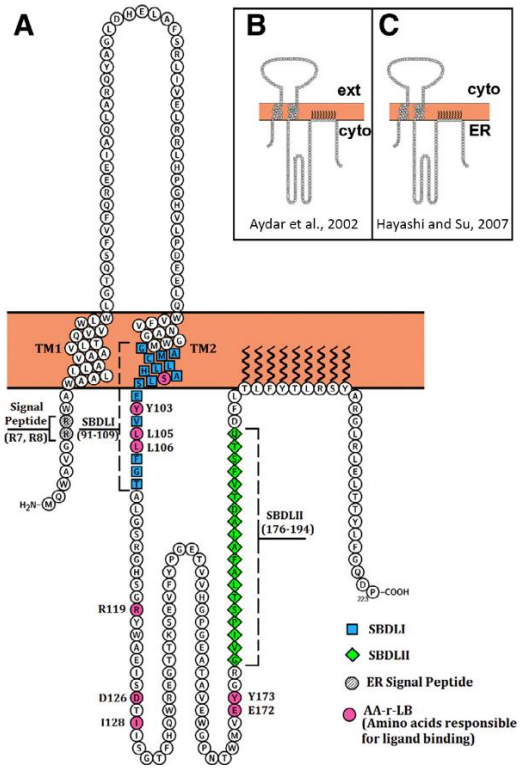


Figure 17. The putative structural model of the sigma-1 receptor (σ1R) (A). σ1R contains two hydrophobic transmembrane regions with the N- and C- terminals in the intracellular side of the plasma membrane (B) or in the endoplasmic reticulum (ER) lumen (C). Circles represent amino acids and the numbers correspond to the serial number of the residues. Ext, extracellular space; Cyto, cytoplasm; ER, lumen of the endoplasmic reticulum; TM1 and TM2, transmembrane alpha-helices; SBDLI and SBDLII, steroid-binding domains I and II; AA-r-LB, amino acid residues participating in the binding of the σ1R ligands (Bolshakova *et al.*, 2016).

3.3 Anatomical and subcellular distribution

At the **anatomical level**, σ1R is ubiquitously expressed in mammalian tissues and is widely distributed in **peripheral organs**, such as digestive tract (Samovilova and Vinogradov, 1992), liver (Hellewell *et al.*, 1994), kidney (Hellewell *et al.*, 1994), heart (Ela *et al.*, 1994), sexual organs (Jansen *et al.*, 1992) and skin (Sánchez-Fernández *et al.*, 2013). It is also extensively present in different areas of the **nervous**

system, where it is concentrated in specific brain areas involved in memory, emotion and sensory and motor functions, such as the hippocampus, hypothalamus, olfactory bulb, several cortical layers like the PFC, PAG, locus coeruleus or rostroventral medulla (RVM) (McCann *et al.*, 1994; Alonso *et al.*, 2000). In addition to the brain, σ1R is also abundant in the spinal cord, mainly in the superficial layers of the dorsal horn, and in the DRG (Alonso *et al.*, 2000; Bangaru *et al.*, 2013).

The location of σ1R in the **subcellular compartment** is dynamic, and it is found in several membranes (Hayashi and Su, 2005a, 2005b). Binding experiments with σ1R radioligands showed that the receptor is especially enriched in the microsomal membrane (McCann and Su, 1990; Cagnotto *et al.*, 1994), suggesting that σ1Rs are mainly located in the endoplasmic reticulum membrane. This result was further confirmed by immunohistochemical studies, which showed the existence of σ1R in the endoplasmic reticulum in neurons (Alonso *et al.*, 2000) and in glial cells (Palacios *et al.*, 2003; Hayashi and Su, 2005a; Jiang *et al.*, 2006). Moreover, the cloned amino acid sequence of the σ1R has a double-arginine endoplasmic reticulum retention signal on the N-terminus (Figure 17). At this level, σ1R is located at the interface with the mitochondria at the **mitochondria-associated endoplasmic reticulum membrane**, from where it is redistributed, upon activation, to other subcellular locations like the plasma or the nuclear membranes (Morin-Surun *et al.*, 1999; Hayashi and Su, 2001). This relocation possibly increases the number or type of proteins that can be targeted by the σ1R (Zamanillo *et al.*, 2013).

3.4 Mechanisms of action of the sigma-1 receptor

σ1R is a **ligand-regulated chaperone** that interacts with other proteins to modulate their activity. It is apparently devoid of its own specific signalling machinery but it amplifies or reduces the inter-organelle signalling provoked by its target proteins (receptor, ion channel or enzyme) (Su and Hayashi, 2003; Tsai *et al.*, 2009; Su *et al.*, 2010). Under normal physiological conditions, most target proteins are not affected by σ1R, but when they become conformationally unstable, disturbed or stressed, the σ1R chaperone can assist and modulate their activity (Hayashi *et al.*, 2000; Su and Hayashi, 2003; Su *et al.*, 2010) (summarized in Figure 18).

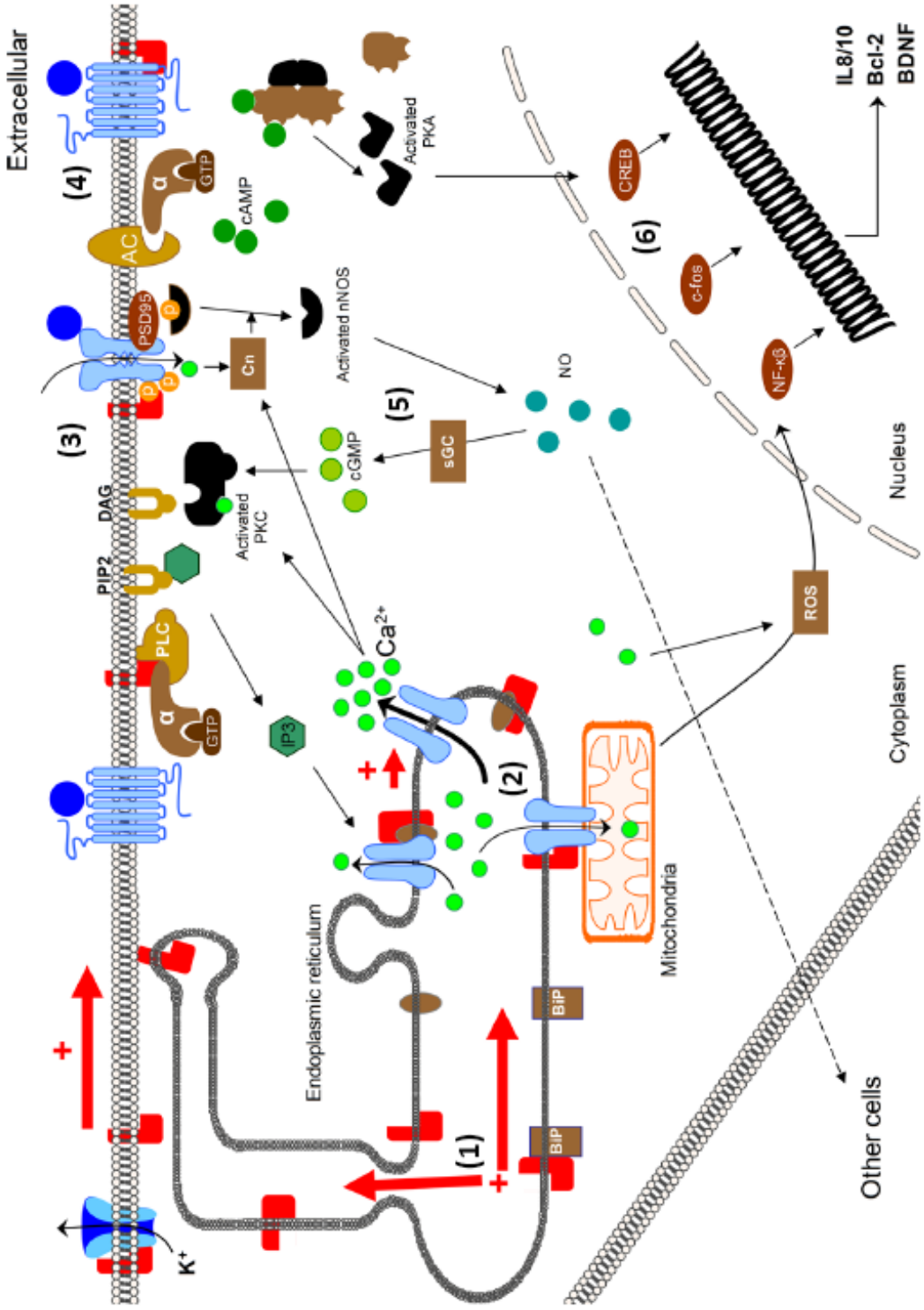
The best characterised σ1R chaperoning effect occurs at the **endoplasmic reticulum**, where inositol-1,4,5-triphosphate (**IP_3**) receptors mediate the efflux of Ca^{2+} from the endoplasmic reticulum into the mitochondria. Under normal resting conditions, σ1R forms a complex with the binding immunoglobulin protein. Under pathological conditions and in the presence of high concentrations of cytosolic IP_3, there is a dramatic drop of Ca^{2+} concentration at the endoplasmic reticulum and the σ1R becomes activated. It dissociates from binding immunoglobulin protein and interacts with the unstable IP_3 receptors, thus preventing IP_3 receptor degradation and ensuring the proper Ca^{2+} influx into the mitochondria, which plays a central role in energy production (Hayashi and Su, 2007; Tsai *et al.*, 2009; Zamanillo *et al.*, 2013) (Figure 18).

Once σ1R is located in the **plasma membrane**, it can interact with other receptors to generate heteromers, or forming homodimers with

itself (Chu and Ruoho, 2015). When σ1R is activated, it stimulates phospholipase C to hydrolyse phosphatidylinositol 4,5-biphosphate, producing diacylglycerol and **IP_3** (Morin-Surun *et al.*, 1999). IP_3 then binds to IP_3 receptors in the endoplasmic reticulum to promote the efflux of Ca^{2+} to the cytoplasm. It has also been reported that σ1R activation facilitates the phosphorylation of the NR1 subunit of the **NMDAR** at the protein kinase C (PKC)-dependent Ser^{890} and Ser^{896} and the PKA-dependent Ser^{897} (Kim *et al.*, 2008; Roh *et al.*, 2008), hence favouring the activation of the receptor and potentiating the NMDAR currents. In fact, a direct physical interaction of the σ1R with the C terminal of the NMDA-NR1 subunit has been described (Balasuriya *et al.*, 2013; Rodríguez-Muñoz *et al.*, 2015b). In addition, σ1R also modulates NMDAR activity through an indirect mechanism. The activation of σ1R inhibits the **small conductance Ca^{2+}-activated potassium channels**, which in turn potentiates NMDAR currents (Martina *et al.*, 2007). Activated σ1R also regulates the activity of **voltage-gated Ca^{2+} channels and potassium (K^+) channels** (Wilke *et al.*, 1999a; Aydar *et al.*, 2002), leading to a decreased influx of Ca^{2+} and efflux of K^+. G protein-coupled receptors have also been involved with σ1R. In particular, a physical interaction with σ1R has been demonstrated for the **cannabinoid receptor CB1R** and the **MOR** (Kim *et al.*, 2010; Sánchez-Blázquez *et al.*, 2014). In addition, the activation of σ1R diminishes the association of the **neuronal nitric oxide synthase** (**nNOS**) with the NR2 subunit of the NMDAR by reducing the recruitment of nNOS to the membrane fraction and its interaction with the postsynaptic density protein-95 (Yang *et al.*, 2010) (Figure 18).

σ1R activation produces consequences at the **cytosolic** level, although its free form has not been found in the cytoplasm. The σ1R-induced rise of intracellular Ca^{2+} results in a decreased phosphorylation of **nNOS**. As a consequence, the activity of this enzyme increases notably and forms **nitric oxide** (**NO**), which can diffuse freely to other cells and stimulates the soluble guanylate cyclase to produce cGMP. This leads to PKC activation, which phosphorylates and consequently activates the NMDAR and the **extracellular-signal regulated kinases** (**ERK**) (Roh *et al.*, 2011) (Figure 18).

At the **nucleus**, σ1R activation has been proposed to modulate several transcription factors, such as the reactive oxygen species-induced **nuclear factor-κB**, **cAMP response element-binding protein** (**CREB**) and **c-fos**. Consequently, σ1R transcriptionally regulates the gene expression of several proteins related to inflammation, nociception, neuronal survival, synaptogenesis and neurogenesis, such as nNOS, inducible nitric oxide synthase, the antiapoptotic protein Bcl-2, BDNF and interleukins 8/10 (Yang *et al.*, 2007; Meunier and Hayashi, 2010; Hayashi *et al.*, 2011) (Figure 18).


Extracellular
(4)
α
GTP
AC
cAMP
Activated PKA
PSD95
P
Cn
Activated nNOS
(3)
(5)
NO
DAG
PIP2
Activated PKC
cGMP
sGC
PLC
α
GTP
IP3
Ca2+
(2)
ROS
CREB
(6)
c-fos
NF-kß
IL8/10
Bcl-2
BDNF
Nucleus
Cytoplasm
Endoplasmic reticulum
BiP
BiP
Mitochondria
(1)
K+
Other cells

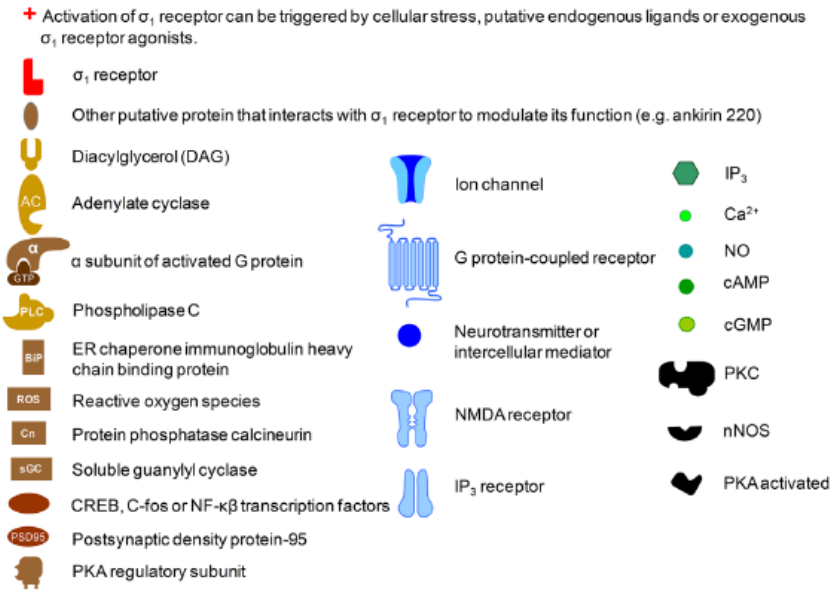


Figure 18. Signal transduction pathways modulated by σ1R activation. (**1**) Activation of σ1R in the endoplasmic reticulum (ER) allows itself to dissociate from the chaperone binding immunoglobulin protein (BiP) and to redistribute to peripheral endoplasmic membranes or to the plasma membrane to bind ion channels, receptors or protein kinases. (**2**) At the endoplasmic reticulum, σ1R binds IP_3 receptor to enhance Ca^{2+} signalling from the endoplasmic reticulum into the mitochondria to increase ATP production. The IP_3 receptor interaction could be inhibited or facilitated by coupling to other proteins (e.g. ankyrin) modulating the Ca^{2+} efflux from the endoplasmic reticulum. (**3**) At the plasma membrane, σ1R regulates the activity of components of the plasma membrane-bound signal transduction such as phospholipase C (PLC) and protein kinase C (PKC) and modulates the activity of neurotransmitter receptors and ion channels, including K^+, Ca^{2+} channels and NMDA receptors. (**4**) σ1R also interacts with G protein-coupled receptors, such as the cannabinoid receptor CB1 and the mu-opioid receptor. (**5**) At the cytoplasm, increased cytosolic Ca^{2+} reduces the phosphorylation of the neuronal nitric oxide synthase (nNOS), resulting in an increase in its activity. The nitric oxide (NO) generated from nNOS stimulates cGMP production via sGC, which in turn leads to an increase in PKC activity. In addition, the diffusible NO produced can diffuse to affect other cells. (**6**) At the nucleus, σ1R activation controls transcriptional regulation of gene expression of the antiapoptotic protein Bcl-2, brain-derived neurotrophic factor (BDNF) or interleukins 8/10 (IL8/10) by the reactive oxygen species (ROS)-induced nuclear factor-κB (NF-κB), by cAMP response element-binding protein (CREB) or by c-fos, respectively (Zamanillo *et al.*, 2013).

3.5 Modulation of the sigma-1 receptor

σ1R displays chaperone activity to regulate inter-organelle signalling while it is simultaneously modulated by ligands in an **agonist/antagonist** manner. However, the discovery and characterization of σ1R ligands have been complicated due to the wide spectrum of molecules with diverse structures that have affinity for this receptor.

3.5.1 Agonists versus antagonists

Historically, the distinction between agonists and antagonists for many σ1R ligands has laid primarily on the pharmacological response from rodent behaviour assays (Su *et al.*, 2010), but the difference at the molecular and cellular level remains unresolved. Currently, the discovery of new selective ligands with a defined functionality presents some difficulties (Zamanillo *et al.*, 2012):

- σ1R exerts a modulatory action on several receptors, ion channels and enzymes (Su and Hayashi, 2003), rather than an easily estimated direct effect.
- σ1R is mainly located intracellularly (Alonso *et al.*, 2000), hence the hydrophobicity of ligands is a major determinant to predict the potency of σ1R ligands *in vivo*.
- σ1R activity can vary depending on the conformational state of the target proteins, given that σ1R is a chaperone protein that only exerts its activity under pathological conditions (Hayashi *et al.*, 2000). Hence, the nature of the disease provides the selectivity of σ1R ligands.

- Some σ1R ligands do not show the classical linear dose-response curve in behavioural, biochemical and electrophysiological studies, as their effects disappear when used at high doses (Maurice *et al.*, 1994; Bergeron *et al.*, 1995; Hayashi *et al.*, 2000; Dhir and Kulkarni, 2008).
- Many of the widely accepted σ1R antagonists (BD1063, BD1047 or NE-100) bind also at nanomolar affinities to the σ2R. Thus, the effects of these nonselective ligands could result in apparent discrepancies due to the activation of other σ receptor subtypes at high doses.
- The assay conditions and readouts used notably affect the outcome. Indeed, it has been shown that known σ1R agonists and antagonists, whose functional nature was identified based on other readouts, produced the same effect inhibiting K^+ currents (Wilke *et al.*, 1999a, 1999b; Zhang and Cuevas, 2005), whereas two identified selective σ1R agonists induced opposite effects modulating Ca^{2+} influx (Hayashi *et al.*, 2000).

Despite the listed obstacles, several useful approaches are being used to discern whether a σ1R ligand is an agonist or an antagonist:

- *In vivo* test. The antinociceptive activity in animal models such as formalin or capsaicin tests has been often used for establishing the antagonist nature of a σ1R ligand (Cruz M. Cendán *et al.*, 2005; Entrena *et al.*, 2009a)
- Phenytoin assay. Phenytoin is a low-potency allosteric modulator of σ1R that modifies the binding of σ1R ligands depending on their agonist or antagonist nature. Phenytoin increases the

affinity of putative agonists while keeping unmodified or poorly decreasing the affinities of antagonists (Cobos *et al.*, 2005, 2006).

- Fluorescence resonance energy transfer studies. It has been reported that the binding of agonists to the σ1R leads to an increased separation between the N- and C-termini, whereas antagonists promote their approximation. The fluorescence resonance energy transfer assay can reveal the intrinsic nature of σ1R ligands by detecting these ligand-mediated conformational changes of the receptor (Gómez-Soler *et al.*, 2014), which correlate well with the antinociceptive *in vivo* effects of the compounds.

3.5.2 Endogenous ligands

Although the endogenous ligands for the σ1R have not been unequivocally defined, currently **neurosteroids**, such as dehydroepiandrosterone (DHEA), pregnenolone, progesterone and their sulphate esters, are considered the most likely naturally-occurring σ1R endogenous ligands (Su *et al.*, 1988; Maurice *et al.*, 2001; Hayashi and Su, 2004; Moriguchi *et al.*, 2013). They are synthesized in the CNS and peripheral tissues and are able to exert a modulatory effect on neuronal excitability, in which the interaction with σ1R could contribute (Monnet and Maurice, 2006). Whether neurosteroids are the endogenous σ1R ligands remains controversial because their affinities do not seem to be sufficient for endogenous ligands (Schwarz *et al.*, 1989; Hayashi and Su, 2004), yet many studies still support this idea. In different experimental paradigms, DHEA and pregnenolone sulfate behaved as other known σ1R agonists, whereas

progesterone acted as an antagonist (Su *et al.*, 1988; Maurice *et al.*, 2001). The exogenous administration of neurosteroids produced an inhibition of *in vivo* σ1R radioligands (Maurice *et al.*, 1996; Waterhouse *et al.*, 2007) and showed activity in behavioural evaluations that were blocked by σ1R antagonists (Maurice *et al.*, 2001; Monnet and Maurice, 2006). It has also been reported the existence of other putative endogenous ligands, such as the natural hallucinogen N,N-Dimethyltryptamine (DMT) (Fontanilla *et al.*, 2009), sphingosine (Ramachandran *et al.*, 2009) or the endogenous peptide NPY (Roman *et al.*, 1989) (Table 3).

Table 3. Summary of endogenous ligands known to interact with the σ1R (Adapted from Zamanillo *et al.*, 2012).

Compound	σ1R affinity [Ki nM]	Function on σ1R	Pharmacological actions	References
NPY	~10	Agonist?	Anti-amnesic, anticonvulsant	(Roman *et al.*, 1989; Ault et al., 1997; Maurice *et al.*, 2001)
L-*threo*-sphingosine	20	Agonist?	Endogenous amine involved in lipid signalling	(Ramachandran *et al.*, 2009)
Sphinganine	70	Agonist?		
N,N-dimethyl-sphingosine	120	Agonist?		
Progesterone	130	Antagonist	NMDAR negative/$GABA_A$ positive modulator	(Su *et al.*, 1988; Maurice *et al.*, 2001)
D-*erythro*-sphingosine	140	Agonist?	Endogenous amine involved in lipid signalling	(Ramachandran *et al.*, 2009)
Pregnenolone sulfate	980	Agonist	NMDAR positive/$GABA_A$ negative modulator	(Su *et al.*, 1988; Maurice *et al.*, 2001)
DHEA	5200	Agonist	$GABA_A$ negative modulator	(Moriguchi *et al.*, 2013)
DMT	14750	Agonist	5-HT_{2A} receptors agonist, psychedelic drug	(Fontanilla *et al.*, 2009; Su *et al.*, 2009)

3.5.3 Exogenous ligands

For several years, many structurally-diverse drugs have shown moderate/low to high affinity for σ1R, but with low selectivity. These ligands include compounds with a broad range of therapeutic and pharmacological applications, comprising **antipsychotics** (e.g. haloperidol, chlorpromazine), **antidepressants** (e.g. fluvoxamine, sertraline, clorgyline), **antitussive** (carbetapentane, dextromethorphan, dimemorfan), drugs for the **treatment of neurodegenerative disorders** such as Parkinson's disease (amantadine) or Alzheimer's disease (memantine, donepezil), and **drugs of abuse** (cocaine, methamphetamine), among others (reviewed by Hayashi and Su, 2004; Cobos *et al.*, 2008; Zamanillo *et al.*, 2012). However, it is still uncertain which of the pharmacological actions exerted by these compounds are mediated by the σ1R (Narita *et al.*, 1996; Sánchez and Meier, 1997; O'Dell *et al.*, 2000; Hayashi and Su, 2004). Moreover, more selective and high-affinity σ1R drugs have been developed, which are now considered prototypical σ1R ligands. (+)-pentazocine and PRE084 are examples of σ1R agonists, while BD1063, BD1047 or NE100 are some of the antagonists developed (reviewed by Hayashi and Su, 2004; Cobos *et al.*, 2008; Zamanillo *et al.*, 2012). Of special interest for this thesis is the antagonist **S1RA** (also named **E-52862** or **MR309**), which was recently developed and exhibits an exceptional selectivity for σ1R (Romero *et al.*, 2012), and **SIMU**, which is a multimodal compound that acts both as a σ1R antagonist and MOR agonist. The number of reported σ1R ligands is rapidly increasing as these ligands provide valuable research tools to

investigate the characteristics and function of the σ1R. Some of the most common σ1R agonists and antagonists are listed in Table 4.

Table 4. Summary of exogenous ligands that interact with the σ1R. (A), Under active development; (D) discontinued; (L), launched; OCD, Obsessive-compulsive disorder; ADHD, Attention deficit/hyperactivity disorder (Adapted from Zamanillo *et al.*, 2012).

Compound	σ1R affinity [Ki nM]	Function on σ1R	Pharmacological actions	Clinical development: indications	References
Benzomorphans					
(+)-SKF-10.047	597	Agonist	NMDAR ligand (PCP site)		(Maurice *et al.*, 1996; Hayashi and Su, 2004)
(-)-Pentazocine	807	Agonist	K_1 agonist, μ_1 μ_2 ligand, low-affinity δ and κ_3 opioid ligand		(Chien and Pasternak, 1995a; Vilner and Bowen, 2000)
Antipsychotics					
Haloperidol	6.44	Antagonist	Dopamine D_2 and D_3 antagonist, σ2R agonist	Psychosis (L); Schizophrenia (L); Tourette's disease	(Jaen *et al.*, 1993; Maurice *et al.*, 2001; Hayashi and Su, 2004)
BMY-14802	66	Antagonist	5-HT_{1A} agonist	Psychosis (D); Schizophrenia (D)	(Matos *et al.*, 1996; Matsumoto and Pouw, 2000)
Eliprodil	132	Antagonist	NMDAR antagonist, α_1-AR ligand	Schizophrenia (D); head injury (D); cerebrovascular ischemia (D); Parkinson's disease (D)	(Hashimoto and London, 1995)
Chlorpromazine	453		Dopamine D_2 antagonist	Psychosis (L)	(Matsumoto and Pouw, 2000; Hayashi and Su, 2004)
Rimcazole	2380	Antagonist	Dopamine transporter inhibitor	Psychosis (D); breast, lung and prostate cancer	(Matsumoto and Pouw, 2000; Matsumoto *et al.*, 2001)

Antidepressants					
Cutamesine (SA4503)	4.6	Agonist	Acetylcholine release enhancer	Depression (A); stroke (A)	(Lever *et al.*, 2006)
Fluvoxamine	36	Agonist	Selective 5-HT reuptake inhibitor	Depression (L); OCD (L); Social phobia (L)	(Narita *et al.*, 1996; Hayashi and Su, 2008)
Sertraline	57	Antagonist ?	Selective 5-HT reuptake inhibitor	Depression (L); OCD (L); Post-traumatic stress (L); Panic disorder (L); Social phobia (L); Premenstrual syndrome (L)	(Bermack and Debonnel, 2005; Hayashi and Su, 2008; Nishimura *et al.*, 2008; Ishima *et al.*, 2014)
Fluoxetine	240	Agonist	Selective 5-HT reuptake inhibitor	Depression (L); OCD (L); Panic disorder (L); Bulimia nervosa (L); Obesity (L); Premenstrual syndrome (L); Fibromyalgia	(Narita *et al.*, 1996; Hayashi and Su, 2008)
Citalopram	292		Selective 5-HT reuptake inhibitor	Depression (L); Panic disorder (L); Mood disorder; Huntington's disease; Bipolar disorder	(Narita *et al.*, 1996; Hayashi and Su, 2008)
Imipramine	343	Agonist	Monoamine reuptake inhibitor (TCA)	Depression (L); Enuresis (L); Dyspepsia	(Narita *et al.*, 1996; Hayashi and Su, 2008)
Desipramine	1987		Monoamine reuptake inhibitor (TCA)	Depression (L); Gastroesophageal reflux disease	(Narita *et al.*, 1996; Hayashi and Su, 2008)
Antitussives					
Carbetapentane	128	Agonist	Muscarinic antagonist, σ2R agonist	Cough (L)	(Calderon *et al.*, 1994; Matsuno *et al.*, 1996; Maurice *et al.*, 2001)
Dimemorfan	151	Agonist		Cough (L); Epilepsy (L)	(Chou *et al.*, 1999; Wang *et al.*, 2003; Shin *et al.*, 2005)
Dextromethorphan	205	Agonist	NMDAR allosteric antagonist	Cough (L); Rett's syndrome; Diabetic macular oedema	(Maurice *et al.*, 2001; LePage *et al.*, 2005; Shin *et al.*, 2005)

Parkinson's and/or Alzheimer's disease					
Donopezil	14.6	Agonist	Cholinesterase inhibitor	Dementia (L); ADHD; Ischemic stroke; Cocaine dependence; Autism; Down's syndrome; Neurological disorders; Fragile X syndrome	(Kato *et al.*, 1999; Maurice *et al.*, 2006; Meunier *et al.*, 2006)
Memantine	2600	Agonist?	NMDAR antagonist, antiviral properties	Spasticity (L); Dementia (L); Cancer therapy associated disorders; Cognitive disorders; Depression; Heroin dependence; Autism	(Peeters *et al.*, 2004; Chen and Lipton, 2006)
Amantadine	7440	Agonist?	NMDAR antagonist, antiviral properties	Influenza A (L); Parkinson's disease (L)	(Peeters *et al.*, 2004; Chen and Lipton, 2006)
Anticonvulsants					
Phenytoin	Not applicable	Allosteric modulator	Delayed rectifier K^+ channels blocker, T-type Ca^{2+} current inhibitor, Na^+ current inhibitor	Arrhythmia (L); Epilepsy (L); Neuropathic pain (L)	(Rush and Elliott, 1997; Nobile and Lagostena, 1998; Todorovic and Lingle, 1998; Cobos *et al.*, 2005, 2006)
Drugs of abuse					
Cocaine	2000	Agonist	Monoamine transporters inhibitor, psychostimulant		(Sharkey *et al.*, 1988; Matsumoto *et al.*, 2002; Rothman and Baumann, 2003)
Methampheta mine	2160		Preferential dopamine transporter inhibitor, psychostimulant		(Nguyen *et al.*, 2005; Fleckenstein *et al.*, 2007; Chao *et al.*, 2017)
MDMA	3057		Preferential SERT inhibitor, psychostimulant	Post-traumatic stress (A)	(Green *et al.*, 2003; Brammer *et al.*, 2006)

Other σ1R ligands					
CM-31747; SR-31742A	0.4		C8-C7-sterol isomerase ligand	Prostate cancer (D); Immunological disorders (D); Rheumatoid arthritis (D); Schizophrenia (D)	(Poncelet *et al.*, 1993; Bourrié *et al.*, 2002)
BD-1047	0.9	Antagonist	A-AR ligand		(Matsumoto *et al.*, 1995; McCracken *et al.*, 1999b; Maurice *et al.*, 2001)
NE-100	1.5	Antagonist		Schizophrenia (D)	(Chaki *et al.*, 1996)
BD-1063	9	Antagonist			(Matsumoto *et al.*, 1995; McCracken *et al.*, 1999a; Brammer *et al.*, 2006)
Siramesine	17	Antagonist	α_1-AR ligand	Anxiety disorder (D); Cancer	(Perregaard *et al.*, 1995)
S1RA (E-52862, MR309)	17	Antagonist		Pain; Neuropathic pain (A)	(Romero *et al.*, 2012; Gris *et al.*, 2014)
PRE-084	44	Agonist			(Maurice *et al.*, 1999)
DTG	77		σ2R agonist		(Kedjouar *et al.*, 1999; Matsumoto and Pouw, 2000; Maurice *et al.*, 2001)
(+)-3-PPP	79	Agonist	σ2R agonist, NMDAR ligand, dopaminergic agonist		(Walker *et al.*, 1990; Höfner and Wanner, 2000; Hayashi and Su, 2004)
SIMU	118	Antagonist	μ-opioid receptor agonist (Ki = 64 nM)		

3.5.4 The selective sigma-1 receptor antagonist S1RA

In 2012, Laboratories Esteve developed the new chemical entity 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine, which was named **S1RA** or **E-52862** (Díaz *et al.*, 2012) (Figure 19). S1RA shows high σ1R affinity in humans (Ki = 17 nM) and guinea pigs (Ki = 23.5 nM), whereas its affinity for σ2R is not significant (Ki > 1000 nM for guinea pig and 9300 nM for rat), exhibiting a good σ1/ σ2 selectivity ratio (>550). Moreover, while many other σ1R ligands have also reported high selectivity against σ2R, 5-HT_{1A}, 5-HT_6, 5-HT_7, α_{1A}, α_2, and NMDAR (Oberdorf *et al.*, 2008), S1RA is a highly selective compound, lacking significant affinity for another 170 additional molecular targets, including receptors, transporters, ion channels and enzymes (Díaz *et al.*, 2012; Romero *et al.*, 2012).

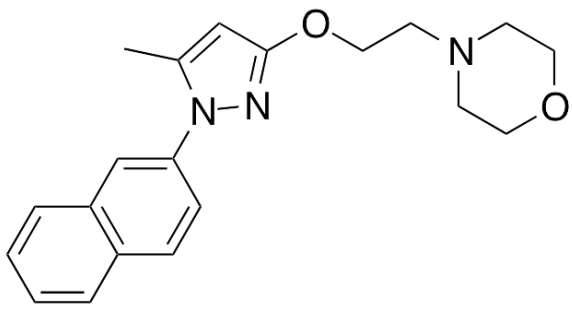


Figure 19. Molecular structure of the selective σ1R antagonist S1RA/E-52862. N, Nitrogen atoms; O, Oxygen atoms (Díaz *et al.*, 2012).

The functional activity of S1RA was evaluated using the phenytoin assay, where S1RA produced a small shift to lower-affinity values when incubated in the presence of the allosteric modulator (Ki without phenytoin / Ki with phenytoin = 0.8), thus suggesting antagonistic properties. The antagonistic nature of the compound was

further confirmed in mouse models of pain, in which S1RA exerted a clear dose-dependent analgesic effect on capsaicin-induced mechanical hypersensitivity, on both phases of formalin-induced pain and in the partial sciatic nerve ligation model (Romero *et al.*, 2012). Besides, the possible interference of S1RA with motor coordination and thus with the nociceptive responses was discarded testing the motor performance with the rotarod test (Díaz *et al.*, 2012; Romero *et al.*, 2012).

S1RA penetrates the blood-brain barrier and binds σ1R in the CNS, showing a significant correlation between the extent of CNS receptor occupancy and the antinociceptive effects elicited by S1RA in different pain models (Romero *et al.*, 2012). Additionally, its pharmacokinetic profile characterized in mice showed that after oral administration, the compound achieved a peak concentration in the plasma, forebrain and spinal cord at 0.5 h postdosing (Díaz *et al.*, 2012), being the levels higher in the CNS than in plasma (Romero *et al.*, 2012).

Safety evaluations indicated a low potential for drug-drug interactions, and no teratogenic, genotoxic, phototoxic or skin irritation effects were found at doses associated with preclinical analgesic activity (Díaz *et al.*, 2012). S1RA is currently undergoing phase II clinical trials for neuropathic pain and represents a potential first-in-class analgesic.

3.5.5 The dual mu-opioid receptor agonist – sigma-1 receptor antagonist SIMU

SIMU is a new chemical entity synthesized by Laboratories Esteve as an analgesic compound for the treatment of moderate to severe

chronic pain. It is a dual molecular compound binding to both human MOR as an agonist (Ki = 64 nM) and to σ1R as an antagonist (Ki = 118 nM). SIMU has shown high selectivity for these two receptors, as it failed to exhibit significant affinity for over more than 180 other molecular targets.

SIMU has been the selected compound after successfully completing selectivity and liability assays and early *in vivo* analysis. SIMU showed an efficacy profile comparable to oxycodone or morphine in different acute pain models revealing a better safety profile in terms of gastrointestinal side effects, CNS-related side effects, development of tolerance to the analgesic effect and naloxone-precipitated physical withdrawal. Altogether, this makes SIMU suitable to selectively antagonize σ1R and activate MOR, and to study its potential as a pain-relieving compound.

3.6 Therapeutic interest of the sigma-1 receptor

The diversity of compounds that bind to σ1R indicated an extensive contribution and pharmacological significance of the receptor in many diseases. Indeed, given the broad spectrum of modulatory effects reported for σ1R ligands and the widespread distribution of the receptor in the CNS and peripheral organs, drugs interacting with the σ1R appear to be useful in a large number of therapeutic fields. Many of the proposed indications are in the neurological field, such as schizophrenia, depression and anxiety, drug addiction, cognitive deficits, neurodegenerative disorders and pain (Cobos *et al.*, 2008; Maurice and Su, 2009; Zamanillo *et al.*, 2013; Nguyen *et al.*, 2017), but some unrelated indications such as cardioprotection (Bhuiyan and

Fukunaga, 2011) or cancer (Aydar *et al.*, 2004; Spruce *et al.*, 2004) have also been suggested. Although the therapeutic area with the greater number of patents is the psychotic disorder, the interest on the development of σ1R ligands for the treatment of schizophrenia has decreased, whereas the interest on pain treatments based on σ1R interaction has progressively grown (Zamanillo *et al.*, 2012) (Figure 20).

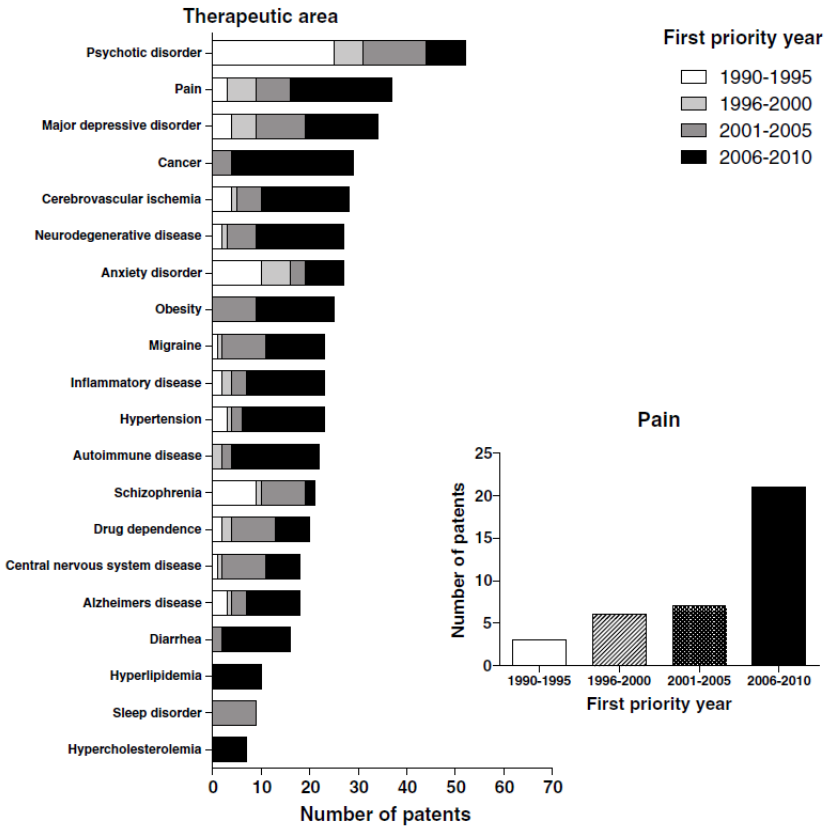


Figure 20. Patent activity surrounding σ1R ligands by therapeutic area. Source: WIPO-World Intellectual Property Organization (January 2011) (Zamanillo *et al.*, 2012).

3.6.1 Sigma-1 receptor and pain

σ1R is expressed in important areas of the nervous system for pain control such as the DRG, superficial layers of the spinal cord, the PAG and the RVM (Alonso *et al.*, 2000). Initially, probably because of their early confusion with opioid receptors, the interaction between opioid antinociception and the effects of σ1R drugs was explored in models of acute nociceptive pain (Chien and Pasternak, 1993). More recently, it has been shown that σ1R also plays a role in the modulation of pain behaviours in the absence of opioids in certain rodent models (Díaz *et al.*, 2009).

3.6.1.1 Sigma-1 receptor modulation of pain in acute and chronic pain conditions

σ1R ligands do not affect thermal and mechanical nociception in physiological conditions, as observed in the tail-flick, the hot plate and the paw pressure test in rodents (Marrazzo *et al.*, 2006; de la Puente *et al.*, 2009; Sánchez-Fernández *et al.*, 2013). However, σ1R plays a key role in modulating pain behaviour under sensitizing conditions (Zamanillo *et al.*, 2013), as studied using σ1R knockout mice and selective σ1R antagonists.

σ1R knockout mice, which perceive and respond normally to stimuli of different nature in naïve conditions (Cruz Miguel Cendán *et al.*, 2005; de la Puente *et al.*, 2009; Nieto *et al.*, 2012; González-Cano *et al.*, 2013; Gris *et al.*, 2014), have been widely used to study the involvement of the receptor in several pain conditions. Mice lacking σ1R showed more than 50% reduction of pain responses in both phases of the **formalin** test when compared to wild-type animals

(Cruz Miguel Cendán *et al.*, 2005), and failed to develop mechanical hypersensitivity after intraplantar administration of **capsaicin** (Entrena *et al.*, 2009b), indicating that σ1R is crucial for the full expression of formalin and capsaicin-induced pain. In addition, in a **visceral pain** model induced by intracolonic capsaicin, mice lacking σ1R showed a reduction in the number of pain behaviours when compared to wild-type mice (González-Cano *et al.*, 2013). In models of **neuropathic pain**, cold and mechanical hypersensitivity were strongly attenuated in σ1R knockout mice treated with paclitaxel (Nieto *et al.*, 2012) or exposed to partial sciatic nerve ligation (de la Puente *et al.*, 2009) or to spinal cord contusion (Castany *et al.*, 2018). Interestingly, while mice with a spinal cord contusion lacking σ1R also showed attenuation of thermal hyperalgesia (Castany *et al.*, 2018), it was fully developed after partial sciatic nerve ligation regardless of the genotype (de la Puente *et al.*, 2009). Similarly, in the carrageenan or the complete Freund's adjuvant (CFA)-induced **inflammatory pain**, the genetic inactivation of σ1R did not prevent the acquisition of thermal and punctate mechanical hypersensitivity (Gris *et al.*, 2014), whereas it prevented the paw pressure-induced mechanical hyperalgesia (Tejada *et al.*, 2014).

Investigations based on the use of **pharmacological antagonists for the σ1R** also contributed to determine the role of this receptor in pain sensitization. The antagonist haloperidol, S1RA and other σ1R antagonists inhibited **formalin**-induced pain (Cruz M. Cendán *et al.*, 2005; Kim *et al.*, 2006; Romero *et al.*, 2012; Lan *et al.*, 2014) and intraplantar **capsaicin**-induced sensitization in mice (Oberdorf *et al.*,

2008; Entrena *et al.*, 2009a, 2009b; Romero *et al.*, 2012). **Visceral pain** induced by intracolonic capsaicin was also inhibited by different σ1R antagonists, including S1RA (González-Cano *et al.*, 2013). Nociceptive responses have also been reversed using σ1R antagonists in different animal models of **neuropathic pain**, such as chronic compression of the DRG (Son and Kwon, 2010), the migraine model induced by intracisternal injection of capsaicin (Kwon *et al.*, 2009), trigeminal and diabetic neuropathy (Gris *et al.*, 2016), chemotherapy-induced neuropathic pain (Nieto *et al.*, 2012; Gris *et al.*, 2016), the partial sciatic nerve ligation (Romero *et al.*, 2012; Bura *et al.*, 2013) or the spinal cord contusion model (Castany *et al.*, 2018, 2019). Finally, blockade of the σ1R also produced an antinociceptive effect in the carrageenan and CFA-induced **inflammatory pain** (Gris *et al.*, 2014).

Overall, the σ1R appears to play a key role in pain hypersensitivity and in modulating nociception under certain pain conditions. Nevertheless, from a mechanistic point of view, it is still unclear the site of action and the possible mechanisms underlying the effect of σ1R antagonism treatment. Several evidences pointed to **spinal** Ca^{2+}-dependent second messenger cascades and enhanced NMDA responses as key mechanisms underlying the σ1R antinociceptive effects. *In vitro* electrophysiological recordings of isolated spinal cords revealed that the wind-up amplification responses that normally arise following repetitive stimulation of nociceptive afferent C-fibres were inhibited in σ1R knockout mice (de la Puente *et al.*, 2009) and after pharmacological blockade of the σ1R (Romero *et al.*, 2012; Mazo *et al.*, 2015). In addition, neurochemical approaches have been used to

investigate the effects of σ1R on spinal neurotransmitters and the NMDAR signalling. Data from microdialysis in the ipsilateral dorsal horn of awake, freely moving rats showed that pharmacological inactivation of σ1R reduces the formalin-evoked glutamate release and enhances noradrenaline spinal levels (Vidal-Torres *et al.*, 2014), suggesting an inhibition of the glutamatergic nociceptive inputs and an increase of the descending inhibitory inputs to the spinal cord. Regarding the modulation of NMDAR function by σ1R ligands, it is well documented that σ1R agonists increase while antagonists decrease NMDAR currents and Ca^{2+} flow through the channel (Monnet *et al.*, 1990; Yamamoto *et al.*, 1995; Bergeron *et al.*, 1996). Studies using formalin-induced nociception and several models of neuropathic pain showed increased phosphorylation levels of ERK and the NMDAR in the spinal cord, which were reduced in σ1R knockout mice and/or after administration of σ1R antagonists (Kim *et al.*, 2006; Roh *et al.*, 2008; de la Puente *et al.*, 2009; Son and Kwon, 2010; Nieto *et al.*, 2012; Castany *et al.*, 2018, 2019).

Some preclinical studies support a role of **supraspinal** σ1R in the modulation of pain sensitization. S1RA also attenuated formalin-induced pain behaviours when injected intracerebroventricularly (Vidal-Torres *et al.*, 2014). Moreover, intracisternal or systemic BD-1047 inhibited intracisternal capsaicin-evoked headache pain (Kwon *et al.*, 2009) and the nociceptive responses in the orofacial formalin model (Roh and Yoon, 2014), respectively. This σ1R antagonist reduced NMDAR phosphorylation (Kwon *et al.*, 2009), and decreased the number of cFos-immunoreactive cells and the phosphorylation of

p38 MAPK in the trigeminal nucleus caudalis (Roh and Yoon, 2014) in these pain models.

Increasing evidences suggest that activity from the periphery is essential, not only to initiate but also to maintain pain symptoms (Richards and McMahon, 2013). Interestingly, σ1R expression in DRG is roughly an order of magnitude higher than in several CNS areas involved in pain signalling (Sánchez-Fernández *et al.*, 2014), thus pointing to a functional role of **peripheral** σ1R in pain modulation. Indeed, intraplantar administration of σ1R antagonists in the inflamed paw was sufficient to completely reverse the hypersensitivity induced by carrageenan (Tejada *et al.*, 2014) or formalin (Vidal-Torres *et al.*, 2014). Besides, the intraplantar injection of the σ1R agonist PRE-084 abolished the antinociceptive effect of systemic antagonists (Tejada *et al.*, 2014). In addition, σ1R blockade prevented the paclitaxel-induced mitochondrial damage in the peripheral saphenous nerve (Nieto *et al.*, 2014), and it has also been reported an alteration of the σ1R expression in peripheral tissues after spinal nerve injury (Bangaru *et al.*, 2013) or thrombus-induced ischemic pain (Kwon *et al.*, 2016).

3.6.1.2 Sigma-1 receptor modulation of opioid-induced analgesia

Investigations on the role of **σ1R on opioid antinociception** began in 1993 in Pasternak's laboratory, where it was demonstrated that the selective σ1R agonist (+)-pentazocine attenuated morphine antinociception, whereas the non-selective σ1R antagonist haloperidol greatly enhanced this opioid effect (Chien and Pasternak, 1993). Later on, the observations on opioid modulation were supported by studies using other σ1R ligands and other opioid

receptor ligands (δ- and κ- in addition to μ-opioid receptors) in both thermal and mechanical acute nociception (tail-flick or paw pressure test, respectively) (Chien and Pasternak, 1994, 1995a, 1995b; Mei and Pasternak, 2002, 2007; Marrazzo *et al.*, 2006; Sánchez-Fernández *et al.*, 2013, 2014; Vidal-Torres *et al.*, 2013). These results suggested an anti-opioid sigma mechanism in which σ1R exerted a tonic inhibitory control on the opioid receptor-mediated antinociception, which could be pharmacologically counteracted by using σ1R antagonists to increase the response to opioids. However, it is important to notice that the antagonist BD-1047 prevented, while NE-100 did not potentiate the analgesic effect of a κ-opioid receptor agonist during heat (Prezzavento *et al.*, 2008) or chemical (Hiramatsu *et al.*, 2002) acute nociception, respectively. These results suggest that σ1R modulation of opioid antinociception might be ligand- and model-dependent.

Interestingly, the increase in opioid potency appears to be limited to the analgesic effect, but not to its **side effects**. When co-administered with MOR agonists, σ1R antagonists enhanced its antinociceptive effect in the tail-flick or the paw pressure tests, whereas the reward effect of morphine was attenuated (Vidal-Torres *et al.*, 2013), and opioid-induced antinociceptive tolerance and inhibition of gastrointestinal transit, hyperlocomotion or mydriasis were not modified (Chien and Pasternak, 1994; Vidal-Torres *et al.*, 2013; Sánchez-Fernández *et al.*, 2014). In the same line, the non-analgesic effects of morphine on locomotion and gastrointestinal transit were unaltered by the genetic inactivation of σ1R (Sánchez-Fernández *et*

al., 2013). Altogether, these results suggest that the modulatory effect of σ1R on opioid analgesia can be dissociated from other opioid effects (Vidal-Torres *et al.*, 2013).

Interestingly, the modulatory effects on opioid analgesia did not seem to depend on **spinal** σ1R, given that intrathecal administration of σ1R ligands did not modify the antinociceptive effect of systemic or spinal morphine (Mei and Pasternak, 2002; Vidal-Torres *et al.*, 2019). Moreover, although individually-administered morphine and S1RA increased noradrenaline spinal levels, they failed to modify the spinal concentrations of this neurotransmitter when drugs were combined (Vidal-Torres *et al.*, 2019). This result discarded the modulation of noradrenaline-dependent descending inhibition as the mechanism underlying the potentiation of opioid analgesia mediated by the σ1R antagonism.

Several evidences pointed to a key role of **supraspinal** σ1R in the modulation of opioid analgesia, since such modulation also occurred when σ1R and/or MOR ligands were administered intracerebroventricularly (Mei and Pasternak, 2002; Marrazzo *et al.*, 2006; Rodríguez-Muñoz *et al.*, 2015b; Vidal-Torres *et al.*, 2019). Indeed, some brain regions including PAG, RVM and the locus coeruleus have been identified as supraspinal sites where σ1R ligands might exert their modulatory effects on opioid analgesia in the tail-flick test (Mei and Pasternak, 2007). Intracranial co-administration of morphine and the σ1R agonist (+)-pentazocine into PAG, RVM or the locus coeruleus diminished the opioid analgesia, whereas σ1R blockade by haloperidol enhanced morphine antinociception only

when co-injected in the RMV (Mei and Pasternak, 2007). However, another study recently showed that S1RA directly injected into the RVM failed to increase the effects of systemic morphine (Vidal-Torres *et al.*, 2019). Therefore, further studies are required to understand the role of σ1R in specific brain areas modulating opioid analgesia.

Peripherally, intraplantar opioid agonists showed increased antinociception in σ1R knockout mice and when they are locally combined with σ1R antagonists (Sánchez-Fernández *et al.*, 2013, 2014). Furthermore, the enhanced analgesia was completely reversed by the peripherally-restricted opioid antagonist naloxone methiodide (Sánchez-Fernández *et al.*, 2014).

σ1R has been shown to be physically associated with MOR, and σ1R antagonists potentiate opioid-induced G protein-coupled transduction without influencing opioid receptor binding (Kim *et al.*, 2010). It has been widely recognized the existence of a **bidirectional crosstalk** between **MOR** and **NMDAR**, in which the stimulation of MOR results in enhancement of NMDAR conductance, whereas NMDAR activation ultimately reduces MOR function (Garzón *et al.*, 2012). This cross-regulation is involved in nociceptive transmission and in the development of morphine tolerance (Mao, 1999; Trujillo, 2002; Inoue *et al.*, 2003; Rodríguez-Muñoz *et al.*, 2012). Recent studies indicated that such interaction required the **σ1R**, which cooperated with the **histidine triad nucleotide-binding protein 1** (**HINT1**) to modulate the negative influence of NMDARs on MOR activity (Rodríguez-Muñoz *et al.*, 2015a, 2015b). It has been proposed that σ1R associates with NMDAR-NR1 subunit in a Ca^{2+}-dependent manner and when activated

by σ1R agonists. Then, σ1R keeps HINT1 bound to MOR, favouring its positive regulation upon NMDARs. The activated glutamate receptors induce Ca^{2+}/calmodulin-dependent kinase II activity, which in turn phosphorylate MOR and reduce its association with G-proteins, thus promoting a loss of the antinociceptive capacity of MOR agonists. In contrast, σ1R antagonists detach σ1R from NMDAR and allow the transfer of HINT1 from MOR to NMDAR. This results in the disruption of the cross-talk between both receptors, hence releasing MOR from the negative modulation by NMDARs and endorsing opioid analgesia (Rodríguez-Muñoz *et al.*, 2015a, 2015b) (Figure 21).

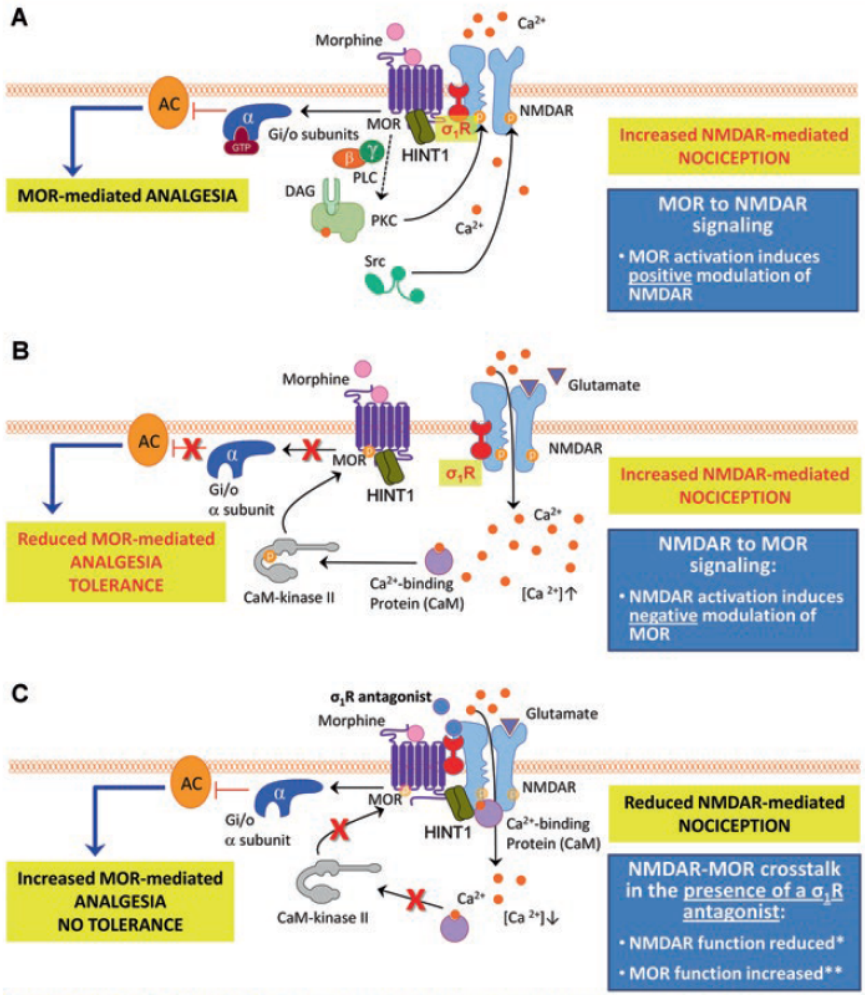


Figure 21. Proposed mechanism for σ1R antagonists to enhance opioid analgesia based on the modulation of the μ-opioid receptor (MOR)-NMDAR crosstalk by σ1R. (**A**) σ1R is associated with NMDAR and maintains HINT1 bound to MOR, favouring the positive modulation of MOR on NMDAR. Thus, upon MOR activation, NMDARs are phosphorylated, increasing their activity. (**B**) As a consequence of increased Ca^{2+} influx through NMDARs, the Ca^{2+}/calmodulin-dependent kinase II (CaM-kinase II) becomes activated and phosphorylates MORs, which reduces MOR-mediated analgesia and the response to subsequent morphine challenges (promotes tolerance). (**C**) The absence of σ1R or treatment with a σ1R antagonist to detach σ1R from NMDA-NR1 subunit induces a reduction on NMDAR function, hence impairing its negative feedback on MORs. Therefore, this is the proposed mechanism by which σ1R antagonists enhance opioid analgesia by releasing MORs from the negative influence of NMDARs (Merlos *et al.*, 2017).

OBJECTIVES

Objective 1

To investigate the role of σ1R in the nociceptive and neurochemical alterations associated to osteoarthritis pain and its participation on opioid tolerance.

Article #1

Sigma-1 receptor modulates neuroinflammation associated with mechanical hypersensitivity and opioid tolerance in a mouse model of osteoarthritis pain

Mireia Carcolé, Sami Kummer, Leonor Gonçalves, Daniel Zamanillo, Manuel Merlos, Anthony H. Dickenson, Begoña Fernández-Pastor, David Cabañero*, Rafael Maldonado*

British Journal of Pharmacology, (2019)

Objective 2

To study the involvement of σ1R in the emotional and cognitive manifestations of osteoarthritis pain

Article #2

Blockade of the sigma-1 receptor relieves cognitive and emotional impairments associated to chronic osteoarthritis pain

Mireia Carcolé, Daniel Zamanillo, Manuel Merlos, Begoña Fernández-Pastor, David Cabañero*, Rafael Maldonado*

Frontiers in Pharmacology, 10:468 (2019)

Objective 3

To elucidate the efficacy of SIMU, a dual compound acting as σ1R antagonist and MOR agonist, and the participation of central and peripheral MOR on its antinociceptive effects during osteoarthritis pain.

Supplementary results

A novel compound acting over MOR and σ1R relieves osteoarthritis pain in mice: participation of MOR

Mireia Carcolé, Begoña Fernández-Pastor, David Cabañero, Rafael Maldonado

RESULTS

Article #1

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Carcolé M, Kummer S, Gonçalves L, Zamanillo D, Merlos M, Dickenson AH, et al. Sigma-1 receptor modulates neuroinflammation associated with mechanical hypersensitivity and opioid tolerance in a mouse model of osteoarthritis pain. British journal of pharmacology. 2019;176(20):3939–55. DOI: 10.1111/bph.14794

Article #2

Blockade of the sigma-1 receptor relieves cognitive and emotional impairments associated to chronic osteoarthritis pain

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Carcolé M, Zamanillo D, Merlos M, Fernández-Pastor B, Cabañero D, Maldonado R. Blockade of the Sigma-1 Receptor Relieves Cognitive and Emotional Impairments Associated to Chronic Osteoarthritis Pain. Frontiers in pharmacology. 2019;10:468–468. DOI: 10.3389/fphar.2019.00468

Supplementary results

A novel compound acting over MOR and σ1R relieves osteoarthritis pain in mice: participation of MOR

Mireia Carcolé, Begoña Fernández-Pastor, David Cabañero, Rafael Maldonado

(a) The simultaneous blockade of σ1R and stimulation of MOR was effective alleviating osteoarthritis pain.

SIMU showed greater acute and chronic antinociceptive efficacy than EST-A in a murine model of osteoarthritis pain.

SIMU and EST-A are multimodal drugs which act as antagonists of σ1R and agonists of MOR. To assess its therapeutic potential in chronic osteoarthritis pain, we evaluated the sensitivity in response to static mechanical pressure 30 minutes after drug administration on CD1 mice intra-articularly injected with MIA. The single administration of either SIMU or EST-A showed acute effects decreasing the MIA-induced mechanical allodynia 30 min after i.p. administration in a dose-dependent manner (Figure 22A). However, SIMU revealed greater analgesic efficacy (ED_{50} ≈ 6 mg/kg) than EST-A (ED_{50} ≈ 32 mg/kg), as reflected by a lower median effective dose. Thus, SIMU was 5 times more potent than EST-A alleviating osteoarthritis pain.

To assess the analgesic effect of these drugs during a chronic administration, mice were repeatedly treated for 2 weeks, twice a day by i.p. route, at doses showing equivalent acute antinociceptive effects (7 mg/kg for SIMU and 50 mg/kg for EST-A). Mechanical allodynia was assessed with the von Frey test before (PRE) and 30 min after (POST) the first daily dose. The chronic SIMU treatment induced a slight recovery of the mechanical thresholds assessed before the daily administration of the drug (Figure 22B). This recovery was significant since the seventh day of treatment ($p < 0.05$ for MIA – PRE-treatment vs. MIA – PRE-treatment at day 1) and became more prominent at day 10 ($p < 0.001$ for MIA – PRE-treatment vs. MIA –

PRE-treatment at day 1). Moreover, repeated SIMU did not show a loss of analgesia over time, whereas osteoarthritic mice strongly developed tolerance to the antinociceptive effect of EST-A, revealed by the absence of analgesic efficacy from the seventh until the last day of treatment ($p < 0.01$ for MIA – POST-treatment vs. MIA – POST-treatment at day 1; Figure 22C). Hence, the simultaneous blockade of σ1R and stimulation of MOR by SIMU induced an antiallodynic effect after acute and chronic administrations.

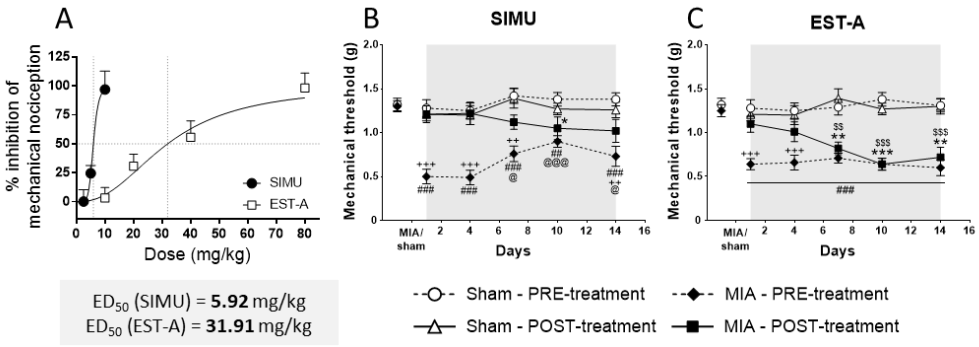


Figure 22. The σ1R antagonist and MOR agonist SIMU produces acute and long-lasting normalization of mechanical thresholds during osteoarthritis pain. (**A**) SIMU and EST-A showed analgesic efficacy after acute administrations, but lower doses were needed to induce acute pain relief with SIMU than with EST-A. (**B**) The SIMU-induced antinociception was maintained for the whole duration of the chronic treatment, and it produced a slight recovery of mechanical thresholds measured before the daily doses (PRE values). (**C**) Mice repeatedly treated with EST-A developed a strong analgesic tolerance. Data is expressed as mean ± SEM (n = 8 animals per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for MIA-POST vs. Sham-POST; ## $p < 0.01$, ### $p < 0.001$ for MIA-PRE vs. Sham-PRE; ++ $p < 0.01$, +++ $p < 0.001$ for MIA-PRE vs. MIA-POST; @ $p < 0.05$, @@@ $p < 0.001$ for MIA-PRE vs. MIA-PRE day 1; $$ $p < 0.01$, $$$ $p < 0.001$ for MIA-POST vs. MIA-POST day 1 (3-way repeated measures ANOVA plus Fisher least significant difference test). MIA, monoiodoacetate; SEM, standard error of the mean.

(b) The analgesic efficacy of SIMU was dependent on peripheral and central MORs.

To assess the participation of central and peripheral MOR on the antinociceptive effect of SIMU, different lines of MOR knockout mice were used. Osteoarthritis pain was induced by intra-articular MIA injection into the knee joint of male and female wild-type (WT), constitutive MOR knockouts (Total KO), and conditional knockout mice lacking the receptor either in the peripheral neurons expressing the sodium channel Nav1.8 (Nav1.8 KO) (Weibel *et al.*, 2013) or in GABAergic neurons of the forebrain (Dlx5/6 KO) (Charbogne *et al.*, 2017). During the first week after the MIA injection, sensitivity in response to static mechanical pressure was assessed to compare the development of mechanical hypersensitivity between the different genotypes. Mice were treated s.c. twice a day for 14 days with either vehicle or SIMU (5 mg/kg) starting the treatment 7 days after MIA or sham injection, and mechanical sensitivity was tested before (PRE) and 30 min after (POST) administration (Figure 23).

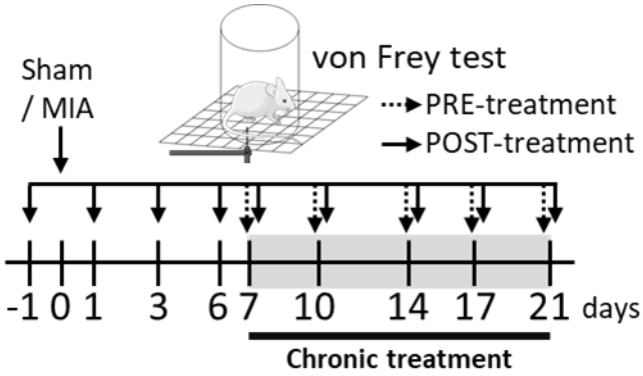


Figure 23. Schematic representation of the experimental design. Wild-type (WT), constitutive MOR knockouts (Total KO), and peripheral and central conditional MOR knockouts (Nav1.8 KO and Dlx5/6 KO, respectively) mice received an intra-knee injection of MIA or saline (sham) and were treated subcutaneously with vehicle or SIMU (5 mg/kg) twice a day from day 7 to day 21 after the intra-articular injection. Mechanical allodynia was assessed under basal conditions, at days 1, 3 and 6, and before (PRE) and 30 min after (POST) the first daily dose at days 7, 14, 17 and 21.

Male mice with complete or peripheral lack of MOR showed a differential development of mechanical hypersensitivity after MIA injection.

The intra-knee injection of MIA led to a marked decrease of the withdrawal thresholds to mechanical stimuli when compared to sham in male (Figure 24A) and female (Figure 24B) mice of all genotypes ($p < 0.001$ for MIA vs. sham). This mechanical allodynia was shown from the first day after MIA injection until the initiation of the repeated treatments. However, male mice showed differential sensitivity between genotypes. Animals with a total absence of MOR showed reduced mechanical sensitivity compared to WT or conditional KO mice ($p < 0.05$ for Total KO vs. WT; $p < 0.001$ for Total KO vs. Nav1.8 KO; $p < 0.01$ for Total KO vs. Dlx5/6 KO; Figure 24A). On the other hand, mice without MOR in peripheral neurons exhibited greater

mechanical allodynia that WT animals ($p < 0.05$ for Nav1.8 KO vs. WT; Figure 24B). Therefore, although all mice developed osteoarthritis pain, our results suggest that the lack of peripheral or central MOR might impact on the MIA-induced pain sensitivity.

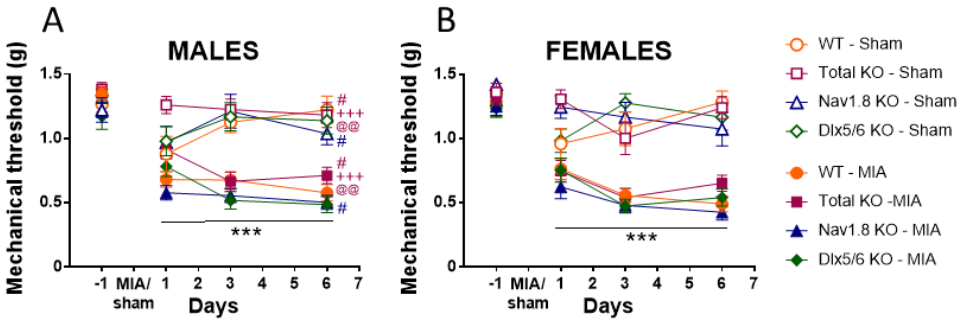


Figure 24. The complete lack of MOR in male mice induced hyposensitivity to mechanical stimuli, while the absence of the peripheral receptors produced enhanced pain responses Mice from all genotypes developed mechanical hypersensitivity after MIA injection, as observed by the decreased mechanical thresholds observed in both male (**A**) and female (**B**) mice. Besides, male mice (**A**) with a complete lack of MOR showed a reduced sensitivity after intra-knee injections, whereas mice lacking peripheral MOR presented increased responses to mechanical stimuli compared to WT animals. Mice lacking central MOR showed similar mechanical allodynia than WT animals. Female mice (**B**) did not exhibit differences between genotypes. Data is expressed as mean ± SEM (n = 8 - 11 animals per sham group / 16 – 30 animals per MIA group). *** $p < 0.001$ for MIA vs. Sham; # $p < 0.05$ vs. WT; +++ $p < 0.001$ vs. Nav1.8 KO; @@ $p < 0.01$ vs. Dlx5/6 KO (3-way repeated measures ANOVA plus Fisher least significant difference test). MIA, monoiodoacetate; SEM, standard error of the mean; WT, wild-type.

SIMU alleviated osteoarthritis pain in control WT and total KO mice, but not in Nav1.8 and Dlx5/6 KO mice.

Vehicle or SIMU were repeatedly administered twice a day for 14 days to male and female mice from all genotypes, starting the treatment 7 days after sham or MIA intra-knee injection. During vehicle treatment, male and female mice lacking MOR completely or in the forebrain did

not show alterations in their normal mechanical thresholds after the sham injection compared to WT animals (Figure 25A, B and C, Figure 26A, B and C). In agreement with the previous results, male Nav1.8 KO mice exhibited increased mechanical allodynia compared to WT and total KO mice ($p < 0.05$ for Nav1.8 KO vs. WT; $p < 0.001$ for Nav1.8 KO vs. Total KO; Figure 25A and C). Therefore, the lack of MOR in peripheral neurons expressing Nav1.8 induces over-sensitization of the normal mechanical thresholds that remains until day 21 after sham injection. The MIA-induced mechanical hypersensitivity observed in previous results was persistent during the repeated treatment with vehicle and was maintained until the end of the experimental protocol. Total KO male mice still exhibited higher withdrawal thresholds than WT and conditional KO mice ($p < 0.01$ for Total KO vs. WT; $p < 0.05$ for Total KO vs. Nav1.8 KO; $p < 0.01$ for Total KO vs. Dlx5/6 KO; Figure 25D and F). Female mice did not show differences in the MIA-induced hypersensitivity when compared to WT animals, but total KO mice did exhibit lower allodynia than Dlx5/6 KO animals ($p < 0.05$ for Total KO vs. Dlx5/6 KO; Figure 26C). As expected, the repeated treatment with SIMU alleviated the MIA-induced pain in WT mice, both male and female, as seen by the increased mechanical thresholds showed after the daily injections ($p < 0.001$ for MIA-SIMU POST vs. MIA-SIMU PRE; Figure 25I and Figure 26I). Surprisingly, SIMU administrations also reduced the mechanical allodynia in male and female mice completely lacking MOR ($p < 0.01$ for MIA-SIMU POST vs. MIA-SIMU PRE; Figure 25I and Figure 26I). Interestingly, total KO female mice showed a recovery of the mechanical thresholds assessed before the daily SIMU

administrations, as revealed by a gradual normalization of pain sensitization in the PRE values ($p < 0.01$ for Total KO vs. Total KO at day 7; Figure 26Figure 26G). On the other hand, when there was an absence of MOR only in the peripheral nociceptors, this dual compound showed no effect relieving osteoarthritis pain (Figure 25I and Figure 26I). These results support that the peripheral MORs have a crucial role, not only in the σ1R modulation of opioid analgesia but also in σ1R antinociception itself. Finally, SIMU chronic administration induced antinociceptive effects in Dlx5/6 KO mice only at the beginning of the treatment, but such effect was lost over time, revealed by the reduced mechanical thresholds observed from the eighth when compared to the first day of treatment ($p < 0.01$ for Dlx5/6 KO vs. Dlx5/6 KO at day 7; Figure 25H and Figure 26H). Overall, these results imply that peripheral MORs are key to observe an analgesic effect of SIMU, whereas central MORs are important for the maintenance of such effect. The unexpected presence of SIMU antinociception in total MOR KO mice, which lack MOR at both peripheral and central levels of the pain pathway, pointed to a possible alteration in the σ1R signalling in these constitutive KO mice.

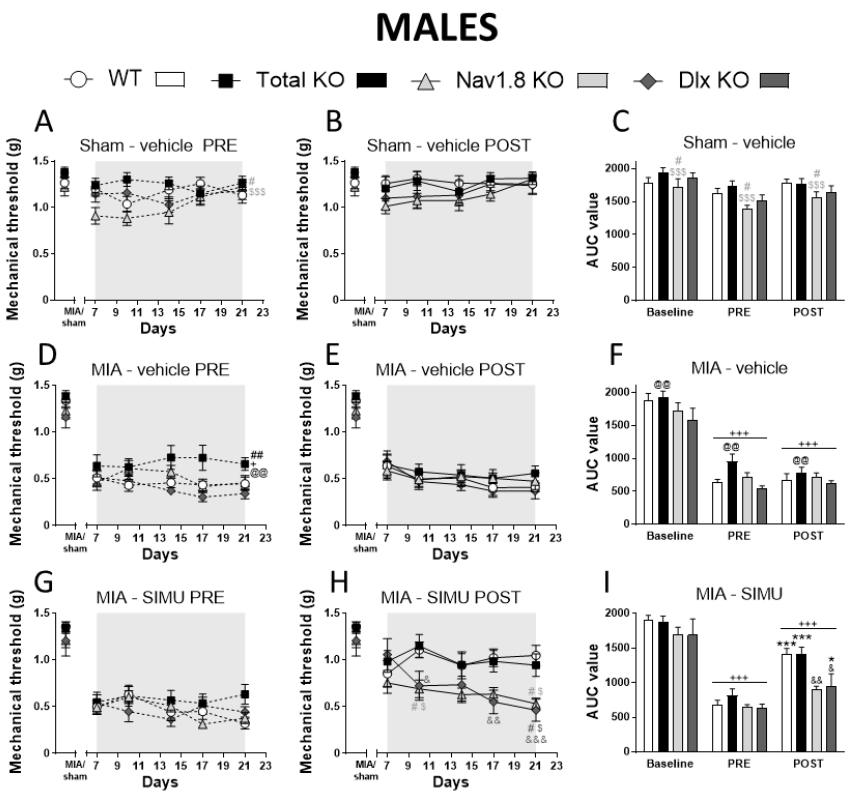


Figure 25. SIMU alleviated osteoarthritis pain in control WT and total MOR KO male mice, whereas it did not alter mechanical hypersensitivity in Nav1.8 KO mice and it only showed initial antinociceptive effects in Dlx5/6 KO animals. (**A, B, C**) Nav1.8 KO mice presented hypersensitization after sham-injection, whereas WT, total and Dlx5/6 KO showed no alterations of normal mechanical sensitivity in the PRE (**A**) and POST (**B**) time-course and the overall values measured with the area under the curve (AUC; **C**). (**D, E, F**) Mice of all genotypes treated with vehicle developed mechanical allodynia after MIA injection; however animals completely lacking MOR exhibited reduced sensitization. (**G, H, I**) SIMU treatment produced antinociception in WT and total KO mice, but not in Nav1.8 or Dlx5/6 KO animals. Data is expressed as mean ± SEM (n = 8 - 15 animals per group). For figures A-B, D-E, G-H: # $p < 0.05$, ## $p < 0.01$ vs. WT; \$ $p < 0.05$, \$\$\$ $p < 0.001$ vs. Total KO; + $p < 0.05$ vs. Nav1.8 KO; @@ $p < 0.01$ vs. Dlx5/6 KO; & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ vs day 7 (2-way repeated measures ANOVA plus Fisher least significant difference test). For figures C, F, I: # $p < 0.05$, \$\$\$ $p < 0.001$ vs. Total KO; @@ $p < 0.01$ vs. Dlx5/6 KO; +++ $p < 0.001$ vs. Baseline; & $p < 0.05$, && $p < 0.01$ vs. WT-POST; * $p < 0.05$, *** $p < 0.001$ vs. PRE (2-way repeated measures ANOVA plus Fisher least significant difference test). MIA, monoiodoacetate; SEM, standard error of the mean; WT, wild-type.

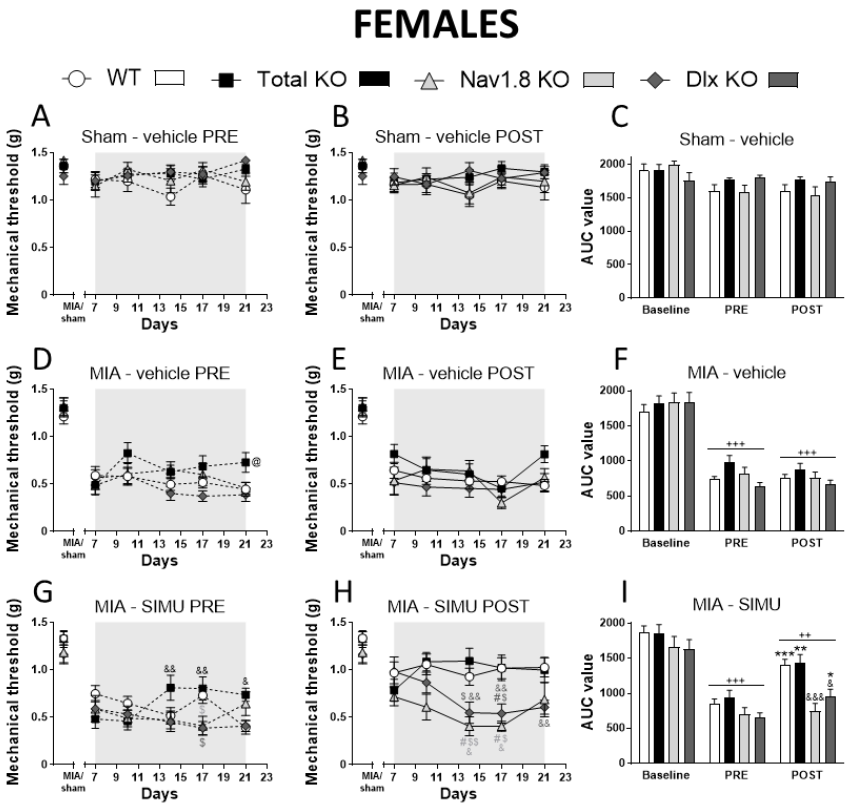


Figure 26. SIMU treatment showed analgesic efficacy in WT and total MOR KO female mice during osteoarthritis pain, but not in conditional KO animals. Normal mechanical thresholds were not affected in any genotype after an intra-knee injection of saline, neither in the PRE (**A**) and POST (**B**) time-course nor in the area under the curve (AUC) values of the overall treatment (**C**). (**D, E, F**) Vehicle-treated mice of every genotype developed persistent mechanical hypersensitivity after MIA. (**G, H, I**) Nav1.8 KO mice did not show alleviation of MIA-induced pain after SIMU, whereas WT and total KO animals presented a complete pain relief and Dlx5/6 KO mice only showed mitigation of osteoarthritis pain at the beginning of the repeated treatment. Data is expressed as mean ± SEM (n = 8 - 13 animals per group). For figures A-B, D-E, G-H: # $p < 0.05$ vs. WT; \$ $p < 0.05$, \$\$ $p < 0.01$ vs. Total KO; @ $p < 0.05$ vs. Dlx5/6 KO; & $p < 0.05$, && $p < 0.01$ vs day 7 (2-way repeated measures ANOVA plus Fisher least significant difference test). For figures C, F, I: ++ $p < 0.01$, +++ $p < 0.001$ vs. Baseline; & $p < 0.05$, &&& $p < 0.001$ vs. WT-POST; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. PRE (2-way repeated measures ANOVA plus Fisher least significant difference test). MIA, monoiodoacetate; SEM, standard error of the mean; WT, wild-type.

Blockade of peripheral MOR in WT animals induced a reversion of the analgesic effect of SIMU.

The next step was to further comprehend the analgesic efficacy of SIMU in mice completely missing MOR and the lack of such effect when MOR was absent only in the periphery or the forebrain. For this purpose, Naloxone methiodide (NX-ME), a MOR antagonist that does not cross the blood-brain barrier, was used to block the peripheral opioid receptors in WT mice, whereas Naloxone was administered to both conditional mice (Nav1.8 and Dlx5/6 KO) to antagonize all MORs and thus pharmacologically mimic a total KO animal. These MOR antagonists were co-administered with SIMU the last day of the previous experimental protocol. The dual compound administered alone alleviated pain in WT mice, but not in the conditional KOs ($p < 0.001$ for Pre SIMU vs. SIMU; Figure 27A and B), as observed previously on the last day of the repeated treatment. In WT mice, when SIMU was injected together with NX-ME, the analgesic effect was inhibited (Figure 27A and B), thus indicating that the antinociceptive effect of SIMU in WT animals relies on the peripheral MOR population. However, the complete blockade of MOR in both conditional KO mice did not mimic the analgesic effect of SIMU seen in the total KO animals (Figure 27A and B), suggesting adaptive alterations in these mutant mice.

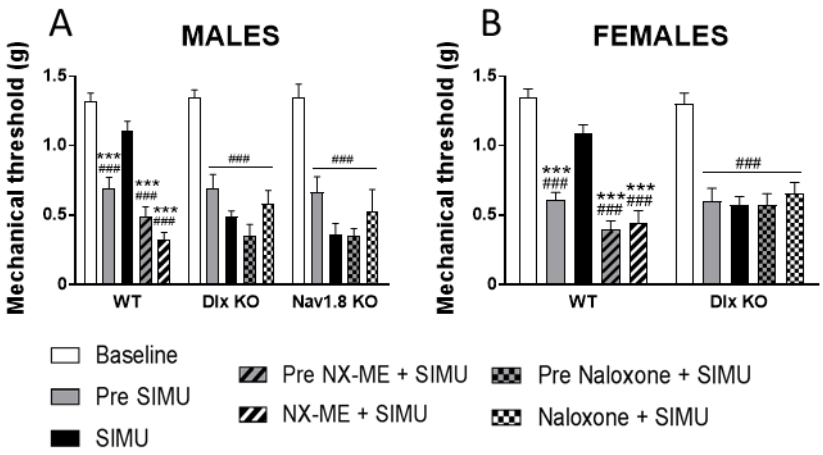


Figure 27. Peripheral MORs are crucial for the analgesic effect of SIMU in WT animals. The co-administration of SIMU with Naloxone methiodide (NX-ME) reverted the antinociceptive efficacy of the dual compound in both male (**A**) and female (**B**) mice. The MIA-induced mechanical allodynia of conditional KO mice remained unaltered after SIMU or SIMU in combination with Naloxone. Data is expressed as mean ± SEM (n = 8 animals per group). ### $p < 0.001$ vs. Baseline; *** $p < 0.001$ vs. SIMU (2-way repeated measures ANOVA plus Fisher least significant difference test). MIA, monoiodoacetate; SEM, standard error of the mean; WT, wild-type.

σ1R expression was altered in mice completely or partially lacking MOR.

Our previous results pointed to possible adaptative molecular changes in the total KO mice which might explain whether SIMU produced analgesia in these animals but not in the conditional KOs. Therefore, we analysed the expression of the σ1R by qPCR at different levels of the pain pathway. For this purpose, the thalamus, spinal cord and DRG were extracted the day following the end of the repeated treatment. MIA-induced osteoarthritis pain and chronic SIMU did not affect the σ1R expression at the supraspinal, spinal nor peripheral level (Figure 28A, B and C). Mice with a complete absence of MOR

expressed higher levels of σ1R at the peripheral DRG and the thalamus ($p < 0.05$ for Total KO vs. WT; Figure 28A and C), whereas the mRNA levels of the receptor were unchanged in the spinal cord (Figure 28B). Interestingly, while animals lacking MOR at the periphery presented increased σ1R expression in the DRG ($p < 0.001$ for Nav1.8 KO vs. WT; $p < 0.01$ for Nav1.8 KO vs. Dlx5/6 KO; $p < 0.05$ for Nav1.8 KO vs. Total KO; Figure 28C), mice without MOR in the forebrain exhibited higher σ1R levels in the thalamus ($p < 0.05$ for Dlx5/6 KO vs. WT; Figure 28A). Spinal σ1R was not modified in any of the conditional MOR KO (Figure 28B). These results indicate that levels of σ1R are enhanced in the central and the peripheral nervous system when lacking the opioid receptor in such areas. The unaltered levels of spinal σ1R suggest that this receptor population is not involved in the SIMU analgesic effect in the constitutive KO mice nor in the lack of antinociception in the conditional KOs. On the other hand, the increased expression of σ1R in both central and peripheral regions of the pain pathway might explain the efficacy of SIMU in the total MOR KO mice. This result agrees with the absence of the SIMU analgesic effect when all MORs were pharmacologically blocked in the conditional KOs, which do not share the same σ1R expression pattern than the constitutive KO mice.

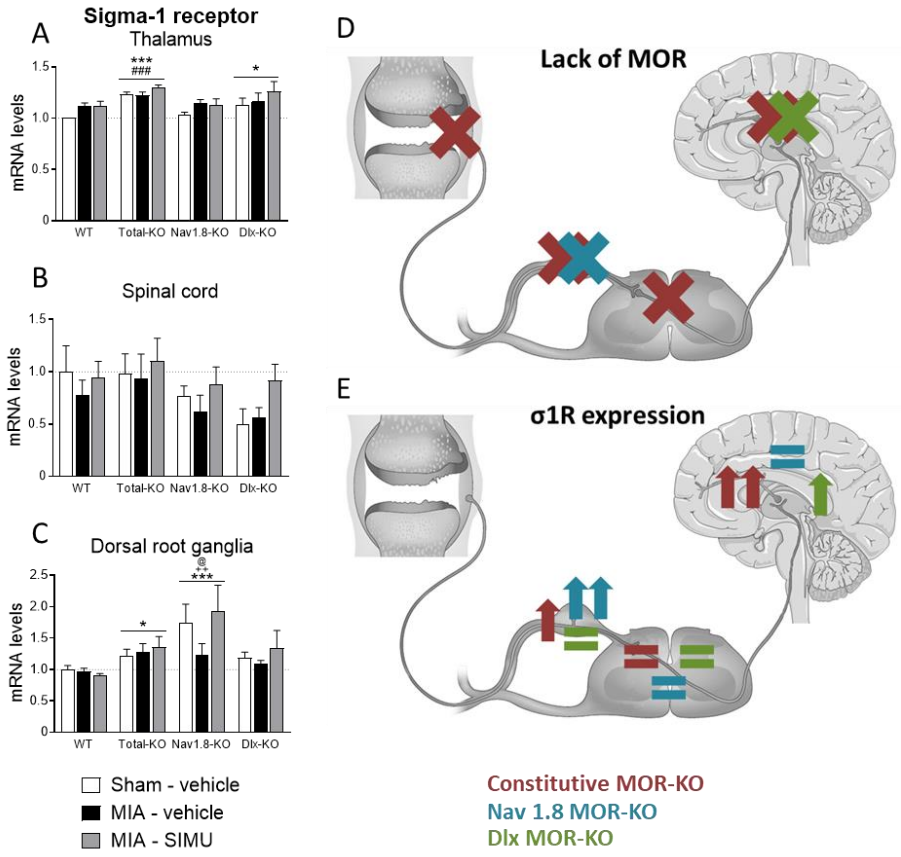


Figure 28. Sigma-1 receptor (σ1R) expression is enhanced in areas of the nervous system lacking mu-opioid receptor (MOR). (**A**) σ1R levels in the thalamus are increased in total and Dlx5/6 KO mice. (**B**) Spinal σ1R expression is unaltered regardless of the genotype. (**C**) Total and Nav1.8 KO animals showed higher levels of σ1R in the DRG. MIA injection and SIMU treatment did not alter the σ1R expression in any of the tissues (**A**, **B**, **C**). (**D**) Crosses represent the lack of MOR in the different regions of the pain pathway in constitutive (red), peripheral (blue) and central (green) MOR KO animals. (**E**) Arrows symbolize the changes on the σ1R expression, whereas the equal symbol represents unaltered mRNA levels in constitutive (red) and conditional (blue and green) KO mice. Data is expressed as mean ± SEM (n = 5 animals per group). * $p < 0.05$, *** $p < 0.001$ vs. WT; ### $p < 0.001$ vs. Nav1.8 KO; @ $p < 0.05$ vs. Total KO; ++ $p < 0.01$ vs. Dlx5/6 KO (2-way ANOVA followed by Fisher least significant difference test). MIA, monoiodoacetate; SEM, standard error of the mean; WT, wild-type.

DISCUSSION

The overall purpose of this doctoral thesis was to explore the role of σ1R and its pharmacological blockade on osteoarthritis pain to clarify the possible therapeutic interest of this novel pharmacological target. Using a mouse model of this chronic pain condition, we have focused our attention in the contribution of σ1R alone or combined with opioid therapy, at different stages of the disease and in several behavioural pain manifestation, as well as in pain-associated biochemical alterations at the periphery, spinal and supraspinal level.

The acute blockade of the σ1R on the nociceptive manifestations of osteoarthritis pain

S1RA did not modify mechanical sensitivity of sham mice nor the responses of the contralateral paws of MIA-injected animals, which exhibit normal mechanical thresholds (La Porta *et al.*, 2013; Negrete *et al.*, 2017). This suggests that normal transmission and perception of sensory inputs remain intact following antagonism of σ1R. In agreement, responses of σ1R KO mice to mechanical and thermal stimuli were found to be indistinguishable from those of WT animals in the absence of sensitization (de la Puente *et al.*, 2009; Entrena *et al.*, 2009b; Nieto *et al.*, 2014). This is in accordance with the chaperone activity of σ1Rs, which increase their affinity for their target ion channels, receptors or kinases only when these are conformationally unstable under pathological conditions demanding the assistance of σ1R chaperones (Hayashi and Su, 2007; Tsai *et al.*, 2009; Su *et al.*, 2010). Therefore, the biochemical action of σ1Rs is modulatory in nature and the consequences of their stimulation may only be manifested when another biological system is first activated.

Under MIA-induced sensitization, S1RA dose-dependently inhibited the mechanical hypersensitivity associated with osteoarthritis. This result confirms and extends the spectrum of analgesic activity revealed in previous studies, where S1RA showed efficacy alleviating a broad range of acute and chronic pain conditions (Nieto *et al.*, 2012; Romero *et al.*, 2012; Gris *et al.*, 2014, 2016; Castany *et al.*, 2018). This effect was produced by the interaction of S1RA with the σ1R since the σ1R agonist PRE-084 blocked S1RA acute antinociception. S1RA selectivity was previously demonstrated using pharmacological and genetic approaches (Gris *et al.*, 2014; Sánchez-Fernández *et al.*, 2014). Interestingly, the σ1R antagonist exhibited different efficacy depending on the stage of the osteoarthritis pain sensitization. Acute antiallodynic effects of S1RA were more prominent 15 days than 1 day after the injection of MIA. Such intra-model variance might be due to the differential contribution of inflammation and nerve injury at distinct stages of osteoarthritis. The initial stage of the disease is mainly considered nociceptive pain related to damage and inflammation in the joints, but changes related to neuropathic conditions may occur in the nervous system over time (Thakur *et al.*, 2014). Thus, neuropathic mechanisms are often involved in the pain perception of late osteoarthritis stages (Ohtori *et al.*, 2012; Power *et al.*, 2018). Preclinical evidence has also described the appearance of sensory abnormalities in the MIA model of osteoarthritis. Increased levels of the nerve injury marker ATF-3 in DRG neurons and microgliosis in the spinal cord were found from day 8 after pain induction (Ivanavicius *et al.*, 2007; Orita *et al.*, 2011; Thakur *et al.*, 2012), as well as upregulation of galanin and neuropeptide Y in the

DRG (Im *et al.*, 2010), a typical pattern of neuropathy. Moreover, the medial meniscus destabilization model showed similar changes of neuropeptides in the DRGs (Im *et al.*, 2010), but it did not exhibit overexpression of the ATF-3 (Inglis *et al.*, 2008). Hence, our results indicate that S1RA exhibits greater acute analgesia when the neuropathic component of osteoarthritis is fully established. In agreement, previous results showed higher efficacy of S1RA alleviating neuropathic pain than models of inflammatory pain, although a side-by-side study has not been yet conducted (Romero *et al.*, 2012; Gris *et al.*, 2014).

Central sensitization in mice with osteoarthritis pain was revealed by *in vivo* electrophysiological recordings of spinal WDR neurons. These recordings showed facilitated firing frequencies in response to mechanical, but not to thermal peripheral stimuli. These results agree with a previous work revealing MIA-induced exaggerated responses evoked by mechanical and electrical stimulation (Harvey and Dickenson, 2009). This increased excitability of spinal WDR neurons has also been reported in other pain models (Leem *et al.*, 2010; Quinn *et al.*, 2010; F.-Y. Liu *et al.*, 2011; Aby *et al.*, 2018), and is manifested by an enlargement of receptive field size, increased spontaneous activity, decreased thresholds for the generation and propagation of action potentials and an increase in C-fibre response duration (Latremoliere and Woolf, 2009). Interestingly, we observed that the application of S1RA into the exposed spinal cord reduced these facilitated responses in osteoarthritic mice. Regarding cellular excitability, application of σ1R antagonists alone in *in vitro*

preparations or in intact naïve animals has no effect on ion currents (Tchedre *et al.*, 2008; Zhang *et al.*, 2009; Kourrich *et al.*, 2013; Pan *et al.*, 2014). However, under pathological conditions, activated σ1R regulates glutamate receptors as well as Na^+, K^+ and Ca^{2+} ion channels, pointing to an alteration of neuronal excitability that could be modulated by the blockade of the receptor. Indeed, previous *ex vivo* electrophysiological studies showed that S1RA inhibited the amplified spinal responses that would normally arise from repeated nociceptor stimulation (Romero *et al.*, 2012). This modulatory role of σ1R on spinal excitability was also demonstrated *ex vivo* with spinal cords of σ1R KO mice, which exhibited reduced wind-up responses when compared to WT animals (de la Puente *et al.*, 2009). Therefore, our results revealed that acute S1RA reduces mechanical allodynia involving inhibition of the spinal central sensitization associated with osteoarthritis pain.

We also investigated pain-related molecular alterations that might be involved in the acute S1RA analgesia induced in the osteoarthritis model. Our results showed an early over-expression of BDNF and NPY at DRG, as well as increased TNFα levels in the spinal cord only one day after MIA injection. In agreement, it has been previously reported that BDNF mRNA expression shows a maximal increase in the DRG the day following nerve injury and these elevated levels are sustained for at least two weeks (Uchida *et al.*, 2013). Interestingly, it has been proposed that BDNF released from primary sensory neurons does not significantly contribute to acute pain, but it is necessary for the transition from acute to chronic pain (Sikandar *et al.*, 2018). Several

researchers have also shown prominent up-regulation of NPY expression in primary afferent fibres of nerve-injured animals (Benoliel *et al.*, 2001; Hökfelt *et al.*, 2007; Son *et al.*, 2007), and this synthesis *de novo* has been explained as an adaptive response to the hyperalgesia-induced excitatory signalling (Munglani *et al.*, 1995). Furthermore, the increased expression of TNFα in the DRG has been previously observed immediately after injury and has been suggested to initiate the cytokine-mediated cascade that generates hypersensitivity (Ohtori *et al.*, 2004). Interestingly, acute administration of S1RA did not inhibit these pain-related molecular alterations, suggesting that the σ1R modulation of the excitability of spinal neurons is independent of the modification of BDNF, NPY and TNFα levels.

Chronic administration of a σ1R antagonist in the nociceptive, emotional and cognitive manifestations of osteoarthritis pain

Our results demonstrated that the repeated treatment with the σ1R antagonist S1RA promoted a gradual recovery of sensitivity without inducing the development of analgesic tolerance. Furthermore, the σ1R agonist PRE-084 also blocked this prolonged effect induced by S1RA, demonstrating that the long-term restoration of the mechanical thresholds is also σ1R-dependent. Interestingly, such sustained recovery was observed with all the S1RA doses tested, even with those that had no or poor analgesic effect when administered acutely. Furthermore, the antiallodynic effect was maintained for several days

after interrupting the repeated treatment. The efficacy of S1RA has been previously investigated in other mouse pain models using chronic treatments, but the mechanical thresholds were not tested before the daily dose of the compound (Romero *et al.*, 2012; Bura *et al.*, 2013). Surprisingly, these long-lasting restorative effects also differ according to the stage of the osteoarthritis pain sensitization. Contrary to the acute effect, the gradual pain recovery required longer exposure to S1RA to reach baseline levels at later stages of osteoarthritis. Apart from the presence of a neuropathic component as the disease progresses, there is a differential time-dependent contribution of the peripheral and central nervous system in osteoarthritis pain. It has been shown that the inhibition of primary afferent sensory neurons attenuates nociception at early, but not late stages of osteoarthritis in different animal models of the disease (Miller *et al.*, 2017; Haywood *et al.*, 2018), indicating an increased central contribution for the maintenance of this pain state. At this late time-points, there have been observed markers of central sensitization indicative of the transition from acute to chronic pain mechanisms (Sagar *et al.*, 2011). Altogether, this might explain the stronger S1RA analgesic effort required to fully recover the baseline mechanical thresholds at late time-points of the osteoarthritis pain development. However, contrary to NSAIDs that loss its analgesic efficacy after two weeks of pain induction (Fernihough *et al.*, 2004; Ivanavicius *et al.*, 2007; Rashid *et al.*, 2013), S1RA maintains acute and long-lasting effects at both points of the disease.

In addition to the effect of S1RA over MIA-induced mechanical hypersensitivity, the σ1R antagonist also inhibited the gait alterations associated with osteoarthritis pain. In agreement with these results, previous studies using the MIA model in rodents showed that celecoxib and morphine reduced mechanical allodynia along with gait abnormalities (Ferland *et al.*, 2011; Ferreira-Gomes *et al.*, 2012), suggesting an appropriate correlation between both nociceptive parameters. Such correlation has also been described in neuropathic pain rodent models, where decreased mechanical thresholds were accompanied by altered walking patterns (Vrinten and Hamers, 2003), as well as in higher-order mammals with osteoarthritis pain (Haussler *et al.*, 2007; Frost-Christensen *et al.*, 2008; Moreau *et al.*, 2011; Cake *et al.*, 2013). Contrary, some authors proposed that gait abnormalities could be a consequence of pain in inflammatory models, but not in nerve injury-related pain (Piesla *et al.*, 2009), and suggested that spontaneous neuropathic pain in mice cannot be assessed using gait analysis (Mogil *et al.*, 2010). However, it has been proposed for osteoarthritis pain that some altered parameters, such as paw print area, represent a good measure of pain, whereas others like the angle between paws were exclusively influenced by the structural damage of the joint as indicated by its correlation with cartilage destruction (Boettger *et al.*, 2009). In our study, taking into account that S1RA inhibited both mechanical allodynia and gait alterations without normalizing the structural damage observed in the histological assessment, we can assume that the reduced paw print area and maximal contact area were a consequence of an unwillingness of the animal to bear weight on the injured limb. Thus, the effect of S1RA on

the normalization of these gait parameters is probably due to the reduced pain perception observed after the repeated treatment. Considering that patients with knee osteoarthritis also show compensatory gait alterations (Kaufman *et al.*, 2001; Sun *et al.*, 2017), the σ1R antagonism could represent an appropriate therapeutic option for the management of the altered walking patterns associated to osteoarthritis pain.

It has been widely reported that chronic pain conditions such as osteoarthritis pain are frequently accompanied by co-morbid cognitive alterations (Sturgeon *et al.*, 2016; Innes and Sambamoorthi, 2018). In agreement, we found memory deficits associated with the injection of MIA. Previous studies have also shown cognitive impairments in other chronic pain models (Zhao *et al.*, 2006; Kodama *et al.*, 2011; Liu and Chen, 2014) and specifically in rodent models of osteoarthritis pain (La Porta *et al.*, 2015; Negrete *et al.*, 2017). The overlap between the neuroanatomical substrates implicated in both pain control and cognitive functions provides information about the development of memory deficits in patients with chronic pain (Moriarty and Finn, 2014). However, the specific causal mechanisms underlying the cognitive impairments associated with chronic pain are still unclear. It has been proposed a model of pain-related cognitive dysfunction based on human imaging studies and preclinical evidence of pain-induced alterations on brain morphology, neuronal excitability, glial cells and cytokine release, enzymes and neurotrophic factors (Moriarty *et al.*, 2011). Taking all together, these authors proposed that pain uses cognitive resources, alters neural plasticity

and affects expression and activity of neuromediators. Furthermore, several studies have also investigated the connectivity between brain regions related to pain and cognition. Reduced connectivity between the mPFC and the hippocampus or the thalamus has been associated with impaired memory performance in rodent models of neuropathic (Cardoso-Cruz *et al.*, 2013a) or inflammatory pain (Cardoso-Cruz *et al.*, 2013b). We observed that these MIA-induced cognitive deficits were significantly reduced by the repeated administration of S1RA, suggesting that the blockade of σ1R plays a protective role in the impairment of long-term memory associated with osteoarthritis. Interestingly, the acute treatment with S1RA before the cognitive task also induced a memory improvement in this model of osteoarthritis pain. Given that acute pain interrupts attention (Boyette-Davis *et al.*, 2008), the acute pain relief by S1RA may affect the test performance by disrupting the negative impact of pain on attention. It has been elucidated that selective σ1R ligands, either agonists or antagonist, failed to improve or impair the learning, consolidation or retention phases of the mnemonic process in naïve animals (Hashimoto *et al.*, 2007; Antonini *et al.*, 2011), but σ1R activation reduced memory deficits associated with Alzheimer disease (Maurice *et al.*, 1998; Antonini *et al.*, 2011), schizophrenia (Hashimoto *et al.*, 2007), or scopolamine treatment (Hiramatsu *et al.*, 2002). However, the pathogenesis of these neurological disorders widely differs from the chronic pain mechanisms, and the role of the σ1R on this specific type of memory deficit induced by persistent pain has not been studied. Our data suggest that the blockade of σ1R improves cognitive functions under chronic pain states.

Chronic pain conditions have also been found to co-occur with emotional manifestations. We observed increased anxiety-like responses in mice with osteoarthritis pain, in agreement with previous studies of inflammatory (Schellinck *et al.*, 2003; Chen *et al.*, 2013) or neuropathic pain (Benbouzid *et al.*, 2008; Matsuzawa-Yanagida *et al.*, 2008; La Porta *et al.*, 2016). However, anxiety-like behaviour was present 3 weeks after MIA, but not at earlier time points, contrary to a previous study showing that anxiogenic responses were already established 11 days after the induction of osteoarthritis pain (La Porta *et al.*, 2015). Due to the strong behavioural tolerance that occurs after one single exposure to the elevated plus maze (File *et al.*, 1990; Holmes and Rodgers, 1998), in our study, we assessed the early and late anxiety-like behaviour with two different tests. However, both paradigms have been shown to equally detect anxiolytic and anxiogenic responses in rodents, being the results from the elevated plus maze and the zero maze comparable (Braun *et al.*, 2011). Thus, such discrepancy could not be explained by the different tests used, but it might be due to the chronic treatment performed in our study, where mice received an intraperitoneal injection twice a day for the whole duration of the experiment. This might have produced an overall increase in anxiety responses, thus reducing the differences between groups. Furthermore, it has been shown that the onset of anxiety-like behaviours does not correspond with the timeline for mechanical hypersensitivity, suggesting that chronic pain might promote alterations in brain areas involved in affective responses which over time may lead to emotional comorbidities (Narita *et al.*, 2006; Suzuki

et al., 2007; Seminowicz *et al.*, 2009; Sellmeijer *et al.*, 2018). In fact, 25 days after the intra-knee injection of MIA we observed depressive-like responses in animals with osteoarthritis pain, in agreement with previous studies investigating inflammatory and neuropathic pain (Hasnie *et al.*, 2007; Suzuki *et al.*, 2007; Norman *et al.*, 2010; Negrete *et al.*, 2017).

The common neurobiological alterations for chronic pain and depression have been widely investigated, and it has been reported that neuroplasticity crucially affects the occurrence and the development of both disorders and may involve the same brain structures, neurotransmitters and signalling pathways (Nekovarova *et al.*, 2014; Sheng *et al.*, 2017; Humo *et al.*, 2019). Considering this mechanistic overlap between pain and depression, it is not surprising that analgesic drugs, such as opioids (Mague *et al.*, 2003; Tenore, 2008), have been proposed as a treatment for chronic pain-induced depression, while antidepressants like selective serotonin reuptake inhibitors (SSRIs) (Tasmuth *et al.*, 2002; Nagata *et al.*, 2009; Gebhardt *et al.*, 2016) or tricyclic antidepressants (Rowbotham *et al.*, 2005; Kopsky and Keppel Hesselink, 2012) showed antinociceptive effects under chronic pain conditions. This is in agreement with other studies demonstrating that the reduction of serotonin levels in the brain not only worsened depressive symptoms (Booij *et al.*, 2005; van Steenbergen *et al.*, 2012) but also increased the sensation of pain (Supornsilpchai *et al.*, 2006; Wei *et al.*, 2010). Strikingly, memory deficits are also associated with depression (Kizilbash *et al.*, 2002), and antidepressants have also been proposed to affect cognitive

functions (Monleón *et al.*, 2008), whereas enhancement of cognitive performance can alleviate depression (Knapp *et al.*, 2002). Interestingly, several antidepressants showed moderate to high affinity for σ1R sites (Schmidt *et al.*, 1989; Itzhak *et al.*, 1991; Narita *et al.*, 1996), which increased the interest for σ1R ligands as a treatment for depressive states. Evidence suggests that some SSRIs like fluvoxamine or fluoxetine are σ1R agonists, whereas others like sertraline may act as antagonists of σ1R (Nishimura *et al.*, 2008; Ishima *et al.*, 2014; Hashimoto, 2015). In agreement, we observed that the repeated treatment with S1RA abolished the MIA-induced depressive-like state, but not the anxiety-like behaviour. Previous studies also showed a distinct contribution of the σ1R modulating depressive and anxiety responses, since σ1R KO animals exhibited increased immobility time in the forced swimming test, but a normal performance in the elevated plus maze (Sabino *et al.*, 2009). Therefore, the antagonism of σ1R could be effective in reducing the depressive-like symptoms associated with osteoarthritis pain.

We have assessed the osteoarthritis pain perception using nociceptive tests, but also gait analyses parameters, cognitive function and anxiety- and depressive-like behaviours, thus extending the research beyond the boundaries of sensory aspects to comorbidities that frequently afflict patients with osteoarthritis pain (Moriarty and Finn, 2014; Sharma *et al.*, 2016). While the sensory features of chronic pain conditions have been extensively studied in experimental models, the comorbid psychiatric disorders were not addressed by preclinical research until the end of last century (Kontinen *et al.*, 1999). Since

then, a great number of behavioural paradigms have been introduced in the field of experimental pain research (Leite-Almeida *et al.*, 2015). Importantly, self-perceived measures of quality of life, which include physical health, vitality, social functions, emotional problems and mental health correlate better with pain than with radiologic changes in osteoarthritis patients (Imamura *et al.*, 2008; Goldenberg, 2010). Therefore, the assessment of emotional and cognitive manifestations of chronic osteoarthritis pain in basic research is crucial to improve face validity and to better predict the efficacy of analgesic drugs in humans.

The histological analysis showed cartilage destruction in the MIA-injected mice, as previously described in several models of osteoarthritis (Bove *et al.*, 2006; La Porta *et al.*, 2013; Negrete *et al.*, 2017; Farrán *et al.*, 2018; Tawonsawatruk *et al.*, 2018). Importantly, the beneficial effects of S1RA on the nociceptive, cognitive and emotional behaviours of osteoarthritic mice were not accompanied by a normalization of the structural alterations in the knee joints, pointing to a centrally mediated control of pain by the σ1R. Low expression levels of σ1R have been reported in chondrocytes and bone marrow when compared to its expression in the peripheral and central nervous system (Expression atlas, 2019), thus agreeing with the absence of effect of σ1R ligands over cartilage destruction. Furthermore, it is widely recognised that the degree of pain in patients with osteoarthritis poorly correlate with the extent of joint damage (Lawrence *et al.*, 1966; Dieppe, 2004; Bedson and Croft, 2008). In fact, it is considered that central sensitization is essential for

osteoarthritis pain and it strongly contributes to such discordance (Finan *et al.*, 2013; Arendt-Nielsen *et al.*, 2015), suggesting that centrally acting drugs targeting central sensitization would be crucial for an appropriate management of osteoarthritis pain (Woolf, 2011). Therefore, the relief of mechanical hypersensitivity and pain-associated comorbidities after the treatment with S1RA probably relies on its modulatory role on the central nervous system and is independent of the site of the primary lesion.

We also explored pain-related molecular alterations at late stages of osteoarthritis, and we observed pronounced increases of IL1β and NPY mRNA levels in the spinal cord and DRG, respectively, as well as an enhancement of the microglial marker Iba1. In addition, the over-expression of BDNF and TNFα was sustained in DRG and spinal cord, respectively. Hence, in our experimental conditions, the knee joint injury induced persistent changes in neuroinflammatory mediators, possibly contributing to the osteoarthritic phenotype. In agreement, it has been reported that increased BDNF/TrkB signalling in the spinal cord may contribute to chronic pain by activating microglial cells (Zhou *et al.*, 2011), which in turn are the major source of cytokines like IL1β and TNFα (Hanisch, 2002; Welser-Alves and Milner, 2013). Interestingly, it has been observed a TNFα-dependent infiltration of macrophages into the DRG correlating with pain behaviours (Segond von Banchet *et al.*, 2009; Miller *et al.*, 2012). However, we observed unchanged levels of Iba1 in DRG at late stages of osteoarthritis. Overall, the observed pain-related changes were reported to increase glutamate release and stimulate the glutamatergic system (Takeuchi,

2013; Vaz *et al.*, 2015). Accordingly, we observed that mice with osteoarthritis also showed increased phosphorylation of the NR1 and NR2B subunits of the NMDA receptors and an over-expression of mGluR5, which is associated with excessive levels of glutamate in the nervous system (Wang *et al.*, 2012). Our results showed that repeated treatment with S1RA reduced the up-regulated levels of NPY, agreeing with the evidence that NPY has affinity for the σ1R and its effects via this receptor are blocked by σ1R antagonists (Bouchard *et al.*, 1997; Meurs *et al.*, 2007). Furthermore, we found that chronic S1RA inhibited microgliosis and the over-expression of pro-inflammatory cytokines and BDNF. These findings are consistent with the high levels of σ1R reported in microglia (Gekker *et al.*, 2006), and agree with the effects of the σ1R antagonist BD1047 attenuating spinal microgliosis in a model of bone cancer pain (Zhu *et al.*, 2015). In the same line, recent experiments in mice with neuropathic pain demonstrated normalization of TNFα and IL1β spinal levels after genetic or pharmacological inactivation of σ1R (Castany *et al.*, 2018, 2019). Furthermore, over-expression of σ1R has been shown to potentiate BDNF actions and, consequently, enhance glutamate release (Yagasaki *et al.*, 2006). The effects of S1RA on proinflammatory cytokines and BDNF were only observed after the repeated treatment, in agreement with a previous study showing that a single injection of a σ1R agonist did not alter BDNF protein levels, whereas 2- or 4-weeks chronic administrations tended to increase BDNF in the hippocampus (Kikuchi-Utsumi and Nakaki, 2008). Interestingly, the BDNF effects facilitating neuronal activity are dependent on the mGluR5 (Gibon *et al.*, 2016), which we also found to be normalised by the chronic

treatment of S1RA in osteoarthritic mice. Hence, while the effects of acute administration of σ1R antagonists have been associated with reduced phosphorylation of NMDAR-NR1 subunit (Kim *et al.*, 2006, 2008; Yoon *et al.*, 2010; Rodríguez-Muñoz *et al.*, 2015b; Zhu *et al.*, 2015), we showed that the long-term effect of S1RA chronic treatment is independent of such phosphorylations, but involves regulation of metabotropic glutamate receptors. Therefore, repeated treatment with the σ1R antagonist inhibits the over-expression of neuroinflammatory mediators and glutamate receptors involved in chronic osteoarthritis pain.

At the supraspinal level, we also found increased microgliosis in the mPFC produced by the injection of MIA. This result agrees with previous work showing enhanced microglial density in the infralimbic mPFC of nerve-injured rats (Chu Sin Chung *et al.*, 2017). Microgliosis and the consequent overproduction of pro-inflammatory cytokines were also accompanied by depressive-like behaviours during neuropathic pain (Xu *et al.*, 2017). It has been proposed that microglial alterations in cortical regions underlie the pain-induced emotional and cognitive impairments (Panigada and Gosselin, 2011). Indeed, an increased production of proinflammatory cytokines has been widely described in patients with depressive and anxiety disorders (Müller, 2013; Bai *et al.*, 2014; Lotrich, 2015; Vogelzangs *et al.*, 2016; Farooq *et al.*, 2017; Hou *et al.*, 2017), and a differential expression of IL1β has been reported 10 and 24 days after nerve injury in the brainstem, the thalamus and the PFC (Apkarian *et al.*, 2006), agreeing with the late onset of anxiety that we observed in

osteoarthritic mice. Our data also showed that the antagonism of σ1R significantly reduced the microgliosis in the mPFC, in agreement with the S1RA effect over the spinal microglia. It is well known that σ1R modulates several signal transduction pathways, including the production of ATP, reactive oxygen species or mitogen-activated protein kinases (Zamanillo *et al.*, 2013; Hayashi, 2015; Zhao *et al.*, 2017). All these molecules have been identified as effective signals for microglial migration and activation (Biber *et al.*, 2007; Fan *et al.*, 2017), pointing to an indirect modulatory role of σ1R. In the same line, σ1R activation by methamphetamine induces microgliosis that involves the production of reactive oxygen species and activation of the mitogen-activated protein kinases pathway (Chao *et al.*, 2017). In our studies, the effect of S1RA over the supraspinal anatomical changes was not accompanied by a reduction of anxiety-like behaviour, but it did correlate with the depressive-like behaviour and the cognitive improvement, pointing to an involvement of cortical microglia on both pain comorbidities. Interestingly, the systemic microglial inhibitor minocycline attenuated mechanical hypersensitivity and depressive-like behaviour during neuropathic pain (Xu *et al.*, 2017), whereas intrathecal minocycline reduced mechanical allodynia and anxiety-like behaviour at early, but not late post-operative stages (Li *et al.*, 2016). Furthermore, antidepressant drugs such as SSRIs also showed activity modulating microgliosis and reducing microglial production of pro-inflammatory cytokines (Chung *et al.*, 2011; Tynan *et al.*, 2012; Dubovický *et al.*, 2014; Ohgidani *et al.*, 2016). Therefore, σ1R-regulated cortical microgliosis might be crucial

for the manifestation of cognitive and depressive alterations often present in chronic osteoarthritis pain patients.

Reciprocal modulation of σ1R and MOR during osteoarthritis pain

Although opioids are powerful analgesic drugs, it has been widely recognised that repeated opioid treatment might lead to the development of tolerance to its analgesic effect, driving to the need of increased doses to maintain the same level of analgesia (Ballantyne and Mao, 2003; Raehal *et al.*, 2011). In agreement, the 14-day treatment with morphine induced loss of the antinociceptive effect over time. Furthermore, we revealed a σ1R-dependent modulation of opioid analgesia during chronic osteoarthritis pain, since a single sub-effective dose of S1RA co-administered with morphine restored the analgesic effects of the opioid after tolerance development. Previous preclinical studies have shown increased opioid effects in σ1R KO mice or when combined with several σ1R antagonists in physiological conditions (Chien and Pasternak, 1994; Sánchez-Fernández *et al.*, 2013, 2014). Moreover, S1RA has demonstrated efficacy restoring morphine analgesia in tolerant animals during acute nociceptive or inflammatory pain (Vidal-Torres *et al.*, 2013; Rodríguez-Muñoz *et al.*, 2015b; Montilla-García *et al.*, 2019). It has been postulated that this modulation over the antinociception of opioid drugs relies on the physical and functional association between σ1R and MOR, by which σ1R promotes MOR phosphorylation, decreasing its association with G-proteins and leading to a reduced effect of opioid agonists (Rodríguez-Muñoz *et al.*, 2015a, 2015b). Our data add knowledge to

better understand the role of σ1R modulating morphine analgesia under chronic pain conditions and suggest that σ1R antagonists could be efficient not only alleviating pain by themselves, but also restoring opioid analgesia in tolerant osteoarthritis patients. Considering that opioid tolerance drives to dose escalation and abuse and that S1RA is void of reinforcing effects in physiological conditions (Bura *et al.*, 2013), the blockade of σ1R could represent an appropriate alternative to opioids for chronic pain treatments.

We also investigated the effect of acute and chronic morphine on the molecular alterations associated with osteoarthritis pain and to S1RA analgesia. Interestingly, opposite to the σ1R antagonist, a single administration of morphine inhibited the over-expression of BDNF and NPY at DRG suggesting that the acute effect of the opioid could involve the regulation of neuroinflammatory factors in the peripheral nervous system. On the contrary, chronic morphine treatment did not modify or further increased the neuroinflammatory mediators in the spinal cord and the DRG, including microglia, proinflammatory cytokines and the glutamatergic receptors. These results are in agreement with previous findings revealing that repeated, but not acute morphine administration was associated with enhanced proinflammatory cytokines in the dorsal spinal cord (Johnston *et al.*, 2004). Additionally, chronic morphine also induced enhancement of spinal glial reactivity, BDNF release from microglia and AMPA receptor expression (Raghavendra *et al.*, 2002; Cabañero *et al.*, 2013; Hayashi *et al.*, 2016). Hence, our results showed that repeated morphine contributed to an overall increase of spinal neuroinflammation and

excitability, which we found to be reduced by repeated S1RA. Altogether, these molecular alterations could constitute a common pathway by which the σ1R antagonist provides restoration of opioid analgesia in morphine-tolerant individuals. In fact, it has been shown that acute and chronic blockade of IL1β prolongs and potentiates morphine analgesia (Shavit *et al.*, 2005), and mGluR5 KO mice and systemic or intrathecal administration of mGluR5 antagonists attenuate the development of tolerance to morphine antinociception (Narita *et al.*, 2005; Huang *et al.*, 2019).

Interestingly, we found that the effects of σ1R over MOR were bidirectional since the MOR antagonist naloxone diminished the acute and sustained antinociception induced by S1RA. In addition, morphine tolerant mice showed decreased S1RA efficacy, altogether pointing to a participation of MOR activity in σ1R-induced analgesia. As mention above, it has been proposed a physical and functional association between σ1R and MOR (Kim *et al.*, 2010; Rodríguez-Muñoz *et al.*, 2015a, 2015b). σ1R antagonism promotes the binding between σ1R and MOR protecting the latter from phosphorylation and enhancing its activity. Furthermore, persistent MOR stimulation enhances MOR phosphorylation (Rodríguez-Muñoz *et al.*, 2015a, 2015b) and further increased in our studies the osteoarthritis-related over-expression of neuroinflammatory markers, providing a possible explanation for the reduced analgesia of the σ1R antagonist after opioid tolerance. Therefore, since both naloxone and MOR desensitization attenuated the antinociceptive effects of S1RA, it can be concluded that part of

the analgesic efficacy of the σ1R blockade relies on the enhancement of the endogenous opioid system activity.

Considering the crosstalk between σ1R and MOR, we investigated the analgesic efficacy of SIMU, a dual compound acting as σ1R antagonist and MOR agonist. We observed that SIMU exhibited dose-dependent analgesic effects in animals with osteoarthritis pain. Furthermore, SIMU did not induce tolerance, contrary to EST-A, which possesses different affinities for σ1R and MOR than SIMU. These results suggest that the development of analgesic tolerance is susceptible to be modulated by an appropriate combination of σ1R antagonism and MOR agonism. It has been previously reported that the repeated co-administration of S1RA (40 mg/kg) with morphine (10 mg/kg) did not avoid tolerance development in naïve mice (Vidal-Torres *et al.*, 2013). Interestingly, chronic SIMU induced a slight restoration of the mechanical thresholds, although normal sensitivity was not fully recovered. Therefore, the simultaneous blockade of σ1R and stimulation of MOR by SIMU could represent a promising opioid-based strategy to control osteoarthritis pain avoiding tolerance.

Since the endogenous opioid system plays a crucial role in the control of nociceptive responses at different levels of the pain pathway (Millan *et al.*, 1991), we also assessed the specific participation of MOR in SIMU-induced analgesic effects during osteoarthritis pain. For this purpose, we evaluated the specific contribution of MOR at the level of peripheral nociceptors, central GABAergic forebrain neurons, or throughout the entire organism. We used conditional knockout mice lacking MOR either in the peripheral neurons expressing the

sodium channel Nav1.8 (Weibel *et al.*, 2013) or in GABAergic neurons of the forebrain (Charbogne *et al.*, 2017), as well as constitutive MOR knockout mice (Weibel *et al.*, 2013). Although MIA injection induced mechanical hypersensitivity regardless of the genotype, the constitutive deletion of MOR reduced the sensitivity of osteoarthritic mice, suggesting pronociceptive activity of MOR under this chronic pain condition. Interestingly, increased, unchanged or attenuated nociceptive behaviour has been reported in different full MOR KO lines under different chronic pain condition (Sora *et al.*, 1999; Mansikka *et al.*, 2004; Bohren *et al.*, 2010; Kögel *et al.*, 2011; Wieskopf *et al.*, 2014; Roeckel *et al.*, 2017), suggesting a complex role of the receptor in the pathophysiology of persistent pain. Although this literature reports conflicting results, it has been proposed that MOR may play an antinociceptive role in certain inflammatory pain conditions, whereas there is a maladaptive function of MOR during neuropathic pain (Maldonado *et al.*, 2018). Furthermore, naïve mice completely lacking MOR exhibit decreased anxiety- and depressive-like behaviours (Filliol *et al.*, 2000), which in turn could contribute to the reduced pain perception (Bushnell *et al.*, 2013). Surprisingly, SIMU chronic treatment alleviated osteoarthritis pain not only in control WT but also in total MOR KO mice. The pharmacological blockade of MOR with naloxone did not mimic the effect of SIMU on the constitutive KO animals, probably because of the adaptive alterations of these mutant mice, which showed increased σ1R expression at the peripheral and central levels of the nervous system. Importantly, we used a dose of naloxone (1 mg/kg) that has been reported to precipitate withdrawal in morphine-dependent mice (Boulos *et al.*, 2019) and to completely

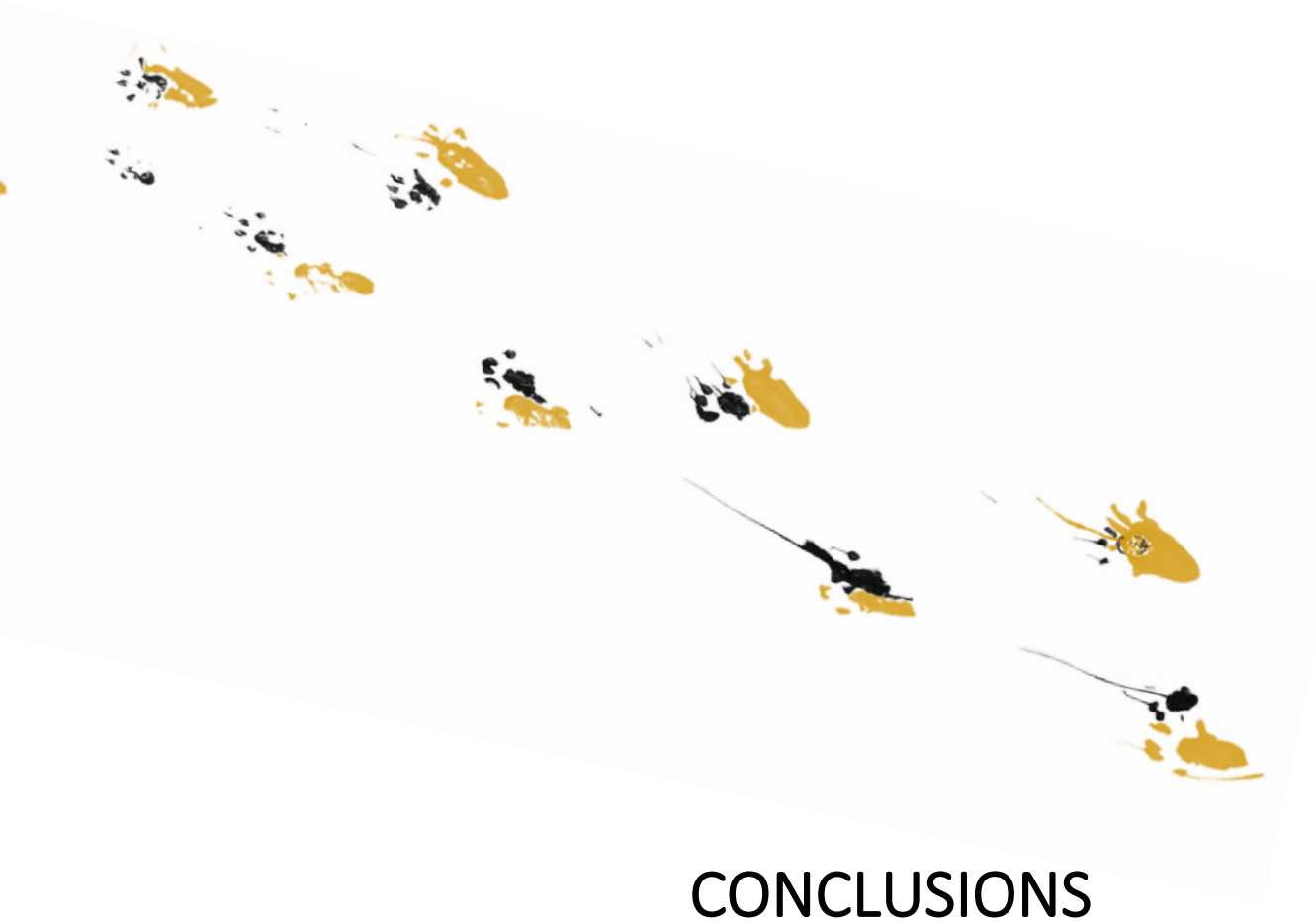
abolish opioid-induced locomotor activity (Eriksen *et al.*, 2016). Interestingly, it has been shown that morphine normally induces microgliosis in the full MOR KO, suggesting that the activation of microglia during repeated morphine treatment might occur through a mechanism different to MOR activation (Corder *et al.*, 2017).

In our chronic pain model, MOR-induced pronociception was not due to GABAergic forebrain neurons nor to Nav1.8+ fibres. Indeed, conditional KO mice lacking MOR in those GABAergic neurons showed similar mechanical sensitivity to WT, whereas animals lacking MOR in Nav1.8+ neurons exhibited increased allodynia. In agreement, a recent work described enhanced mechanosensitivity in conditional Nav1.8-MOR KO animals during inflammatory pain (Severino *et al.*, 2018). Furthermore, SIMU analgesia was abolished in Nav1.8 MOR KO mice pointing to a crucial role of these receptors in the DRG for the analgesic efficacy of SIMU. The pharmacological blockade of peripheral MOR with naloxone methiodide also inhibited the antiallodynic effect of the dual compound in WT animals. It has been previously shown that peripheral MOR inactivation leads to a decreased opioid-induced analgesia (Weibel *et al.*, 2013), and prevents the onset of morphine tolerance (Corder *et al.*, 2017). In agreement, we observed that mice lacking MOR in the GABAergic forebrain neurons, but not in the primary DRG nociceptors showed tolerance development to the analgesic effect of SIMU after its repeated administration.

It has been recently reported that supraspinal and peripheral, but not spinal σ1R antagonism modulates opioid analgesia (Vidal-Torres *et al.*,

2019). In line with these results, we observed that the mRNA levels of the σ1R were unchanged in the spinal cord regardless of the genotype, the surgery or the treatment. In contrast, enhanced levels of σ1R were found in the central or the peripheral nervous system when lacking MOR in such specific areas, pointing to a compensatory over-expression of these interrelated receptors. Interestingly, the σ1R mRNA levels were not modified by the surgery or the treatment in any of the genotypes, in agreement with previous results that reported unaffected transcript levels of σ1R in DRGs from nerve-injured rats when compared to controls (Bangaru *et al.*, 2013). Therefore, our results indicate that the complete lack of MOR leads to molecular changes that facilitate the analgesic effect of SIMU, whereas the studies with the conditional KO mice and the pharmacological MOR blockade suggest that SIMU antiallodynic effect is dependent on peripheral and central MORs.

In conclusion, the present thesis has demonstrated the role of the σ1R in the pathophysiology of osteoarthritis pain and in the development of morphine tolerance under this chronic pain condition. We have proposed a common neurobiological pathway by which the antagonism of σ1R could improve the nociceptive, cognitive and emotional manifestations of chronic osteoarthritis pain and restore opioid analgesia after tolerance development. Hence, σ1R antagonists, alone or as opioid adjuvants, represent a promising therapeutic strategy for the clinical management of osteoarthritis pain and its co-morbid manifestations.



CONCLUSIONS

The main conclusions of the work presented in this thesis can be summarized as follows:

1. The presence of mechanical allodynia in a mouse model of chronic osteoarthritis pain is associated with gait alterations, reduced cognitive performance and increased anxiety- and depressive-like behaviours.
2. Acute administration of S1RA produces a dose-dependent alleviation of osteoarthritis pain through the inhibition of central sensitization at the spinal level, as revealed by electrophysiological approaches.
3. The acute effects of S1RA are independent of the modification of neuroinflammatory mediators.
4. The acute blockade of σ1R shows a greater effect relieving osteoarthritis pain when the neuropathic component associated with late stages of the disease is fully established.
5. Repeated exposure to S1RA promotes a sustained normalization of mechanical sensitivity, which is associated with the inhibition of biochemical alterations critical for osteoarthritis pain.
6. The chronic blockade of σ1R does not induce the development of analgesic tolerance and maintains its long-lasting effects at late stages of osteoarthritis pain, when other analgesic drugs such as NSAIDs tend to lose their efficacy.
7. The analgesic effect of S1RA over mechanical hypersensitivity is accompanied by the inhibition of gait alterations associated with osteoarthritis pain.

8. S1RA does not normalize the structural joint damage, pointing to a centrally mediated control of pain by the σ1R that is independent of the site of the primary lesion.
9. σ1R does not participate in the development of anxiety-like manifestations of osteoarthritis pain, but it contributes to the cognitive impairments and the depressive-like responses associated with the disease.
10. The effect of S1RA over the cognitive and emotional manifestations of osteoarthritis pain is accompanied by a normalization of supraspinal microgliosis in the mPFC, pointing to an involvement of cortical microglia on these pain comorbidities.
11. σ1R is involved in the modulation of opioid-induced analgesia during osteoarthritis pain, since S1RA restores morphine antinociception in tolerant mice.
12. The acute and chronic analgesic effects produced by S1RA during osteoarthritis pain are mediated by its interaction with the σ1R and the participation of endogenous MOR activity, evidencing a crosstalk between σ1R and the opioid system.
13. The σ1R antagonist decreases neuroinflammatory mediators, microglial reactivity and glutamatergic signalling, which are commonly activated by repeated opioid exposure and chronic osteoarthritis pain. This could constitute a common pathway by which the σ1R antagonist provides relief of persistent pain and restoration of opioid analgesia in tolerant individuals.

14. The simultaneous blockade of σ1R and stimulation of MOR by the dual compound SIMU alleviate osteoarthritis pain without inducing development tolerance to its analgesic effect.
15. Animals with a complete absence of MOR exhibit attenuated osteoarthritis pain. This MOR-induced pronociception is not due to primary Nav1.8+ afferent fibres nor to GABAergic forebrain neurons.
16. The complete lack of MOR leads to changes in the expression of σ1R that could facilitate the analgesic effect of SIMU during chronic osteoarthritis pain.
17. The antiallodynic effect of SIMU is dependent on MORs in primary afferent neurons and in GABAergic neurons of the forebrain.
18. The evaluation of different nociceptive, cognitive and affective manifestations of chronic pain is essential to increase the validity of preclinical pain research.
19. We provide new insights to better understand the complexity of chronic osteoarthritis pain, and we suggest the σ1R antagonist, alone or as an opioid adjuvant, as a promising alternative for the management of chronic pain conditions requiring long-term treatments.

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