INVOLVEMENT OF THE ENDOCANNABINOID SYSTEM IN OSTEOARTHRITIS PAIN

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A Ivan

"No llega antes el que va más rápido
sino el que sabe adónde va"
Séneca

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Abstract

Chronic pain is a major clinical problem producing huge economic and social burdens. Currently, chronic pain treatment has limited efficacy and significant side effects. One of the reasons of this unmet clinical need is the insufficient knowledge of the exact mechanisms involved in the generation and maintenance of chronic pain and pain-related comorbidities, such as affective and cognitive disorders that can negatively affect the life quality of patients. It is an important challenge to treat not only the nociceptive symptoms, but also the comorbidities accompanying chronic pain. In the present Thesis, we have validated different behavioral outcomes to evaluate the nociceptive, affective and cognitive alterations promoted by chronic pain in mice. Our work mainly focuses on a particular type of chronic pain that is the osteoarthritis pain. Pain is the principal symptom of osteoarthritis, a degenerative joint disease characterized by articular cartilage degradation. The endocannabinoid system has recently emerged as a new potential therapeutic target for osteoarthritis pain. The endocannabinoid system regulates a wide range of physiopathological processes including articular metabolism, pain, emotions and cognitive functions, and a therapeutic intervention on this system could offer the potential advantage to treat multiple aspects of this disease. We have used behavioral, genetic, pharmacological and biochemical approaches to study the involvement of the endocannabinoid system in different osteoarthritis pain-related alterations in mice, and explored the potential usefulness of the endocannabinoid system components as biomarkers for human osteoarthritis.

Resumen

El dolor crónico es un problema clínico grave con una enorme carga económica y social. Actualmente, el tratamiento del dolor crónico presenta eficacia limitada y efectos adversos significativos. Una de las razones de esta necesidad clínica insatisfecha es el escaso conocimiento de los mecanismos exactos que están involucrados en la generación y mantenimiento del dolor crónico y las comorbilidades relacionadas con el dolor, como son los trastornos afectivos y cognitivos. Estos tienen un impacto negativo sobre la calidad de vida de los pacientes y pueden agravar ulteriormente la percepción del dolor. Por ello, tratar no solamente

los síntomas nociceptivos sino también las cormorbilidades que acompañan el dolor crónico representa un reto importante. En la presente Tesis, hemos validado diferentes modelos conductuales para evaluar las alteraciones nociceptivas, afectivas y cognitivas inducidas por el dolor crónico en ratones. Nuestro trabajo se centra principalmente en un tipo concreto de dolor crónico, el dolor osteoartrítico. El dolor es el principal síntoma de la osteoartritis, una enfermedad degenerativa de las articulaciones caracterizada por la degradación del cartílago. El sistema endocannabinoide ha emergido recientemente como una nueva diana terapéutica para el dolor osteoartrítico. Este sistema endógeno regula una vasta gama de procesos fisiopatológicos, incluyendo el metabolismo articular, el dolor y las funciones emocionales y cognitivas, y una intervención terapéutica sobre este sistema podría ofrecer la ventaja potencial de tratar diferentes aspectos relacionados con esta enfermedad. La combinación de aproximaciones comportamentales, genéticas, farmacológicas y bioquímicas nos han permitido determinar la participación de determinados componentes del sistema endocannabinoide en las diferentes alteraciones relacionadas con el dolor osteoartrítico en ratones. Además, hemos analizado la utilidad potencial de los componentes del sistema endocannabinoide como biomarcadores de la osteoartritis humana.

Abbreviations

2-AG: 2-arachidonoylglycerol

ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs

ACTH: adrenocorticotropic hormone

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AEA: anandamide

ATF-3: activating transcription factor-3

CB1R: cannabinoid receptor 1

CB1KO: knockout mice for cannabinoid receptor 1

CB2R: cannabinoid receptor 2

CB2KO: knockout mice for cannabinoid receptor 2

CB2xP: transgenic mice over-expressing cannabinoid receptor 2 in the central nervous system

cAMP: cyclic adenosine monophosphate

CRH: corticotropin-releasing hormone

CGRP: calcitonin gene related peptide

CNS: central nervous system

COX: cyclooxygenase

DGL: diacylglycerol lipase

DOR: δ (delta) opioid receptor

DRG: dorsal root ganglion

ECS: endogenous cannabinoid system

EPM: elevated plus maze

ERK1/2: extracellular signal-regulated kinase-1 and -2

FAAH: fatty-acid amide hydrolase

FR: fixed ratio

FST: forced swimming test

GABA: gamma-aminobutyric acid

GABA_AR: A-type gamma-aminobutyric acid receptor

GR: glucocorticoid receptors

HPA: hypothalamic-pituitary-adrenal axis

IL: interleukin

KOR: κ (kappa) opioid receptor

LTD: long-term depression

LTP: long-term potentiation

MAGL: monoacylglycerol lipase

MAPK: mitogen-activated protein kinase

MMP: matrix metalloproteinase

MIA: monosodium iodoacetate

MOR: μ (mu) opioid receptor

MRI: magnetic resonance imaging

mPFC: medial prefrontal cortex

NGF: nerve growth factor

NMDAR: N-methyl-D-aspartate receptor

NO: nitric oxide

NSAID: non-steroidal anti-inflammatory drug

ORM: object recognition memory

PAG: periaqueductal grey

PFC: prefrontal cortex

PKA: protein kinase A

PLC-β: phospholipase C-β

PR: progressive ratio

PVN: paraventricular nucleus of the hypothalamus

RVM: rostral ventro-medial medulla

THC: Δ^9-tetrahydrocannabinol

TNFα: tumor necrosis factor α

TRPV1: transient receptor potential vanilloid type-1

TST: tail suspension test

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INTRODUCTION

1 Osteoarthritis

1.1 Epidemiology

Osteoarthritis is the degenerative joint disease showing the highest frequency among all the rheumatic diseases with a social cost of up to 0.5 % of the gross domestic product in developed countries (Puig-Junoy and Ruiz Zamora, 2014). Osteoarthritis was initially described as a primary disease of cartilage, although nowadays it is considered as a disease of the entire joint organ resulting from a complex interplay of genetic, metabolic, biochemical and biomechanical factors. This disease is characterized by the loss of articular cartilage and abnormal changes in the surrounding soft and hard tissues of the joint, including bone. All the joints of the body can be involved and the most affected are the large weight-bearing joints, such as knees and hips, and small peripheral joints, including the hands (Sofat et al., 2011) (Figure 1). Osteoarthritis affects both men and women of all ethnic groups in all geographic locations, although it occurs more frequently in women. Age is the strongest predictor and the extended life expectancy will result in an enhanced occurrence of the disease. Joint trauma and life-style-associated risk factors, such as obesity and excessive joint use in occupational or leisure activities, also contribute to the onset and progression of the disease. In agreement, osteoarthritis most commonly affects the middle-aged and elderly, even though younger people may be affected mainly as a result of injury or over-use.

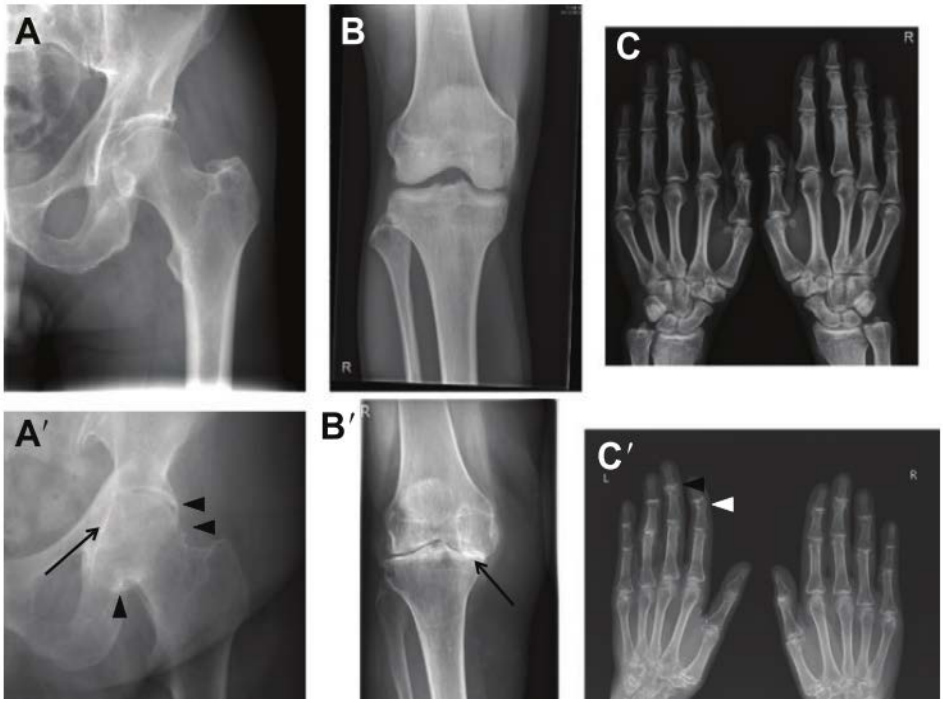

Figure 1. X-ray radiographic images showing structural alterations of the joints most commonly affected by osteoarthritis. (A, B, C) Normal and (A′, B′, C′) severely affected joints of the hip, knee and hand, respectively. Arrows indicate joint space narrowing; arrowheads indicate the presence of osteophytes (bony outgrowths) (Thysen et al., 2015).

The prevalence of osteoarthritis in the world's population over the age of 60 years is very high and varies greatly depending on the disease definition used (radiological or clinical), the joint affected, age, sex and geographical area. It is estimated that the number of people with osteoarthritis will have doubled by 2020 due to the rapidly increasing prevalence of obesity and elderly status (Centers for Disease Control and Prevention, 1994; Hunter, 2011). 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. The total number of years lived with disability caused by knee and hip osteoarthritis world-wide increased by 60.2% between 1990 and 2010, and by 26.2% per 1,000 people, meaning that osteoarthritis has moved up from 15th to 11th in the list of the most frequent causes of disability (Vos et al., 2012). According to the Global Burden of Disease study, progressive ageing of population could make osteoarthritis the 9th cause of disability-adjusted life years in developed countries by the year 2020 (Murray and Lopez, 1997; Puig-Junoy and Ruiz Zamora, 2014).

1.2 Osteoarthritis as a disease of the whole joint

Joints are complex organs in which different tissues functionally cooperate to allow movement between the bones of the skeleton, while limiting the degree and the axes of movement at the same time (Thysen et al., 2015). In the joint, a thin layer of articular cartilage covers the bones providing a smooth and pressure-deformable buffer zone that supports movements. This specialized tissue is composed of articular chondrocytes embedded in a specific extracellular matrix that contains type II collagen and large sulfated proteoglycans, such as aggrecans. The collagen fibers are responsible for resistance against tensile stretch, whereas the abundant negative charges on the macro-molecular proteoglycans attract water molecules that can be shifted within the tissue, giving its capacity to deform and adapt upon loading (Lories and Luyten, 2012). The underlying subchondral bone forms a complex interface with articular cartilage and has a critical role in stress and loading distribution. The joint cavity is further lined by the synovium, a thin

connective tissue composed by synovial fibroblasts and resident macrophages that produces the lubricating synovial fluid. The sub-lining zone is well-vascularized and represents the source of nourishment by diffusion for articular cartilage that is avascular and aneural. Finally, ligaments and the capsule, a strong tension-resistant connective tissue inclosing the joint, provide further strength and limit the range of motion.

Osteoarthritis occurs when the dynamic equilibrium between the breakdown and the repair of joint tissues becomes unbalanced leading to the disruption of the normal homeostasis of the joint (Lories and Luyten, 2011). Despite the identification of many risk factors associated with this disease, the mechanisms of initiation and progression of osteoarthritis are not well understood. Different factors can contribute to the onset of the disease (Figure 2). Repetitive loading or acute trauma and inflammation trigger the articular chondrocytes to become active. These cells start to produce additional extracellular matrix molecules, but also pro-inflammatory cytokines such as interleukins (ILs) and tissue destructive enzymes including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). In the short-term, anabolic signals are able to compete with the destructive cascades, but these protective mechanisms fail in the long-term and progressive loss of cartilage with cell death and depletion of the extracellular matrix evolves (Lories and Luyten, 2012).

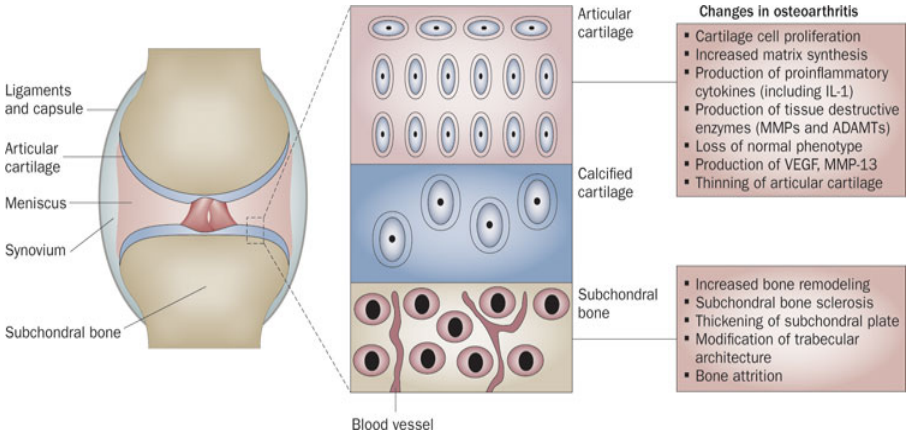

Figure 2. The bone–cartilage unit is at the center of joint function and physiopathology of osteoarthritis. Progressive development of osteoarthritis results in the simultaneous activation of different processes and pathways in the distinct tissues and cells of the joint. Abbreviations: ADAMTs, a disintegrin and metalloproteinase with thrombospondin motifs; IL-1, interleukin-1; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor (Lories and Luyten, 2011).

Progressive cartilage loss, subchondral bone remodeling, formation of osteophytes (bony outgrowths) at the joint margins, synovial inflammation (synovitis), damage/fibrosis of tendons, menisci and capsules, and bone marrow oedema are among the processes that characterize osteoarthritis physiopathology and potentially contribute to joint pain and functional impairment (Thysen et al., 2015) (Figure 3). The initial cartilage degradation is characterized by fibrillations and ulcerations, loss of extracellular matrix and cell death. Fissures in the superficial layer gradually extend into the deeper layers and finally lead to severe loss of cartilage structure and volume. This physiopathological process will then result in secondary changes to the subchondral bone and other tissues of the

joint including menisci, ligaments, periarticular muscle, capsule, and synovium (Thysen et al., 2015).

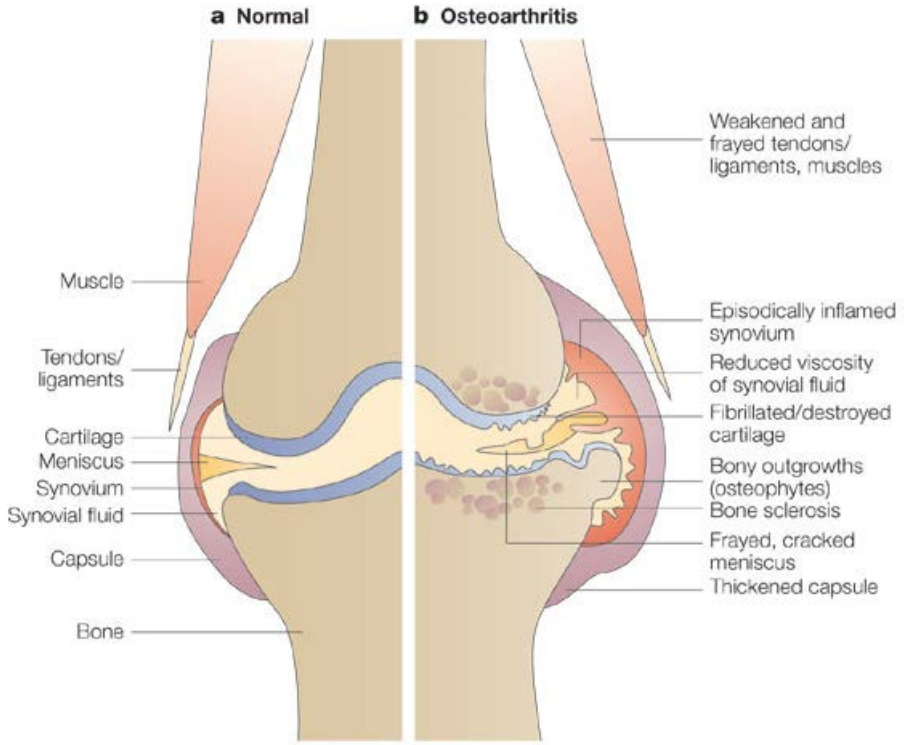

Figure 3. Schematic representation of the joint tissues affected by osteoarthritis (Wieland et al., 2005).

Magnetic resonance imaging (MRI), immunohistochemical and ultrasonography studies in early osteoarthritis patients have demonstrated the presence of synovitis with increased infiltration of macrophages and T cells, and elevated levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and tumor necrosis factor α (TNFα) in the synovial fluid and serum (Tonge et al., 2014) (Figure 4). Inflammation takes part in the osteoarthritis processes, but it

does not seem to be the dominant driving force. Other joint diseases, such as rheumatoid arthritis, are primarily driven by inflammation, whereas damage to cartilage and bone is a secondary phenomenon. Despite the loss of tissue components in osteoarthritis, production of new tissue also occurs especially in the early stages of disease, including fibro-cartilage and attempts by the cartilage to regenerate, as evidenced by increased protein synthesis by chondrocytes (Sofat et al., 2011). All these changes are accompanied by joint remodeling. Therefore, osteoarthritis has been considered as a hypertrophic arthritis in contrast to rheumatoid arthritis (atrophic arthritis), emphasizing that new tissue production and remodeling are characteristic features.

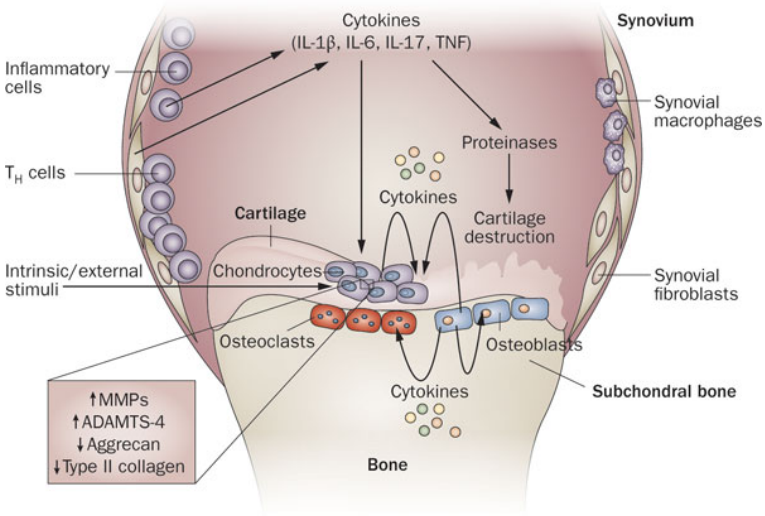

Figure 4. The role of proinflammatory cytokines in the physiopathology of osteoarthritis. The mechanisms involved include down-regulation of anabolic events and up-regulation of catabolic and inflammatory responses resulting in structural damage to the joint (Kapoor et al., 2011).

No sensitive diagnostic techniques beyond classical radiography are currently available for osteoarthritis. Therefore, disease progression cannot be predicted and, as a result, cannot be prevented or halted. Reliable, quantitative and dynamic tests to detect early damage and measure the progress of treatments targeted against joint destruction are required. Although the research aiming to find osteoarthritis biomarkers remains challenging, different structural molecules and fragments derived from bone, cartilage and the synovium have been reported as potential biomarker candidates (Ishijima et al., 2014).

1.3 Osteoarthritis pain: from the joint to the brain

Most of the patients with osteoarthritis describe pain as aching that varies in intensity and is usually worsened by changes in the weather and increased physical activity. Both positive (enhanced/aberrant sensations) and negative neurogical signs (reduced sensations) can be identified in osteoarthritis (Thakur et al., 2014). In agreement, several studies have shown the presence of allodynia (pain in response to normally innocuous stimuli) and hyperalgesia (increased sensitivity to noxious stimuli) in osteoarthritis patients, as well as impaired joint proprioception, loss of cutaneous vibration sensitivity, and hypoaesthesia to punctate mechanical, pin prick and thermal stimuli (Thakur et al., 2014).

It is unclear to date at which stage of the disease the joint becomes painful and very often there is poor correlation between radiological signs (narrow joint space and osteophytes) and the occurrence of pain (Schaible et al., 2009). The majority of patients presents pain and disability after significant loss of cartilage has occurred, but it is

estimated that up to 40% of individuals with radiological damage have no pain (Kidd, 2006). Osteoarthritis pain includes both nociceptive and neuropathic components and is associated with abnormally excitable pain pathways at the peripheral (joint) and central (spinal and supraspinal) levels of the nervous system (Thakur et al., 2014). Intra-articular anesthetic studies in hip and knee osteoarthritis support a peripheral drive to pain in approximately 60-80% of patients (Creamer et al., 1996; Crawford et al., 1998). However, central mechanisms such as dysfunction of descending inhibitory control or altered cortical processing of noxious information may also play an important role (Dray and Read, 2007).

1.3.1 Joint innervation

Knee joints are densely innervated by both sympathetic and sensory nerves (McDougall, 2006). Postganglionic sympathetic fibers terminate near articular blood vessels and regulate joint blood flow. The primary function of sensory nerves is to detect and transmit mechanical information from the joint to the central nervous system (CNS). Large diameter myelinated nerve fibers (Aβ or type II) encode and transmit proprioceptive signals. Corpuscular endings of these fibers were identified in ligaments and fibrous capsule. Pain-sensing nerve fibers are typically less than 5 μm in diameter and are either myelinated with unmyelinated free endings (Aδ or type III) or unmyelinated fibers (C or type IV). These nociceptive fibers are slowly conducting fibers having a high threshold and responding only to noxious mechanical stimuli. These fibers have been

identified in all joint structures, including the capsule, ligaments, menisci, periosteum and subchondral bone, with the exception of the normal cartilage (McDougall, 2006). A large group of C fibers are so-called “silent nociceptors” because they do not respond even to noxious mechanical stimuli of the normal joint. These “silent nociceptors” only respond to mechanical stimulation following tissue injury or inflammation. “Silent nociceptors” are one of the contributing factors responsible for the generation of joint pain (McDougall, 2006). Studies in humans revealed that the direct stimulation of fibrous structures, such as ligaments and capsule, with innocuous mechanical stimuli evokes pressure sensations, whereas pain is elicited when noxious stimuli are applied in these structures (Schaible et al., 2009). No pain is elicited by stimulation of cartilage, and stimulation of normal synovial tissue rarely evokes pain (Schaible et al., 2009). Pain in a normal joint is most commonly elicited by twisting or hitting the joint under physiological conditions.

1.3.2 Peripheral pain mechanisms

Osteoarthritis pain has a strong mechanical component and can be triggered by specific activities, such as climbing stairs. The mechanisms by which mechanical pain is sensed in the joint are poorly understood. However, the recent identification of mechano-gated ion channels on Aδ and C knee joint afferents by electrophysiological studies has provided a first insight into the physiological mechanisms responsible for mechano-transduction in joints (Heppelmann and McDougall, 2005). Moreover, experiments

in rodent osteoarthritis models have identified an important role for the voltage-gated sodium channel Nav1.8 in the noxious mechano-sensation of the joint (Schuelert and McDougall, 2012).

Cartilage is an avascular and aneural tissue, and pain should therefore arise from other joint structures. However, degrading cartilage could represent a major source of factors involved in the pain machinery, including cytokines, H^+ ions, adenosine, and, possibly, extra-cellular matrix fragments. Nocicepive fibers have been reported to express Toll-like receptors, which are pattern-recognition receptors that recognize a variety of damage-associated molecular patterns released during tissue injury and that contribute to pain generation. These receptors might indirectly contribute to pain in osteoarthritis by activating synovial fibroblasts and macrophages or by directly sensitizing nociceptive fibers (Scanzello et al., 2008; Malfait and Schnitzer, 2013). Other potential sources of pain would be modifications in bone tissues, including osteophytes, by impinging on other local joint structures.

Imaging studies in osteoarthritic joints have demonstrated that synovitis and bone marrow oedema can also be important contributors to pain (Sofat et al., 2011). The degree of synovitis detected by MRI correlates with pain levels, even in patients without radiographic signs (Baker et al., 2010). The inflammatory mediators released into the joints by neurons, immunocytes, synoviocytes, and vascular endothelium are crucial for pain sensitization processes (Schaible et al., 2009). These pro-inflammatory mediators include cytokines, chemokines, neuropeptides, prostaglandins, nerve growth factor (NGF) and nitric

oxide (NO), among others (Malfait and Schnitzer, 2013). Nonetheless, the involvement of these processes in osteoarthritis is still unclear. When sensitization of primary afferent fibers for mechanical stimuli occurs, the activation threshold of joint nociceptors is reduced and afferent nerves become hyper-responsive to both normal and noxious types of movement. Spontaneous firing of joint sensory nerves in the absence of any mechanical stimulation has also been described. This phenomenon is consistent with the activation of "silent nociceptors" and accounts for the resting joint pain often experienced by patients (McDougall, 2006).

1.3.3 Central pain mechanisms

The activation of joint fibers is subsequently transmitted via the dorsal root ganglia (DRG) to spinal cord. Central termini of afferent neurons enter the dorsal horn of the spinal cord and make the first synapse with interneurons or projection neurons. Projection neurons relay pain signals to the thalamus and the brainstem, and onwards to higher brain centres where signals are processed and perceived as pain. Two main systems in the brain are responsible for the perception of pain: the lateral and the medial systems of the lateral spino-thalamic tract (Hunter et al., 2009). The lateral system involves the activation of thalamic nuclei in the ventral lateral thalamus and the relay of information to the somatosensory cortex, where the noxious stimulus is analyzed for location, duration, intensity, and quality. The medial system involves the relay of information by other (midline and intralaminar) thalamic nuclei to different regions of the brain. The medial system comprises large

areas of the brain that are responsible for pain perception as well as for other functions, such as affective and cognitive processes, and activation of the descending pain pathways (Hunter et al., 2009).

Continued nociceptor inputs can lead to prolonged hyperexcitability of pain circuits in the CNS, a phenomenon known as central sensitization (Latremoliere and Woolf, 2009). Central sensitization represents an enhancement in the function of neurons and circuits in the nociceptive pathways caused by an increase in membrane excitability and synaptic efficacy, and reduced inhibitory transmission (Latremoliere and Woolf, 2009). Although peripheral mechanisms are involved in osteoarthritis pain, hypersensitivity of the CNS plays a significant role in a subgroup of patients (Lluch et al., 2014). Important factors of central sensitization are the enhanced intra-spinal release of transmitters from sensitized joint afferents as well as the increased excitability of post-synaptic neurons (Schaible et al., 2009). The primary excitatory neurotransmitter in the CNS is glutamate that is also released by Aβ, Aδ, and C fibers in the spinal cord. Glutamate and several modulatory mediators, including the neuropeptides substance P and calcitonin gene related peptide (CGRP), neurotrophins and prostaglandins, are crucially involved in the generation and maintenance of central sensitization (Hunter et al., 2009). The mechanisms that contribute to central sensitization also involve the participation of glial cells (microglia and astrocytes) (Latremoliere and Woolf, 2009). Central sensitization is the result of an enormous plasticity of the CNS that leads to increased spontaneous neuronal activity, reduced activation threshold by peripheral stimuli,

increased responses to supra-threshold stimulation and expansion of receptive fields (Latremoliere and Woolf, 2009). It is manifested as hyperalgesia and allodynia, even in areas outside the initial trigger zone. Indeed, many patients with osteoarthritis describe pain in areas of the body and skin that do not match the innervation territories of peripheral nerves. Primary hip osteoarthritis typically radiates to the knee, thigh or buttocks, whereas knee pain is usually felt in and around the knee, femur and on the upper tibia. This diffuse and radiating pain is called somatic referred pain (Figure 5). The distribution of the referred pain is likely to reflect convergent inputs to spinal cord neurons from primary afferent nerves of the affected joint and from remote tissues (Thakur et al., 2014). Thus, central sensitization of second order nociceptive neurons initiated by ongoing afferent input from the affected joint amplifies convergent afferent inputs from the remote tissue. In some patients, sensory abnormalities can be found in the area of pain referral, including pressure-induced pain and cutaneous mechanical hyperalgesia and allodynia.

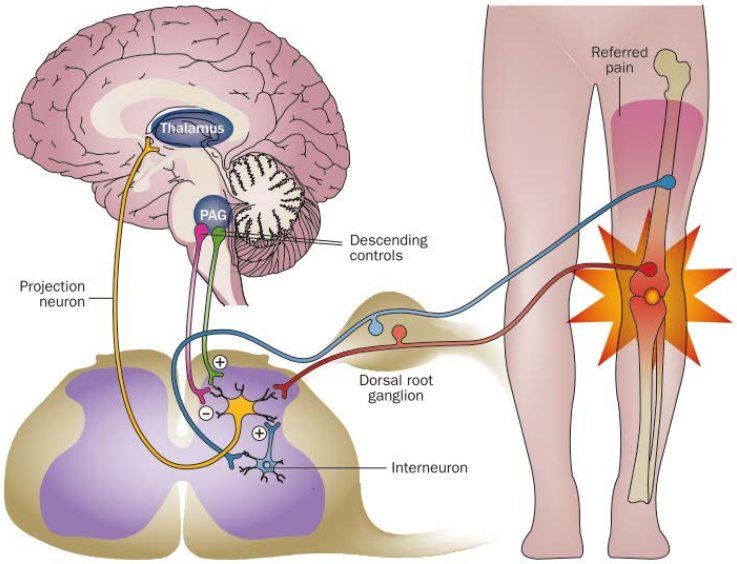

Figure 5. Schematic representation of central pain mechanisms involved in osteoarthritis. Persistent activation of peripheral inputs from one site (red) can lead to the establishment of a referred pain area at a distant site (blue) when central sensitization occurs in second-order processes in the spinal cord (yellow). Ascending sensory messages pass onwards to the thalamus. This process is modulated by excitatory (green) and inhibitory (pink) descending controls projecting to the spinal cord from the periaqueductal grey (PAG) (Thakur et al., 2014).

The plastic changes occurring during central sensitization are not restricted to the spinal cord and also involve supraspinal structures (Lluch et al., 2014). In addition to the ascending pain pathways, descending pathways in the CNS can either facilitate or dampen pain (Malfait and Schnitzer, 2013). Descending signals come from the hypothalamus, amygdala and rostral anterior cingulate cortex, and transmit to the periaqueductal grey (PAG) in the midbrain and the rostral ventro-medial medulla (RVM) in the brainstem. Neurons projecting from the RVM to the dorsal horn of the spinal cord can

directly or indirectly enhance or dampen nociception. Descending inhibitory pathways modulate the response to pain through the release of noradrenaline and serotonin onto the spinal circuits.

Other alterations associated with central sensitization in osteoarthritis patients include altered spinal reflexes, abnormal spatial and temporal summation, impaired descending pain inhibitory mechanisms and enhanced descending pain facilitation (Lluch et al., 2014).

Brain structural alterations have also been described in these patients (Quante et al., 2008; Gwilym et al., 2009; Howard et al., 2012; Malfait and Schnitzer, 2013; Lluch et al., 2014). Thus, a significant decrease in grey matter volume of the thalamus (Gwilym et al., 2010) and specific morphological changes in the cortical grey matter were observed in osteoarthritis patients (Baliki et al., 2011). Brain re-organization in osteoarthritis patients was unique to this condition, enabling to differentiate this “brain signature” from other chronic pain conditions (chronic back pain, complex regional pain syndrome) with high accuracy (Baliki et al., 2011).

The abnormalities in somatosensory perception are often reversible after successful surgery or joint replacement in osteoarthritis patients, and this reversibility further underlines the plasticity of the CNS and, mechanistically, implies that central sensitization is maintained by peripheral inputs from the joint (Malfait and Schnitzer, 2013).

1.4 The neuropathic component of osteoarthritis pain

Pain is currently categorized into nociceptive pain, which results from tissue damage often through inflammatory processes, neuropathic pain, initiated or caused by a primary lesion or dysfunction in the nervous system, and idiopathic pain that has no identified causes (Thakur et al., 2014). Identifying when chronic pain can be classified as neuropathic is important to define an appropriate therapeutic approach. Neuropathic pain is associated with characteristic somatosensory symptoms and pain qualities, including burning pain, paraesthesias, pins and needles, mechanical and thermal hyperalgesia, allodynia, paroxysmal pain and numbness. These features can be used in clinical assessment to distinguish neuropathic pain from chronic nociceptive pain (Thakur et al., 2014). Several screening tools and questionnaires are now available using patient descriptions of the location, intensity, frequency and quality of pain that give indication of the presence of neuropathic characteristics. Among them, the PainDETECT questionnaire has been widely used to aid in this diagnosis (Freynhagen et al., 2006). Studies using PainDETECT have estimated 5–50% prevalence of neuropathic pain in osteoarthritis patients (Thakur et al., 2014). This large range of estimates reflects the great heterogeneity of osteoarthritis population. Although central sensitization alone is not diagnostic for neuropathic pain, it has been demonstrated that osteoarthritis patients obtaining a high score (≥12) in the PainDETECT questionnaire, indicative of more neuropathic components to pain, were six times more likely to show

signs of central sensitization than patients with lower scores (<12) (Hochman et al., 2013).

The MRI technique is not sensitive enough to identify nerve fiber lesions, but immunohistochemistry techniques have identified alterations of the peripheral innervation in articular surface samples obtained from patients undergoing total knee replacement. During osteoarthritis, the innervation territories of different nerve fibers are highly plastic (Hunter et al., 2009). An example of this plasticity is the innervation of normally aneural tissues, such as cartilage, with substance P and CGRP positive nerves (Hunter et al., 2009). Therefore, the cartilage that is "normally" mechanically insensitive could potentially contribute to osteoarthritis pain when it becomes innervated. These peptide-containing nerves may also accelerate disease progression via localized neurogenic inflammatory mechanisms (Hunter et al., 2009). Increased angiogenesis, immune cells infiltration and expression of growth factors have also been demonstrated in osteochondral junctions of joint samples from osteoarthritis patient (Suri et al., 2007). These alterations possibly contribute to the neurovascular infiltration of these structures that in healthy individuals are minimally innervated and avascular. Conversely, a substantial decrease of innervation in the synovial lining layer has also been described in tissues with synovitis (Eitner et al., 2013). The simultaneous loss of innervations in synovial lining together with increased innervation of cartilage and the osteochondral junctions demonstrate that plasticity occurs in intra-articular somatosensory structures during osteoarthritis and further support the presence of a neuropathic component in osteoarthritis

pain (Thakur et al., 2014). The neuropathic component has also been demonstrated in rodent models of osteoarthritis by the expression of a biomarker of nerve damage/neuropathy, the activating transcription factor-3 (ATF-3), in DRG cells, the reduction of intra-epidermal nerve fiber density in plantar hind paw skin, and the microgliosis in the ipsilateral spinal cord (Ivanavicius et al., 2007; Orita et al., 2011).

1.5 Neuronal plasticity during pain: possible contribution in osteoarthritis

An important property of the synapses between neurons contributing to the nociceptive processes is their plasticity, which is the ability to adapt their strength in an activity-dependent manner (Luo et al., 2014). Recent works have recognized that functional and structural synaptic plasticity changes at both excitatory and inhibitory synapses along the nociceptive pathways represent a primary mechanism underlying the shift from physiological to chronic pathological pain (Luo et al., 2014). Emerging evidence demonstrates that maladaptive mechanisms similar to those underlying classical learning and memory can contribute to central sensitization and pain behavior (Tan and Waxman, 2012). Depending on the synapses and the intensity, frequency, and duration of activity, both increases (facilitation, potentiation, or sensitization) and decreases (depression or desensitization) in synaptic function can be elicited at short-term or long-term time scales in the nociceptive pathways (Luo et al., 2014). Short-term synaptic plasticity (short-term depression or potentiation) is referred

to changes in synaptic efficacy occurring over millisec to min and has been described at all levels of central pain processing (Luo et al., 2014). This form of plasticity may serve to amplify and/or filter nociceptive signals, thus modifying nociceptive synaptic transmission involved in the perception of acute pain (Luo et al., 2014). Long-term synaptic plasticity generally involves long-lasting changes in synaptic strength that outlast the duration of the conditioning stimulus for at least 30 min, persists for a few hours, and could even last for days to months. Repetitive activation of synaptic connections can induce two different forms of long-term synaptic plasticity, namely long-term potentiation (LTP) and long-term depression (LTD). LTP is one of the most studied forms of synaptic plasticity initially described in the hippocampus. It constitutes the cellular basis for learning and memory formation, and can also be induced in nociceptive pathways at spinal and supraspinal level (Luo et al., 2014). LTP can be induced and/or expressed by both pre-synaptic mechanisms (increase in transmitter release) and post-synaptic mechanisms (increase in post-synaptic responsiveness). LTP occurs in different types of neurons that release various neurotransmitters. However, an excessive release of glutamate, neuropeptides, such as substance P, and neurotrophic factors, such as brain-derived neurotrophic factor, has been implicated in central sensitization after inflammation and tissue injury (Tan and Waxman, 2012). Indeed, binding of these molecules to their receptors can lead to an intracellular rise in post-synaptic Ca^{2+} concentration, the major trigger for downstream protein kinases involved in LTP. This signaling cascade can ultimately lead

to potentiation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) and N-methyl-D-aspartate (NMDAR) glutamate receptor-mediated responses either by phosphorylation of existing synaptic receptors or by recruitment of additional receptors to the post-synaptic membrane (Luo et al., 2014).

Synaptic plasticity in nociceptive pathways not only spans functional changes but can also involve structural modifications. Dendritic spines are the primary site of excitatory synaptic contacts and are found throughout the nervous system primarily on neurons that receive convergent input, such as hippocampal and cortical pyramidal neurons, and dorsal horn neurons in the spinal cord (Tan and Waxman, 2014). Dendritic spines may develop, reorganize, and mature to maintain long-term synaptic efficacy and their architecture is highly regulated by multiple molecular signaling pathways (Tan and Waxman, 2014). Long-lasting changes in synaptic activity are accompanied by alterations in spine shape, size and number (Bourne and Harris, 2007). A single neuron may contain hundreds of dendritic spines of varied shapes and geometries, which have been generally categorized using simple descriptors in “mushroom”, “thin”, filopodia”, or “stubby” (Hering and Sheng, 2001) (Figure 6). The morphology of individual dendritic spines directly contributes to regulate the local synaptic function. Large mushroom-shaped dendritic spines are associated with mature, stabilized synapses having increased glutamate receptor density, whereas thin or filopodia-like spines are associated with developing or weaker, less-mature synapses. Therefore, the

responsiveness of thin spines to increases and decreases in synaptic activity has led to the suggestion that they are “learning spines”, whereas the stability of mushroom spines suggests that they are “memory spines” that maintain activity-dependent synaptic strength (Bourne and Harris, 2007).

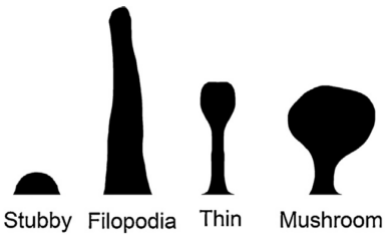

Figure 6. Morphological classification of dendritic spines. Common descriptors include stubby, filopodia, thin and mushroom spines (Tan and Waxman, 2014).

Synaptic enhancement leads to an enlargement of thin spines into mushroom spines and the mobilization of sub-cellular resources to potentiated synapses (e.g. increased membrane expression of AMPAR and NMDAR).

At the cellular level, an increase in dendritic spine density may also represent an increase in excitatory inputs upon a post-synaptic neuron, which may increase the overall excitability of a neural circuit (Bourne and Harris, 2007). Therefore, dendritic spine remodeling provides a structural-based mechanism for modifying or maintaining long-term synaptic function.

Synaptic reorganization in spinal cord pain circuitry is a well-known maladaptive phenomenon that can partially explain central sensitization associated with neuropathic pain (Ji and Woolf, 2001).

Previous reports have demonstrated primary changes in dendritic spines on nociceptive dorsal horn neurons in animal models of spinal cord injury and diabetic neuropathy, including increased spine density and enlargement of the spine head diameter (enhanced mushroom-shaped spines) (Tan and Waxman, 2012; Tan et al., 2012, 2013). These morphological changes accompany electrophysiological and behavioral alterations during neuropathic pain. *De novo* formation or maturation of dendritic spines is the result of the progression from early- to late-phase LTP. Similar plasticity changes in synaptic structure and function have been demonstrated in the mouse somatosensory cortex following peripheral nerve injury (Kim et al., 2012). Such synaptic remodeling causes local hyperexcitability of somatosensory cortex, finally leading to the development of chronic pain (Kim et al., 2012).

These morphological synaptic changes have not been investigated so far in models of osteoarthritis pain. However, it is possible that similar alterations may also occur during osteoarthritis and may affect other pain-related brain areas, and potentially contribute to the different nociceptive, emotional and cognitive symptoms of this disease (see next sections).

1.6 Osteoarthritis pain-related symptoms

1.6.1 The consequences of pain on the emotional state

Osteoarthritis is a chronic long-term condition that has both physical and psychosocial consequences. 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 capability and independence, with limitations of physical activity and reduced social contacts (Smith et al., 2014). This may increase the risk of developing other medical comorbidities that have negative consequences on the quality of life and represent an important cost to society, with significant use of health care resources. Thus, osteoarthritis patients may often suffer sleep disturbances, anxiety, feelings of helplessness and depression that are associated with a general negative attitude towards living with osteoarthritis (Smith et al., 2014). A study in a cohort of lower limb osteoarthritis patients demonstrated that over 40% (at least 2.5 times greater than expected in the general population) suffered from clinically significant anxiety or depression, and the majority of this group had anxiety with depression (Axford et al., 2010). Importantly, the severity of patients' reports of pain correlated with the levels of anxiety and depression (Axford et al., 2010). In agreement, osteoarthritis pain was demonstrated to have strong affective components and to be associated with high activity in brain areas related to emotions and attention, such as the prefrontal cortex (PFC) (Kulkarni et al., 2007; Goldenberg, 2010; Parks et al., 2011). Another study found that the strongest predictors of depression in osteoarthritis were the levels of perceived pain and decreased social contacts (Rosemann et al., 2007). Other predictors included physical limitations, age and body mass index (Rosemann et al., 2007). Moreover, measures of self-perceived quality of life in osteoarthritis patients correlated better

with pain and depression than radiological signs (Goldenberg, 2010).

The causal relationship between pain and emotional alterations is difficult to establish because experiencing pain contributes to a negative affective state and, in turn, a negative affective state magnifies and worsens pain perception. Therefore, persistent reports of elevated pain should prompt all clinicians to consider whether anxiety or depression could be a contributory factor and whether additional treatment is required, including psychosocial and pharmacological approaches.

1.6.2 The consequences of pain on cognitive functions

Chronic pain is commonly associated with the impairment of cognitive functions, representing a major obstacle for clinical pain management (Moriarty et al., 2011). Neuroanatomical and neurochemical substrates involved in cognition and pain processing are closely linked, which suggests that both may modulate one another, reciprocally. A wide array of cognitive domains can be negatively affected by chronic pain, including attention, concentration, speed processing, memory, psychomotor ability, decision-making and executive function (Liu and Chen, 2014). Several theories have emerged regarding the mechanisms mediating cognitive impairment in conditions of persistent pain, although the precise mechanisms have yet to be elucidated. The theory of disruption of attention proposed that pain-related sensory inputs compete for the limited attention resources, thereby affecting the cognitive functions that involves the processing and integration of

other information (Eccleston and Crombez, 1999). Alternatively, another theory proposed that the neurochemical mediators and neuroplastic changes produced under chronic pain may alter neural circuitries and interfere with normal cognitive functioning (Hart et al., 2000). Cognitive deficits have also been found in non-demented osteoarthritis patients and significantly correlated with pain ratings (Tassain et al., 2003; Karp et al., 2006). Notably, mental flexibility and memory are domains at particular risk of impairment, probably because these are already vulnerable areas of cognition in the aging brain (Karp et al., 2006).

1.7 Therapeutic options for osteoarthritis

1.7.1 Current osteoarthritis treatment

Current treatment of osteoarthritis is mainly symptomatic and includes both pharmacological and non-pharmacological approaches. According to the Osteoarthritis Research Society International, this treatment is 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., 2013). Curative treatments are not still available. Current therapies have modest efficacy in most of the cases and leave patients with considerable pain and functional disability (Hunter, 2011) (Figure 7). Non-steroidal anti-inflammatory drugs (NSAIDs) are considered as first-line pharmacological therapy in patients with osteoarthritis with mild to moderate pain (Lee et al., 2004). However, these

compounds have limitations, especially in elder populations because of gastrointestinal side-effects, hepatic and renal toxicity.

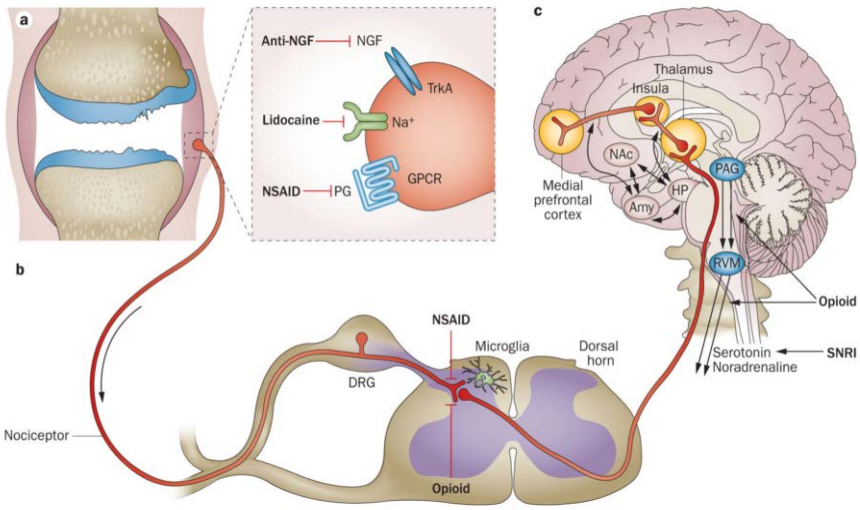

Figure 7. Neuroanatomy of the pain pathway and analgesic targets in osteoarthritis. (A) Various analgesics that are efficacious against joint pain act in the periphery by targeting receptors expressed at nociceptor peripheral terminals. (B) Central sensitization can occur through the strengthening of synapses and through the loss of inhibitory mechanisms. In addition, the activation of microglia contributes to enhanced pain sensitivity. Prostaglandins can also have a sensitizing effect in the dorsal horn, and NSAIDs can thus exert central analgesic actions, in addition to their peripheral actions. Opioids can inhibit incoming pain signals in the dorsal horn. (C) Serotonin–noradrenaline reuptake inhibitors (SNRIs) engage the descending inhibitory pathways. RVM neurons are opioid sensitive, and opioids have an analgesic effect through engaging descending inhibition. Abbreviations: Amy, amygdala; DRG, dorsal root ganglion; GPCR, G-protein-coupled receptor; HP, hippocampus; NAc, nucleus accumbens; NGF, nerve growth factor; PAG, periaqueductal grey; PG, prostaglandin; RVM, rostral ventromedial medulla; TrkA, tropomyosin receptor kinase A, also known as high affinity nerve growth factor receptor (Malfait and Schnitzer, 2013).

Patients who do not respond or cannot tolerate NSAIDs and continue to have severe pain may be considered candidates for other therapeutic approaches, such as opioid therapy. Nevertheless, tolerance, dependence and other adverse effects, including sedation, dysphoria, respiratory depression and constipation, may occur with opioid use.

Topical NSAIDs and capsaicin can be effective as adjunctives and alternatives to systemic administered analgesics in osteoarthritis. In addition, intra-articular corticosteroid administration has been beneficial especially in treating acute pain episodes in patients not responding to oral analgesics (Ravaud et al., 1999). However, the benefit is short-lived and frequent injections can further damage the joint.

Osteoarthritis patients presenting neuropathic pain characteristics are more likely to respond to non standard analgesics than to NSAIDs (Thakur et al., 2014). Among these, tricyclic antidepressants (i.e. amitriptyline), gabapentinoids (gabapentin, pregabalin) and serotonin–noradrenalin reuptake inhibitors (i.e. duloxetine) have demonstrated efficacy in osteoarthritis animal models. In agreement, the analgesic effect of NSAIDs in a rat model of osteoarthritis was maintained during the first two weeks post-induction, but it was highly reduced beyond this time, whereas amitriptyline and gabapentin remained efficacious (Ivanavicius et al., 2007). The analgesic effect of pregabalin, a drug widely used for the management of neuropathic pain, was also demonstrated in an osteoarthritis rodent model with a high degree of nerve damage (Rahman et al., 2009; Thakur et al., 2012). This suggests that,

although inflammation and joint damage cause the initial trigger for pain, sustained exposure to noxious stimuli can cause neuronal plasticity and a subsequent abnormal sensation of pain unrelated to inflammation. However, the negative results obtained in clinical trials with gabapentinoids could be caused by the heterogeneity in the study group, probably not enriched in patients with neuropathic abnormalities, rather than the ineffectiveness of the drug (Thakur et al., 2014). Combination therapy could also be a promising option for the treatment of osteoarthritis pain with neuropathic features (Thakur et al., 2014). In agreement, a small clinical study with knee osteoarthritis patients presenting neuropathic pain characteristics has recently demonstrated that those patients taking a combination of a NSAID and pregabalin reported significantly greater improvement of pain than those taking only one of these drugs (Ohtori et al., 2013).

The selective serotonin-noradrenalin re-uptake inhibitor duloxetine showed a moderate analgesic effect in osteoarthritic rats (Chandran et al., 2009), and recent clinical studies reported that it was an effective and tolerable analgesic in patients with knee osteoarthritis (Micca et al., 2013). Moreover, a monotherapy with a dual mode of action as a μ-opioid receptor agonist and a noradrenalin-reuptake inhibitor, tapentadol, has been confirmed in the treatment of severe osteoarthritis pain (Steigerwald et al., 2013). These non conventional drugs may have a dual beneficial effect on both pain and the pain-related affective alterations, since sensory and affective processing is altered within the CNS during osteoarthritis. However,

the adverse effects associated with many of these drugs would limit their clinical use.

It is widely accepted that the optimal treatment for osteoarthritis combines medication with non-pharmacological therapies. Non-pharmacological interventions are frequently used in the management of patients with osteoarthritis and are currently still considered the first-line treatment (Sarzi-Puttini et al., 2005). Among these non-pharmacological modalities, the most widely proposed include weight reduction, physical therapy, aerobics, muscle strengthening, walking aids, thermal modalities, transcutaneous electrical nerve stimulation, acupuncture, education and self-management (Sarzi-Puttini et al., 2005). In patients with severe osteoarthritis that significantly limits their activities and that does not respond to other treatments, surgery is usually recommended. However, surgery is counseled before the disease causes complications, such as muscle loss and joint deformities. Finally, biological restoration of the articular cartilage has also been explored by stimulating resident hyaline cartilage, biologically enhancing bone marrow progenitors, or by cartilage transplantation (Sarzi-Puttini et al., 2005).

Therefore, osteoarthritis treatment remains an open issue to deal with, and there is an urgent need for more effective drugs and new animal models for better understanding the aetiology and physiopathology of this disease. It is also important to consider the affective and cognitive alterations associated with pain in the preclinical and clinical settings because they critically affect the outcome of the therapeutic approach. A treatment able to improve

pain as well as the emotional and cognitive symptoms would be essential to obtain an effective management of the disease.

1.7.2 Disease-modifying drugs for osteoarthritis

A new strategy for osteoarthritis drug development is focused on modifying the structural progression of the disease. This approach could potentially cause retardation, a complete halt or a reversion in disease progression and even the prevention of disease development (Hunter, 2011). Some disease-modifying osteoarthritis drugs have shown promising results in clinical trials and could represent new therapeutic approaches in the near future. Targeting cartilage matrix degeneration is a well explored area for drug discovery. MMPs were initially seen as attractive drug targets, but, despite several candidates showing good efficacy in preclinical disease models, the development of MMP inhibitors has been limited because of undesirable musculo-skeletal side effects in both osteoarthritis preclinical models and patients (Tonge et al., 2014). In agreement, a Phase II clinical trial involving knee osteoarthritis patients receiving a MMP inhibitor with low affinity for MMP-1 and MMP-7, which were initially considered the major contributors to the musculoskeletal side effects, was unfortunately terminated due to musculoskeletal toxicity. An alternative approach was to target the aggrecanases, members of the ADAMTS family. An ADAMTS4/5 dual inhibitor has been investigated in Phase I studies, although the results have shown poor pharmacokinetics (Tonge et al., 2014). Targeting anabolic pathways to promote cartilage repair is an alternative strategy for preventing cartilage degeneration.

Recombinant fibroblast growth factors and bone morphogenetic protein-7 are currently the focus of several clinical trials (Phase I/II) and include studies evaluating their efficacy in slowing disease progression in a cohort of knee osteoarthritis patients (Tonge et al., 2014).

Bone remodeling has also been taken into consideration in clinical trials that are investigating agents that inhibit bone resorption. Among them, the anti-resorptive bisphosphonates have shown improvements in pain scores, but not disease-modifying efficacy in patients (Phase II), whereas clinical trials with calcitonin were stopped in Phase III due to its association with an elevated risk of prostatic cancer (Tonge et al., 2014). More promising are the recently disclosed Phase III data on strontium ranelate, which demonstrated beneficial effects in both symptoms and structural pathology (Reginster et al., 2013).

The approaches targeting cytokines reported to be elevated in osteoarthritis joints (i.e. IL1β, TNFα) have provided disappointing results in the Phase II clinical studies (Tonge et al., 2014). A non standard NSAID, licofelone, which is a dual cyclooxygenase (COX) and lipoxygenase inhibitor with no reported gastrointestinal or cardiovascular side effects at efficacious concentrations significantly reduced cartilage volume loss in knee osteoarthritis patients (Raynauld et al., 2009). The inducible NO synthase pathway that has a key role in cartilage destruction has been considered as an attractive target for disease-modifying drugs (Tonge et al., 2014). However, despite the promising preclinical results, a recent Phase III study showed that the oral selective

inducible NO synthase inhibitor SD-6010 was well tolerated, but failed to slow the rate of joint space narrowing compared to placebo treatment in a cohort of overweight osteoarthritis patients (Hellio le Graverand et al., 2013).

Finally, the effects of nutritional supplements, such as glucosamine, chondroitin sulphate, avocado-soybean and vitamin D on the progression of osteoarthritis have also been studied in some clinical trials, even though the results are controversial (Akhtar and Haqqi, 2012; Davies et al., 2013; McAlindon et al., 2013).

Therefore, the existing pharmacologic agents do not present convincing disease-modifying efficacy for osteoarthritis. Therapeutic development in this area is challenged by numerous factors including the heterogeneity of the disease in its aetiology and clinical manifestations, the rate of disease progression and the disease stage, and the poor relationship between structural progression and clinical end-points. Therefore, the identification of different disease sub-sets by both genomic/transcriptomic analysis and clinical assessments should help in developing personalized disease-modifying drugs (Tonge et al., 2014). The stratification of osteoarthritis patients has also the potential to reduce candidate drug failure during clinical development since the efficacy of drugs could be masked when tested on heterogeneous populations (Tonge et al., 2014).

1.8 Osteoarthritis animal models

1.8.1 Existing animal models

Several animal models have been developed to study osteoarthritis, contributing to better understand the mechanisms of pathogenesis and to validate new targets for treatment (Little and Zaki, 2012). Nevertheless, these animal models have limitations due to differences in animal anatomy, functionality, dimensions, cartilage repair processes and thickness in comparison with human joints (Lampropoulou-Adamidou et al., 2014). In spite of these limitations, the use of these models presents important benefits because they allow the control of environmental parameters, the cause, the exact time of the disease onset, and permit the collection of tissue samples at any stage of the disease. Therefore, there is a need for continuous refinement of existing models and generation of new ones (Little and Zaki, 2012).

Two categories of osteoarthritis animal models have been extensively used: the induced and the spontaneous models (Lampropoulou-Adamidou et al., 2014) (summarized in Table 1). Osteoarthritis can be induced by surgery or by intra-articular injection of chemical agents in a variety of animal species. Surgical models produce joint instability and alter load bearing, producing similar pathophysiological features to human osteoarthritis associated with traumatic events. The surgical procedures used to induce osteoarthritis include meniscal tear, partial or total meniscectomy, dissection of the medial and/or lateral collateral ligaments, transection of anterior or posterior cruciate ligaments, osteotomy, myectomy, immobilization, patellectomy, articular

groove and trans-articular impact (Bendele, 2001; Longo et al., 2012; Lampropoulou-Adamidou et al., 2014). Another surgical procedure used for the study of the association between low serum oestrogen and the development of osteoarthritis is ovariectomy (Sniekers et al., 2008).

Model	Species
Induced models	
Surgical models	
Anterior cruciate ligament transection	Rat, mouse, dog, rabbit, goat, sheep
Meniscal tear	
Partial meniscectomy	Rat, mouse, dog, guinea pig
Complete meniscectomy	
Ovariectomy	Rat, mouse, rabbit, mouse, sheep
Chemical models	
Monosodium iodoacetate	Rat, mouse
Papain	Rabbit, mouse
Collagenase	Mouse
Quinolone	Mouse
Immunotoxins	Rat, mouse
Spontaneous models	
Naturally occurring	Guinea pig, Syrian hamster, dog, non-human primate
Genetic models	
STR/ort	Mouse
Overexpression of cathepsin K	Mouse
Post-natal expression of constitutively active human collagenase-3	Mouse
Del1	Mouse

Table 1. Animal models of osteoarthritis (Lampropoulou-Adamidou et al., 2014)**.**

Besides the surgical models, chemically induced osteoarthritis models are widely used to assess the therapeutic efficacy of potential agents. They require less invasive procedures, are easy to implement and permit the study of osteoarthritis lesions at different stages. However, the most important limitation of these models is the absence of correlation with the pathogenesis of human osteoarthritis (Lampropoulou-Adamidou et al., 2014). Chemical models include intra-articular injection of substances having deleterious effects on joint homeostasis with consequent destruction of joint structures. These chemical agents can produce inhibition of chondrocyte metabolism, such as papain or monosodium iodoacetate (MIA), damage of ligaments and tendons, such as collagenase or quinolone antibiotics, or selective joint denervation, as the case of immunotoxins (van der Kraan et al., 1989; Miyauchi et al., 1993; van Osch et al., 1994; Salo et al., 1997; Sendzik et al., 2009; van Lent et al., 2012; Lampropoulou-Adamidou et al., 2014). Among them, one of the most used is the intra-articular injection of MIA. This compound inhibits chondrocyte glycolysis (van der Kraan et al., 1989), and produces cartilage degeneration and subchondral bone alterations similarly to the clinical histopathology of osteoarthritis (Guingamp et al., 1997; Janusz et al., 2001). The pain-related behavior developed after a single injection of MIA has been widely described in rats (Fernihough et al., 2004; Sagar et al., 2010b) and mice (Harvey and Dickenson, 2009) demonstrating a functional impairment similar to that observed in human disease. Thus, chemical models are very useful for studying pain mechanisms related to osteoarthritis, although they are not suitable

for the study of disease pathogenesis (Lampropoulou-Adamidou et al., 2014).

A particular category of osteoarthritis models is represented by the spontaneous ones. These models exhibit slow progression and are time-consuming, but are closely related to human degenerative osteoarthritis from the pathophysiological point of view (Lampropoulou-Adamidou et al., 2014). Some laboratory animals such as guinea pigs, Syrian hamsters, dogs and non-human primates can spontaneously develop osteoarthritis. In addition, the use of genetic models has represented an important advance for the study of this disease. These models consist in the modification of particular genes codifying for key proteins that participate in osteoarthritis physiopathology. These transgenic models include STR/ort mice, a mouse strain that spontaneously develops osteoarthritis, Del1 mice that have alterations in cartilage collagen types II and IX, mice over-expressing cathepsin K, an enzyme involved in bone remodeling and resorption, and postnatal expression of constitutively active human collagenase-3 in hyaline cartilage (Lampropoulou-Adamidou et al., 2014).

Most of the current animal models of osteoarthritis mimic the symptoms of the human disorder, although no one completely reproduces the whole variety of symptoms of human osteoarthritis, representing an important limitation in terms of face validity. A gold standard model is still not available and each model has distinct advantages and disadvantages, allowing the knowledge of a small part of human osteoarthritis in terms of natural history, mechanisms and symptoms. Therefore, the use of multiple

osteoarthritis models would be needed, in agreement with the concept of disease stratification, which would implicate a more adequate and precise translation of the data to specific subpopulations of patients with osteoarthritis (Little and Zaki, 2012).

1.8.2 Pain evaluation in osteoarthritis animal models

Several outcome measures are available for the evaluation of the nociceptive-related behavior in animal models of osteoarthritis (Table 2) (Malfait et al., 2013). Most of the techniques used include changes in locomotion, limb incapacitance, mechanical allodynia and hyperalgesia, and spontaneous or evoked joint afferent nerve activity (Little and Zaki, 2012).

Test	Description/ measurements
Electrophysiology	**Recording from neurons in the pain pathway**
Evoked Pain Behaviour	**Evoked response to an external environmental stimulus (mechanical)**
von Frey filament model	Threshold for mechanosensitivity of the paw (mechanical allodynia or referred pain)
Pressure application measurement	Mechanosensitivity at the joint level
Vocalization	Audible or ultrasonic vocalization in response to noxious stimulation of the affected joint
Gait analysis	**Abnormal movement patterns to minimize joint loading and pain**
Incapacitance meter	Static weight bearing
Catwalk apparatus	Dynamic gait analysis
Spontaneous pain behaviours	**Observed behavior in the absence of external stimuli**
Activity-based assessment	Recording of behaviors that includes grooming, feeding, climbing and rearing.

Table 2. Pain assessment in osteoarthritis animal models (Malfait et al., 2013)**.**

However, none of these different outcomes provides a direct measure of spontaneous pain or pain at the joint level like that commonly experienced by patients. Indeed, allodynia is usually evaluated by the von Frey stimulation model in body regions distal from the affected joints (hindpaws), providing a measure of referred pain which does not represent the principal symptomatic outcome in patients. To circumvent this limitation, a pressure application measurement device has been recently developed to apply the mechanical stimulus directly to the joint and measure mechanical sensitivity at this level (Malfait et al., 2013). This device has been mainly used in rat models of joint inflammation and osteoarthritis (Malek et al., 2015). Another limitation of the preclinical pain evaluation in osteoarthritis animal models is represented by the lack of studies investigating the behavioral alterations often associated with pain, including affective and cognitive dysfunctions.

2 The endocannabinoid system

2.1 Overview

Cannabis sativa is one of the most ancient medicinal plants. The main psychoactive constituent in cannabis, Δ^9-tetrahydrocannabinol (THC), was isolated in the mid-1960s (Mechoulam and Gaoni, 1965). At least other 70 structurally related "phytocannabinoid" compounds have been identified since this milestone discovery (Mechoulam and Parker, 2013) and the development of synthetic cannabimimetic drugs has aided in the pharmacological characterization of an endogenous cannabinoid system (ECS) (Figure 8). The discovery of endocannabinoids, which mimic some of the effects of synthetic cannabinoids *in vivo*, their receptors, as well as their synthetic and metabolizing enzymes, has prompted preclinical studies to explore the role of the ECS in physiological and pathological conditions. These studies have been facilitated by the introduction of mice deficient in cannabinoid receptors or the endocannabinoid degrading enzymes, as well as selective cannabinoid receptor ligands and inhibitors of endocannabinoid metabolism. The results of these studies have implicated the ECS in a variety of physiopathological processes in the nervous systems and peripheral organs. However, the clinical use of synthetic cannabinoids or medicinal plant extracts have been largely empirical and limited to a few specific indications related to pain, wasting disorders, and chemotherapy-induced nausea and vomiting, due to their undesirable psychoactive effects (see next sections) (Pacher and Kunos, 2013).

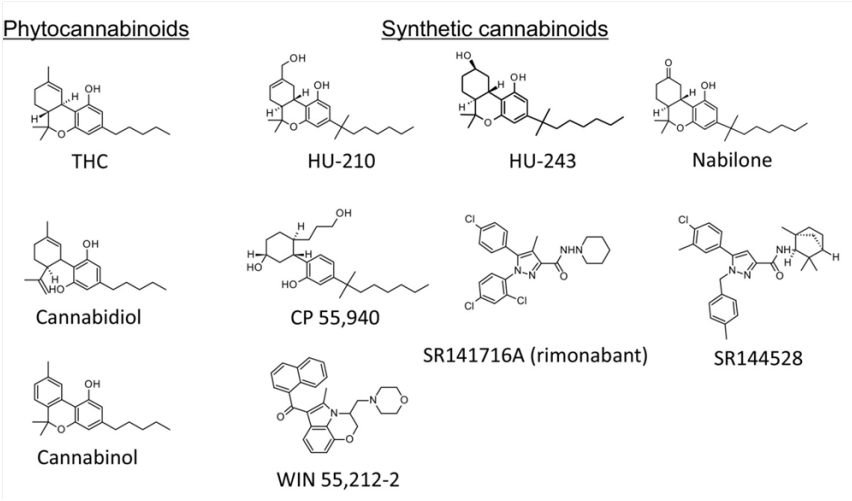

Figure 8. Chemical structure of representative phytocannabinoids and synthetic cannabinoids (Maldonado et al., 2011)**.**

In the last decade, the ECS has emerged as a new potential therapeutic target for osteoarthritis. Indeed, compelling evidence suggests that a therapeutic intervention on this system could offer the advantage to target multiple aspects of this disease due to the important role of the ECS in the control of different processes involved in the osteoarthritis physiopathology.

2.1.1 Cannabinoid receptors

Cannabinoids exert their pharmacological effects through the activation of at least two main cannabinoid receptors, the cannabinoid receptor 1 (CB1R) and 2 (CB2R). Both are G-protein-coupled receptors with seven transmembrane domains associated with the inhibitory $G_{i/o}$ protein (Childers and Deadwyler, 1996). Nevertheless, several evidences support the existence of other receptors that bind cannabinoid ligands, such as GPR55 (Baker et

al., 2006). Moreover, GPR3, GPR6 and GPR12 that are sphingosine-1-phosphate lipid receptors (Yin et al., 2009), together with the transient receptor potential vanilloid type-1 (TRPV1) (Di Marzo and De Petrocellis, 2010), are other potential cannabinoid-like receptors that could explain some of the non-CB1R/CB2R mediated effects. Cannabinoids differ in their affinity for cannabinoid receptors, and some of the most studied cannabinoids with their different affinity for CB1R and CB2R are summarized in Table 3.

Cannabinoid Receptor Ligand	K_i	
	CB_1	CB_2
	nM	
(−)-Δ^9-THC	5.05–80.3	3.13–75.3
HU-210	0.06–0.73	0.17–0.52
CP55940	0.5–5.0	0.69–2.8
R-(+)-WIN55212	1.89–123	0.28–16.2
Anandamide	61–543	279–1940
2-AG	58.3, 472	145, 1400
Agonists with higher CB_1 than CB_2 affinity		
ACEA	1.4, 5.29	195, >2000
Arachidonylcyclopropylamide	2.2	715
R-(+)-methanandamide	17.9–28.3	815–868
Noladin ether	21.2	>3000
Agonists with higher CB_2 than CB_1 affinity		
JWH-133	677	3.4
HU-308	>10000	22.7
JWH-015	383	13.8
AM1241	280	3.4
Rimonabant (SR141716A)	1.8–12.3	514–13,200
AM251	7.49	2290
AM281	12	4200
LY320135	141	14,900
Taranabant	0.13, 0.27	170, 310
NESS 0327	0.00035	21
O-2050	2.5, 1.7	1.5
SR144528	50.3–>10,000	0.28–5.6
AM630	5152	31.2
JTE-907	2370	35.9
11-OH-Δ^8-THC	25.8	7.4
Ajulemic acid	5.7, 32.3	56.1, 170.5
Cannabinol	120–1130	96–301
Cannabigerol	81	2600
Cannabidiol	4350–>10,000	2399–>10,000
N-Arachidonoyl dopamine	250	12,000
Virodhamine	912	N.D.

Table 3. Affinity expressed as K_i values of CB1R/CB2R ligands for the *in vitro* displacement of a tritiated compound from specific binding sites on rodent or human CB1R and CB2R (Pertwee et al., 2010).

The distribution of the two cannabinoid receptors in the CNS and peripheral tissues is rather different (Pertwee et al., 2010). CB1R is the most abundantly expressed metabotropic receptor in the brain and its distribution has been well characterized in rodents

(Herkenham et al., 1991; Tsou et al., 1998) and humans (Westlake et al., 1994). CB1R is highly expressed in the hippocampus, basal ganglia, cortex and cerebellum, and is less abundant in other brain areas such as the basal amygdala, medial hypothalamus, solitary nucleus, thalamus and brainstem, including pain-processing areas of the CNS, such as the PAG, RVM, and dorsal horn of the spinal cord (Freund et al., 2003) (Figure 9).

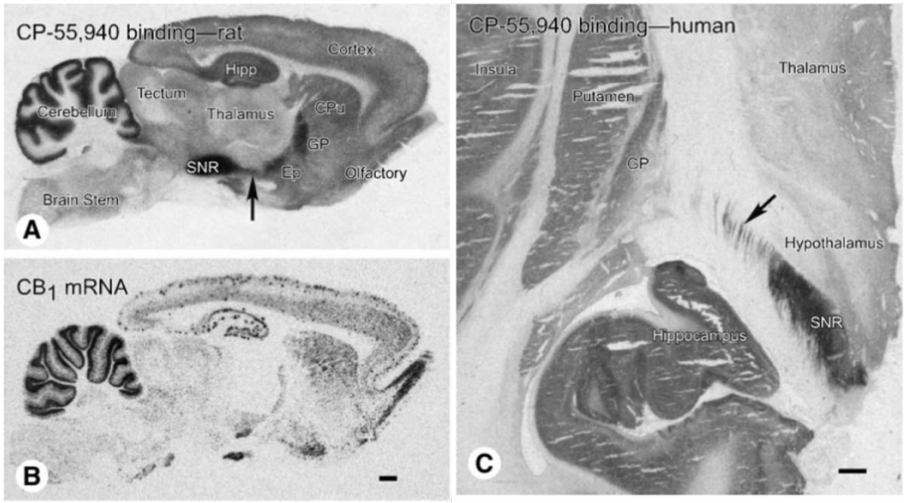

Figure 9. Distribution of CB1R in the brain. Autoradiographic film images showing CB1R localization in rat (A) and human brain (C) marked by the tritiated ligand CP-55,940. Sagittal slide-mounted section of rat brain hybridized with a CB1R-specific oligonucleotide probe (B) shows locations of neurons that express the CB1R mRNA. In both rat and human, high levels of receptor protein are visible in the basal ganglia structures. High binding is also seen in the cerebellum and in the hippocampus, cortex, and caudate putamen; low binding is seen in the brainstem and thalamus (Freund et al., 2003).

In all these areas, CB1R is mainly located on pre-synaptic terminals where they modulate the release of a variety of neurotransmitters,

mainly glutamate and gamma-aminobutyric acid (GABA), but also acetylcholine, noradrenaline, dopamine, serotonin, and cholecystokinin, among others (Howlett, 2002; Pertwee and Ross, 2002; Rodríguez et al., 2005; Szabo and Schlicker, 2005). CB1R is also expressed in peripheral organs, including adipocytes (Cota et al., 2003), liver (Osei-Hyiaman et al., 2006), lungs, smooth muscle, gastrointestinal tract (Calignano et al., 1997), pancreatic cells (Bermúdez-Silva et al., 2008), reproductive organs (Gérard et al., 1991), immune system (Galiègue et al., 1995), peripheral sensory nerves (Hohmann and Herkenham, 1999), sympathetic nerves (Ishac et al., 1996), chondrocytes (Mbvundula et al., 2006; Gómez et al., 2014) and bone cells (Whyte et al., 2012). More recently, CB1R has also been localized in astrocytes (Han et al., 2012) and in a subcellular compartment which is the mitochondria (Bénard et al., 2012).

CB2R is mainly expressed in the immune system cells, including macrophages, neutrophils, monocytes, B-lymphocytes and T-lymphocytes (Munro et al., 1993; Galiègue et al., 1995). Recently, CB2R expression has also been shown in bone cells (Ofek et al., 2006), liver (Julien et al., 2005), somatostatin-secreting cells in the pancreas (Bermúdez-Silva et al., 2008) and keratinocytes (Ständer et al., 2005). The presence of CB2R in primary sensory neurons and its role in the regulation of nociceptor activity has been controversial. However, several immunohistochemical and functional studies have demonstrated CB2R presence in nerve fibers, including those innervating osteoarthritic synovium and digit skin, and its role in nociceptive control (Ständer et al., 2005; Anand

et al., 2008). The presence of CB2R has also been demonstrated in the CNS in astrocytes (Sánchez et al., 2001), microglial cells (Walter et al., 2003), and neurons of spinal cord and brainstem (Van Sickle et al., 2005), among other brain regions (Svíženská et al., 2008). Nevertheless, the expression of CB2R in rat and murine brains was detected at levels much lower than those of CB1R (Svíženská et al., 2008) and the functional activity of CB2R in neurons still remains a controversial issue. Increasing evidence suggests a possible role for this receptor in several central responses, including emotional and rewarding processes (Onaivi et al., 2012).

2.1.2 The endocannabinoids and the enzymes responsible for their metabolism

The most relevant endogenous ligands for cannabinoid receptors are N-arachidonoyl-ethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995). Other putative endocannabinoids have been identified, such as 2-arachidonoylglycerol ether (noladin ether), N-arachidonoyldopamine, and O-arachidonoylethanolamine (virodhamine), although their physiological relevance has not been identified yet (Matias and Di Marzo, 2007) (Figure 10).

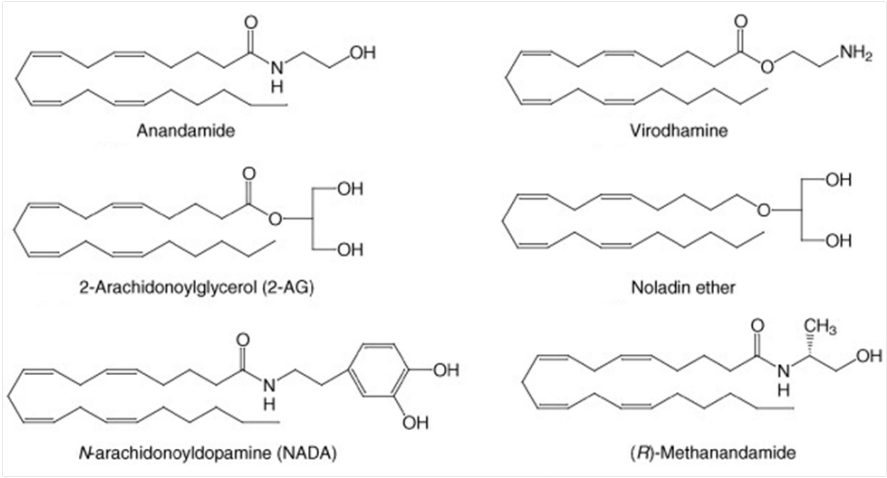

Figure 10. Endocannabinoid structures (Matias and Di Marzo, 2007)**.**

The endocannabinoids are synthesized "on demand", mainly post-synaptically, and act as retrograde messengers regulating the release of a variety of neurotransmitters at the pre-synaptic level (Wilson and Nicoll, 2002). Thus, endocannabinoids are neuromodulators that prevent the presence of excessive neuronal activity and maintain the homeostasis. Both AEA and 2-AG are produced from cell membrane lipids via different biosynthetic pathways. The synthesis of 2-AG from diacylglycerol is mediated by diacylglycerol lipase (DGL), while AEA is synthesized from the precursor N-arachidonoyl-phosphatidylethanolamine by the action of two enzymes, N-acyltransferase and phospholipase D (Di Marzo et al., 1994, 2004). Anandamide is mainly degraded by fatty-acid amide hydrolase (FAAH) (Di Marzo et al., 1994), whereas 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL) (Figure 11) (Dinh et al., 2002). Importantly, alternative metabolic pathways for each endocannabinoid exist (Jhaveri et al., 2007; Guindon and

Hohmann, 2008a). In addition, it is clear that re-uptake of both 2-AG and AEA occurs in the synaptic cleft following their release, and many pharmacological inhibitors of endocannabinoid transport are available, although the specific transporter proteins have not been yet identified (Guindon and Hohmann, 2009).

AEA, as THC, acts as a partial agonist at both CB1R and CB2R (Table 2), but also as endogenous ligand for TRPV1. 2-AG, which is the most abundant endocannabinoid in the brain, acts as a full agonist at both CB1R and CB2R.

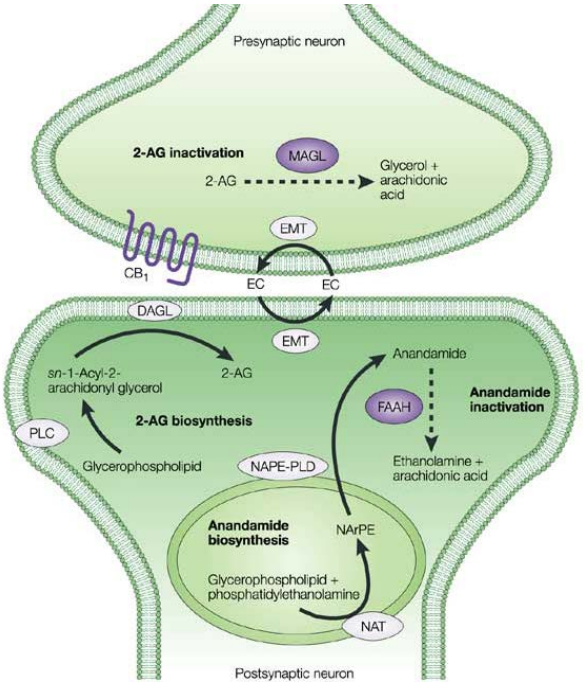

Figure 11. Main pathways involved in biosynthesis and degradation of endocannabinoids (Di Marzo et al., 2004)**.**

2.1.3 Cannabinoid receptor signaling

2.1.3.1 Intracellular pathways downstream cannabinoid receptors

Stimulation of cannabinoid receptors causes a great variety of effects through the activation of numerous signal transduction pathways (Figure 12). CB1R and CB2R mediate their biological effects by activating heterotrimeric $G_{i/o}$ proteins (α, β and γ). This activation leads to the inhibition of adenylyl cyclase activity, and the decrease of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activity (Bosier et al., 2010).

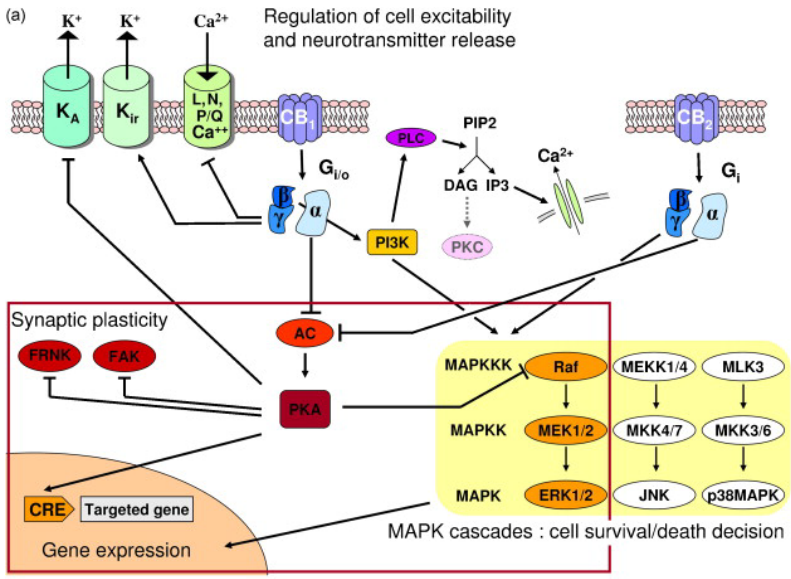

Figure 12. Complexity of cannabinoid receptor signalling. Both CB1R and CB2R are associated with $G\alpha_{i/o}$-dependent inhibition of adenylyl cyclase (AC) activity and Gβγ-dependent activation of the different MAPK cascades. CB1R negatively regulate voltage-gated Ca^{2+} channels and positively regulates inwardly rectifying K^+ channels, thereby inhibiting neurotransmitter release. Cross-talk

between signaling pathways are illustrated by the variety of responses requiring cannabinoid-mediated inhibition of PKA (Bosier et al., 2010).

Moreover, CB1R and CB2R regulate the phosphorylation and activation of different members of the family of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase-1 and -2 (ERK1/2), p38 MAPK and c-Jun N-terminal kinase (JNK) (Bosier et al., 2010). In addition, CB1R can inhibit N- and P/Q-type voltage-gated Ca^{2+} channels and activate A-type and inwardly rectifying K^{+} channels, which negatively regulate neurotransmitter release. CB1R may also induce elevations in intracellular Ca^{2+} through G protein-dependent activation of phospholipase C-β (PLC-β). Finally, the possible formation of heteromers with other G-protein coupled receptors seems to be critical for the regulation of the signal transduction pathways triggered by cannabinoid receptors (Pertwee et al., 2010).

2.1.3.2 Endocannabinoid-mediated short- and long-term synaptic plasticity

The fact that CB1R is one of the most widely expressed G-protein coupled receptor in the brain strongly suggests that it plays an important role in regulating synaptic function. Neuronal activity produces membrane depolarization and the activation of the enzymatic processes that lead to the cleavage of membrane phospholipid precursors for the synthesis of endocannabinoids (Castillo et al., 2012). Once released, endocannabinoids activate pre-synaptic CB1R, leading to the suppression of neurotransmitter release at both excitatory and inhibitory synapses. Therefore,

depending on the nature of the pre-synaptic terminal, endocannabinoids induce either suppression of inhibition or suppression of excitation. This phenomenon could result in a short-term plasticity, as the depolarization-induced suppression of inihibition or excitation, or long-term changes, as the endocannabinoid-mediated excitatory or inhibitory LTD (Castillo et al., 2012) (Figure 13).

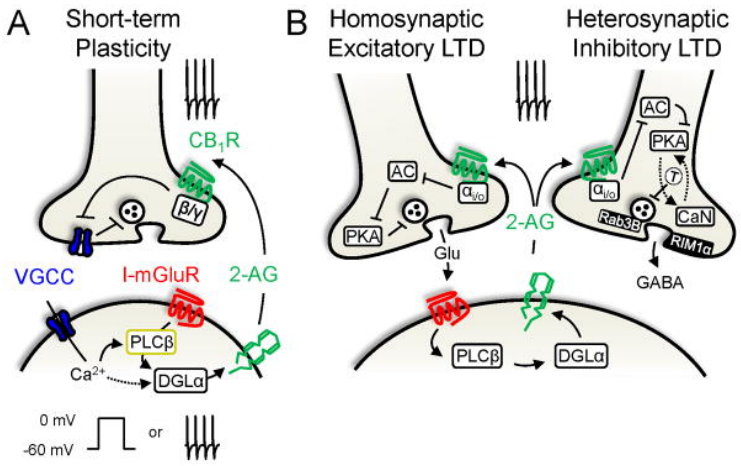

Figure 13. Molecular mechanisms underlying endocannabinoid-mediated short- and long-term synaptic plasticity. (A) The mechanisms of short-term plasticity, in which CB1R is activated for a few seconds, involve both pre-synaptic and post-synaptic activity. Post-synaptic activity evoked by prolonged depolarization triggers Ca^{2+} influx via voltage-gated Ca^{2+} channels (VGCCs). Other Ca^{2+} sources, like NMDARs and internal stores, may also contribute. Ca^{2+} promotes DGLα-mediated 2-AG production. Glutamate (Glu) pre-synaptic activity can also lead to endocannabinoid mobilization by activating post-synaptic group-I metabotropic glutamate receptors (I-mGluRs), which does not require intra-cellular Ca^{2+}. PLCβ can act as a coincidence detector integrating pre- and post-synaptic activity. 2-AG retrogradely targets pre-synaptic CB1R underlying depolarization-induced suppression of inhibition or excitation, both of which are

forms of short-term synaptic plasticity. (B) The induction of excitatory and inhibitory LTD requires probably prolonged activation of CB1R possibly together with concomitant pre-synaptic activity. Patterned pre-synaptic stimulation releases Glu, which activates post-synaptic mGluRs coupled to PLCβ and DGLα. 2-AG homosynaptically targets CB1R localized to excitatory terminals and heterosynaptically engages CB1R at inhibitory terminals. A $G\alpha_{i/o}$-dependent reduction in adenylyl cyclase (AC) and PKA activity suppresses transmitter release (Castillo et al., 2012)

Synaptic plasticity between primary nociceptors and second order dorsal horn neurons has a key role in pain and analgesia, and involves the participation of the ECS (see next sections).

2.2 ECS functions and therapeutic implications

The ECS is involved in a wide range of physiological and pathological processes. This system has a low tonic activity under physiological conditions and it is mainly activated in a phasic manner in order to maintain the homeostatic equilibrium in peripheral tissues and the CNS (Bisogno and Di Marzo, 2010). At peripheral level, the ECS modulates different processes, such as metabolism and energy storage, immune responses, bone remodeling, cardiovascular, respiratory, reproductive and gastrointestinal functions, among others (Grotenhermen, 2005). The ECS plays an important role in multiple aspects of the neural functions, including the control of movement and motor coordination (Rodríguez De Fonseca et al., 2001), learning and memory (Kano et al., 2009), emotion and motivation (Mechoulam and Parker, 2013), reward functions and addictive-like behavior (Maldonado et al., 2011), and pain modulation (Guindon and

Hohmann, 2009), among others. As already mentioned, this system interacts with multiple neurotransmitters, such as GABA, glutamate, acetylcholine, dopamine, histamine, serotonin, norepinephrine, prostaglandins and opioid peptides (Dewey, 1986), and this interaction is responsible for most of the neuronal effects of cannabinoids (Grotenhermen, 2004).

Cannabinoids are gaining more weight in modern medicine due to their promising therapeutic properties and the role of the ECS in the regulation of multiple body functions. Thus, the pharmaceutical companies have shown in recent years a growing interest in the development of novel drugs that target cannabinoid receptors or other components of the ECS for therapeutic intervention. These compounds include synthetic cannabinoid agonists and antagonists, or inverse agonists, non psychotropic phytocannabinoids, as well as endocannabinoid enhancers (Figure 14). Among the agents that enhance the endocannabinoid signaling, there are endocannabinoid cellular re-uptake inhibitors and inhibitors of the hydrolytic enzymes FAAH and MAGL.

The pharmacological modulation of the ECS produces different beneficial effects, including analgesic, immunomodulatory, anti-inflammatory, antiemetic, antiasthmatic, antihypertensive, hypnotic, neuroprotective, antiepileptic and anti-neoplastic effects (Svíženská et al., 2008; Pertwee, 2012). Moreover, cannabinoid agents would be useful for the treatment of glaucoma, spasticity and other movement disorders, eating disorders and drug addiction (Svíženská et al., 2008). Finally, recent works demonstrate the therapeutic potential of cannabinoids in several neuronal disorders that involve

the deregulation of the ECS, including depression and anxiety (Hillard et al., 2012; Micale et al., 2013), schizophrenia (Saito et al., 2013), Alzheimer's disease (Martín-Moreno et al., 2012), Huntington's disease (Sagredo et al., 2012) and autism spectrum disorders such as fragile X syndrome (Busquets-Garcia et al., 2013).

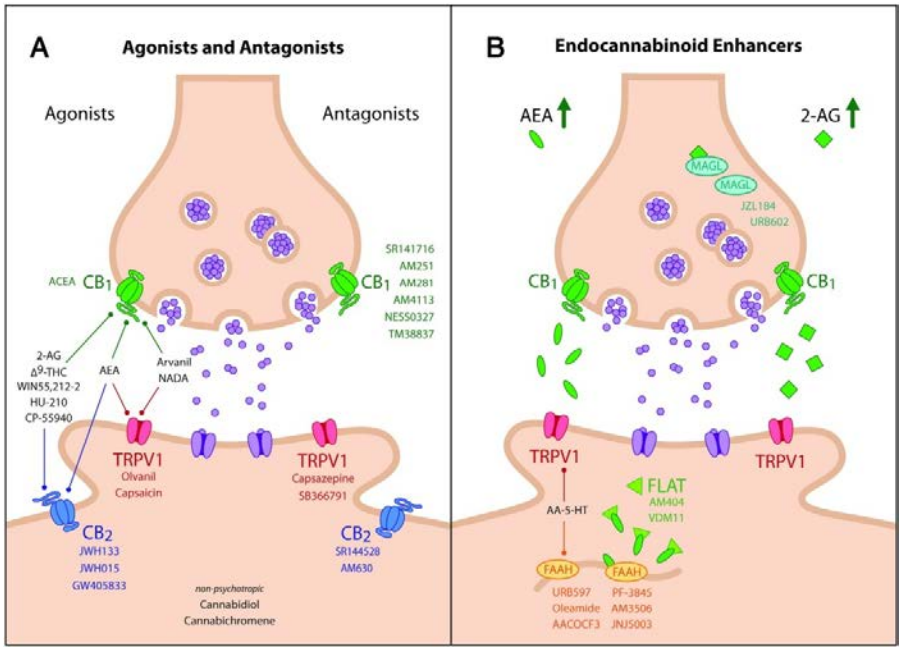

Figure 14. Schematic illustration of the ECS pharmacological modulation (agonists, antagonists and endocannabinoid enhancers). There is currently no consensus about the preponderance of pre-synaptic, post-synaptic or heterosynaptic expression of CB2R and TRPV1. One of the possible localizations in glutamatergic synapse is depicted (Micale et al., 2013).

The differences in cannabinoid receptor distribution also provide important opportunities for a selective therapeutic targeting. Peripheral restricted CB1R agonists or antagonists that do not cross

the blood-brain barrier, selective CB2R agonists and specific modulators of the endocannabinoid activity would represent new therapeutic strategies to circumvent the psychoactive side effects classically attributed to CB1R in the CNS, including dizziness, dry mouth, tiredness/fatigue, drowsiness, disorientation, euphoria, and cognitive alterations.

2.2.1 ECS and pain modulation

2.2.1.1 ECS role in the control of nociceptive transmission: sites of action

The ECS plays an important physiological role in the control of nociceptive responses acting at both central and peripheral levels. The important role of ECS in pain modulation is supported by the dynamic and adaptive changes involving this system in response to both physiological and pathological conditions. In agreement, physiological pain stimuli lead to rapid and transient (sec to min) increases in endocannabinoid levels, whereas pathological conditions lead to much slower and sustained (h to days) modifications of the endocannabinoid tone (Zogopoulos et al., 2013).

Recent findings suggest that the ECS plays an important role in the peripheral regulation of nociception (Agarwal et al., 2007; Clapper et al., 2010). In agreement, CB1R present on nociceptor terminals may mediate the anti-nociceptive and anti-inflammatory actions of locally produced AEA through its inhibitory influence on the release of excitatory neuropeptides (Clapper et al., 2010). CB1R (Bridges et al., 2003; Agarwal et al., 2007) and CB2R (Ständer et

al., 2005; Anand et al., 2008) are expressed in DRG, and their stimulation at this level also decreases nociceptive transmission (Millns et al., 2001; Anand et al., 2008). These receptors are synthesized in the bodies of DRG neurons and transported to their central and peripheral axonal branches. However, immune cells and keratinocytes are also involved in the peripheral CB2R analgesia as CB2R activation reduces the release of pronociceptive molecules from these cells (Ibrahim et al., 2005).

At the central level, the ECS controls nociception through CB1R located at spinal and supraspinal levels. This modulation has been well characterized at the spinal level, where CB1R is mainly found in the dorsal horn (Figure 15), although most of the primary afferent neurons that express CB1R mRNA are non-nociceptive large-diameter fibers (Hohmann and Herkenham, 1999).

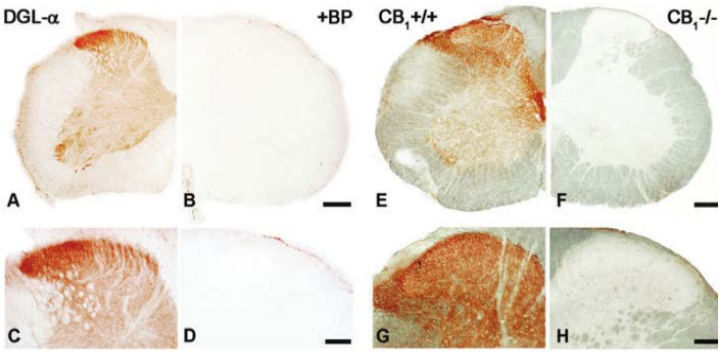

Figure 15. Light micrographs showing the accumulation of DGLα (A, C) and CB1R proteins (E, G) in the dorsal horn of mouse spinal cord. The specificity of the antibodies is indicated by the lack of immunostaining for DGLα (B, D) in the presence of a blocking peptide (BP), and for CB1R (F, H) in CB1R knockout mice ($CB1^{-/-}$) (Nyilas et al., 2009).

However, CB1R is also expressed pre-synaptically on the central terminals of the nociceptive Aδ and C fibers, or local interneurons, and inhibits the release of neurotransmitters involved in pain (Drew et al., 2000; Wilson and Nicoll, 2002; Nyilas et al., 2009). Recent evidence suggests that CB1R residing on the spinal terminals of primary nociceptors critically contributes to an NMDAR-independent form of LTD at these synapses, which requires simultaneous pre- and post-synaptic activity (Kato et al., 2012). This form of LTD is mainly expressed in Aδ fibers, whereas CB1R in C fibers prevents the induction of LTP (Kato et al., 2012). A similar long-lasting depression of nociceptive signal transmission can also be obtained with application of CB1R agonists in the presence of pre-synaptic stimulation (Kato et al., 2012). Moreover, the activation of CB1R on dorsal horn inhibitory interneuron terminals evokes a transient and readily reversible inhibition of synaptic GABA and glycine release. This CB1R-mediated disinhibition contributes to a specific form of secondary hyperalgesia occurring in response to high-intensity C-fibre stimulation (Pernía-Andrade et al., 2009). The predominant activation of one of these two apparently opposing actions occurring on distinct cellular elements of the dorsal horn circuit may depend on the initial activity of the dorsal horn sensory network (Kato et al., 2012).

CB2R has also been recently proposed to participate in pain modulation in the spinal cord (Racz et al., 2008b), although its distribution is difficult to study due to the lack of CB2R specific antibodies.

At the supraspinal level, CB1R stimulation inhibits pain transmission acting on the ascending pathways, mainly at the thalamus level (Martin et al., 1999), and modifies the subjective interpretation of pain by modulating neuronal activity in frontal and limbic structures (Manning et al., 2003; Burston and Woodhams, 2013). Supraspinal ECS modulation of the affective or even cognitive aspects of pain can be dissected from the somatosensory aspects. A recent neuroimaging study on capsaicin-evoked cutaneous pain in human subjects revealed that THC did not affect the intensity of pain sensation, but instead reduced its unpleasantness, in concurrence with altered activity in the anterior cingulate cortex and right amygdala (Lee et al., 2013). Another central mechanism for CB1R antinociception is the modulation of the descending inhibitory pathways. In these pathways, there are different cells ("on cells" and "off cells" in the RVM) that modulate the input of nociceptive information to the upward pain transmission. "On cells" facilitate nociceptive transmission, whereas "off cells" inhibit it (Rea et al., 2007). Microinjection of cannabinoid agonists into the PAG (Martin et al., 1999) and RVM (Martin et al., 1998), as well as the electrostimulation of these areas (Fields et al., 1991), resulted in CB1R-dependent analgesia.

The participation of CB2R in the supraspinal control of pain remains an open issue. However, a recent publication demonstrated the suppression of GABAergic inhibition by CB2R agonism in the medial entorhinal cortex of the rat, providing a pharmacological evidence for functional CB2R at CNS synapses (Morgan et al., 2009).

Supraspinal endocannabinoid signaling is also involved in a phenomenon known as stress-induced analgesia, in which brief exposure to environmental stress (e.g. immersion in cold water, or an electric shock to the paw) reduces the nociceptive responses in a subsequent pain test (Guindon and Hohmann, 2009). A detailed study of this effect revealed the mobilization of both AEA and 2-AG in the PAG, and suggested that 2-AG acting at CB1R was the predominant mechanism (Burston and Woodhams, 2013).

2.2.1.2 ECS role in acute or physiological pain models

Cannabinoids are highly effective against thermal (Khanna et al., 2011), mechanical (Bloom et al., 1977), and chemical stimuli (Sofia et al., 1973; Bloom et al., 1977), and are comparable with opiates (both in potency and efficacy) in producing antinociception in models of acute or physiological pain (Khanna et al., 2011). These antinociceptive effects may differ depending on the assay (e.g. tail flick, hot plate, paw pressure, plantar or radiant heat models), the cannabinoid employed and/or the mechanism used to modify endocannabinoid levels (Guindon and Hohmann, 2009). The exogenous administration of endocannabinoids (AEA and 2-AG), MAGL and FAAH inhibitors, as well as endocannabinoid re-uptake inhibitors produce antinociception mainly through CB1R activation in these different models. Transgenic mice lacking CB1R selectively in nociceptive (expressing Nav1.8) sensory neurons showed exaggerated responses in physiological basal pain sensitivity to noxious heat and mechanical stimuli (Agarwal et al., 2007). This suggests that, despite the important contribution of

central CB1R, peripheral CB1R has an important role in the control of the nociceptive responses (Agarwal et al., 2007). However, CB1R-independent mechanisms also participate in endocannabinoid antinociception (Guindon and Hohmann, 2009). The role of CB2R in mediating endocannabinoid effects in acute nociception is controversial (Guindon and Hohmann, 2009). Only certain assays (e.g. the plantar test) are likely to be sensitive to the detection of CB2R-mediated antinociception in the absence of inflammation or injury. In agreement, baseline responses to heat stimuli in the plantar test were enhanced in mice lacking CB2R, suggesting that CB2R could modulate this type of thermal sensitivity (Ibrahim et al., 2006).

2.2.1.3 ECS role in inflammatory and chronic pain models

Recent studies indicate that the ECS is highly dynamic and is altered under different pathological conditions, including pain states. These alterations include changes in endocannabinoid tone and in the expression of cannabinoid receptors (Sagar et al., 2009).

Cannabinoid agonists, AEA and 2-AG, and/or their modulators produce antinociceptive effects in different inflammatory pain models, including the carrageenan, capsaicin and the complete Freund's adjuvant models (Guindon and Hohmann, 2009). These antinociceptive effects could be due to actions on afferent neurons, but cannabinoids can also act on immune cells by inhibiting the production and release of pro-inflammatory and pronociceptive mediators (Burston and Woodhams, 2013). The involvement of CB1R and CB2R in the physiopathology of inflammatory pain has

also been investigated in mice lacking these receptors (CB1KO and CB2KO, respectively). However, the absence of CB1R or CB2R did not mainly alter the allodynic and hyperalgesic responses in these models (Whiteside et al., 2005; Sain et al., 2009; Naidu et al., 2010; Yu et al., 2010).

CB1R and CB2R activation also produces antinociception in neuropathic pain models (Guindon and Hohmann, 2009). Neuropathic pain can be induced in animals by traumatic nerve injury, toxic insults and metabolic challenges. An up-regulation of spinal CB1R promoting enhanced antinociceptive effects of cannabinoids was revealed in the rat sciatic nerve injury model of neuropathic pain (Lim et al., 2003). However, the constitutive lack of CB1R in mice did not significantly alter the neuropathic pain manifestations in this model (Castañé et al., 2006). In contrast, the specific CB1R loss in peripheral nociceptors enhanced the manifestations of neuropathic pain and reduced the analgesic effects of systemic and local, but not intrathecal, administration of cannabinoids (Agarwal et al., 2007), suggesting that peripheral CB1R is particularly important in this type of chronic pain. CB2R expression was also induced in the spinal cord during neuropathic pain (Zhang et al., 2003) and its important role in the development of neuropathic pain at spinal level has been demonstrated (Racz et al., 2008b). Indeed, the constitutive lack of CB2R in mice induced exacerbated behavioral manifestations of neuropathic pain after sciatic nerve injury that matched with the changes induced in microglia and astrocyte activation in the spinal cord (Racz et al., 2008b). In agreement, the behavioral and histological

manifestations of neuropathic pain were attenuated in transgenic mice over-expressing CB2R in the CNS (CB2xP) (Racz et al., 2008b). The activation of CB2R expressed in spinal microglia seems to be crucial for limiting the interferon-γ-mediated microglial activation and the consequent stimulation of neuroinflammatory pathways involved in the development of this chronic pain state (Racz et al., 2008a). Therefore, targeting the ECS at this level could inhibit both neuronal hyper-excitability and glial cell activation. The pharmacological modulation of endocannabinoid levels by administration of AEA or 2-AG, and FAAH or MAGL inhibitors also suppresses neuropathic pain in rodents (Hohmann, 2002). In agreement with these data, spinal levels of AEA and 2-AG are significantly elevated in the rodent chronic constriction injury model to counteract pain transmission (Petrosino et al., 2007). Moreover, increased endocannabinoid levels were also found in supraspinal areas (e.g. PAG and RVM) during neuropathic pain (Petrosino et al., 2007). Interestingly, desensitization of CB1R in pain-related cortical brain regions has been described, probably as a result of chronically elevated endocannabinoid levels in the brain (Hoot et al., 2010). It remains to be investigated whether pharmacological enhancement of endocannabinoid signaling in these brain regions could have a beneficial effect under these conditions.

The activation of the ECS has been revealed to be of particular interest for the treatment of osteoarthritis pain, as described in the next sections.

2.2.1.4 ECS and endogenous opioid system cross-talk in pain modulation

It is now well recognized that the ECS and opioid system share similar distributions in several brain areas, spinal cord and peripheral sites of pain pathways (Bodnar, 2012). Thus, cannabinoids can interact synergistically with opioid agonists to produce antinociception in different acute and chronic pain models (Welch, 2009). In agreement, THC, or even AEA if protected from degradation, enhances the antinociceptive effects of the opiod agonist morphine (Welch, 2009). This synergism seems to be receptor mediated since it can be blocked by both cannabinoid and opioid receptor antagonists (Welch and Stevens, 1992; Cichewicz et al., 1999). Moreover, the role of endogenous opioid system in cannabinoid antinociceptive effects (Maldonado and Valverde, 2003), as well as the involvement of the ECS in the antinociceptive mechanisms of opioids have been well documented (Desroches et al., 2014) (see next sections). Although the precise molecular and cellular mechanisms underlying these processes are not clearly established, direct receptor-receptor interaction (heteromers) and interaction between intracellular pathways may be involved (Pertwee et al., 2010; Al-Hasani and Bruchas, 2011). Physical and functional interaction between cannabinoid and opioid receptors, which co-localize on the same neurons in several structures, including PAG and dorsal horn, was demonstrated (Hojo et al., 2008).

The endogenous opioid system

The endogenous opioid system consists of three families of opioid peptides that bind to different types of opioid receptors, and the enzymes involved in the cleavage and degradation of these peptides. Three different subtypes of opioid receptors, μ receptor (MOR), δ receptor (DOR) and κ receptor (KOR), have been identified, cloned and characterized at the molecular, biochemical and pharmacological level (Kieffer and Evans, 2009). Another receptor, the nociceptin/orphanin receptor (orphanin-receptor like 1 or ORL1), was initially proposed to be part of the opioid receptor family, but it is now considered to belong to an anti-opioid system by the pharmacological actions arising from its activation (Anton et al., 1996). Opioid receptors are G protein-coupled receptors associated with the inhibitory $G_{i/o}$ protein and are broadly distributed in the nervous system and peripheral tissues. At the peripheral level, they are located in sensory and sympathetic nerve fibers of skin and joints, grastro-intestinal tract, urinary bladder, endocrine and immune system (Stein, 1993). MOR is the opioid receptor with a wider distribution in the brain, mainly in structures related to nociceptive control, motor responses and motivation, while DOR and KOR have a more restricted distribution. In the spinal cord, approximately 60% of opioid receptors are MOR, while 21% are DOR and 19% KOR (Mansour et al., 1995).

Three families of endogenous opioid peptides derived from either pro-opiomelanocortin, pro-enkephalin or pro-dynorphin have also been identified and cloned (Kieffer and Gavériaux-Ruff, 2002). These precursors generate several final active peptides including β-

endorphin, met- and leu-enkephalin, dynorphins and neo-endorphins, respectively. The endogenous opioid ligands exhibit different affinities for each opioid receptor. β-Endorphin binds with higher affinity to MOR than DOR or KOR. The affinity of met- and leu-enkephalin for DOR is 20-fold greater than that for MOR, and dynorphins are the putative endogenous ligands for KOR (Bodnar, 2012). Two enzymes are responsible for the degradation of enkephalins, the main endogenous opioid peptides: the neutral endo-peptidase (neprilysin) and the aminopeptidase N (Roques et al., 2012). Two additional peptides, endomorphin-1 and -2, were proposed as putative MOR selective endogenous opioid ligands (Zadina et al., 1997), although, neither the genes nor the precursor proteins for their endogenous synthesis have been identified.

Based on the anatomical and cellular distribution of its elements, the endogenous opioid system is involved in several physiological responses, such as pain and stress modulation, the control of motivation and reinforcement, and motor and homeostatic adaptive functions, including food intake and regulation of body temperature (Bodnar, 2012). The endogenous opioid system also contributes to the control of some autonomic nervous system functions, such as breathing and gastrointestinal motility. In addition, it also participates in the modulation of immune responses.

The endogenous opioid system plays a crucial role in the control of nociceptive responses at both peripheral and central level, by modulating ascending and descending pain pathways (Fields, 2004; Kapitzke et al., 2005). The activation of opioid receptors has mainly inhibitory functions. In agreement, the reduction of cAMP levels,

together with the inhibition of voltage-gated Ca^{2+} channels and the activation of inwardly rectifying K^+ channels resulting from opioid receptor stimulation lead to the decrease of membrane excitability, the reduction of neurotransmitter release and, ultimately, the reduction of nociceptive transmission.

The involvement of peripheral opioid receptors in analgesia has been demonstrated particularly during inflammatory processes where both opioid receptor expression and efficacy are increased (Kapitzke et al., 2005). Thus, immune cells synthesize and release opioid peptides that bind opioid receptors in the peripheral nerve terminals, thereby reducing nerve excitability and release of inflammatory mediators (Rittner et al., 2008). In the CNS, the endogenous opioid system regulates the nociceptive pathways at both spinal and supraspinal levels. At the spinal level, the endogenous opioid system inhibits nociceptive transmission conveyed by Aδ and C fibers. The opioid receptors are expressed at both pre- and post-synaptic levels. They inhibit the pre-synaptic release of excitatory molecules involved in pain transmission (i.e. glutamate, substance P, CGRP) from the central terminals of nociceptors. Post-synaptically, opioid receptors are located on dendrites of second order spino-thalamic neurons and interneurons, and their activation causes K^+ channel activation with consequent efflux of K^+ and hyperpolarization of projecting neurons. In contrast to this antinociceptive activity, the activation of the endogenous opioid system may have in certain situations pronociceptive effects at the spinal level. Thus, the increase of dynorphin at the spinal level has been linked with the development of hyperalgesia and

allodynia in inflammatory and nerve injury pain models (Dubner and Ruda, 1992; Malan et al., 2000; Laughlin et al., 2001). In these situations, spinal dynorphin causes an increased release of excitatory neurotransmitters, which contribute to amplify pain transmission (Ossipov et al., 2003). At supraspinal level, opioid receptors and peptides are expressed in the main brain areas involved in pain transmission and perception including amygdala, thalamus, hypothalamus, cortex, PAG and RVM (Mansour et al., 1995). Opioid receptors are also abundantly expressed in the limbic system where they control the emotional perception of pain (Bodnar, 2012). The endogenous opioid system plays also a crucial role in the modulation of the descending inhibitory pathways by inhibiting pronociceptive "on cells" and activating antinociceptive "off cells" (Fields, 2004).

The endogenous opioid system in cannabinoid antinociception

Cannabinoid administration (i.e. THC) facilitates the release and/or synthesis of endogenous opioid peptides in different regions of the CNS, including the spinal cord, and at periphery (Maldonado and Valverde, 2003; Ibrahim et al., 2005). This could represent one of the possible mechanisms to explain the interactions between cannabinoid and opioid systems (Maldonado and Valverde, 2003). In agreement, reduced synergistic analgesia produced by cannabinoid and opioid agonists was observed in pro-dynorphin knockout mice or after administering antibodies against dynorphin, or DOR and KOR antagonists, implying that cannabinoids indirectly activate opioid receptors (Manzanares et al., 2006).

Besides this synergism, selective opioid antagonists and knockout mice for specific opioid components have been used to study the specific involvement of this system in cannabinoid antinociception. Thus, contradictory results concerning the ability of naloxone, a preferential MOR antagonist, to block cannabinoid antinociception have been reported, depending on the test and the administration route (Maldonado and Valverde, 2003). Selective DOR antagonists failed to block cannabinoid antinociception, whereas preventing KOR activation was able to reduce it, but mainly at the spinal level, suggesting the possible involvement of KOR in these responses (Maldonado and Valverde, 2003). However, the genetic deletion of a single opioid receptor did not alter acute cannabinoid antinociceptive responses. An attenuation of THC-induced antinociception in the tail-inmmersion test was observed in knockout mice for pre-proenkephalin (Valverde et al., 2000b), and a reduction of THC effects in tail-immersion, but not in hot-plate test (Zimmer et al., 2001), or no modifications at all (Gardell et al., 2002) were reported in knockout mice for pro-dynorphin genes. These data demonstrate that the suppression of opioid receptor activity has no major effects in cannabinoid-induced antinociception. The development of tolerance to THC antinociception was also not modified in these knockout lines. However, the antinociceptive effects of the CB2R agonist AM1241 in the plantar test were suppressed in MOR knockout mice and by naloxone or β-endorphin antiserum administration, indicating the possible participation of MOR in CB2R-mediated analgesia (Ibrahim et al., 2005).

The ECS in opioid antinociception

MOR is selectively involved in the antinociceptive responses produced by morphine (Matthes et al., 1996). However, it was recently demonstrated that the CB1R antagonist AM251 counteracts morphine-induced antinociception in an inflammatory pain model (da Fonseca Pacheco et al., 2008; Pacheco et al., 2009) and in the tail-flick test in mice (Pacheco et al., 2009). These observations led to the hypothesis that MOR activation could induce local release of endocannabinoids, and that the subsequent peripheral (da Fonseca Pacheco et al., 2008) or central (Pacheco et al., 2009) activation of CB1R and/or CB2R could contribute to the antinociceptive effects of morphine. A role for the ECS in MOR functions was recently described in the brainstem (Páldyová et al., 2008), where the systemic administration of the CB2R antagonist SR144528 attenuates MOR gene expression and activity (Páldy et al., 2008; Páldyová et al., 2008).

The absence of CB1R did not modify either the antinociceptive effects induced by selective DOR and KOR agonists, but, in contrast, attenuated stress-induced analgesia mediated by opioid mechanisms (Valverde et al., 2000a).

2.2.1.5 Cannabinoids and pain: clinical data

Cannabinoids, including THC, have effects on both sensory (intensity, quality) and affective (unpleasantness, suffering) components of pain (Lee et al., 2013). However, the development of cannabinoid agonists as analgesics has been hampered due to psychotropic and debilitating side effects, and the prejudice

generated by the recreational use of marijuana. The most common adverse events associated with the use of cannabis are headache, dry eyes, burning sensation in areas of neuropathic pain, dizziness, numbness, cough, and effects on memory and motor control. These unwanted effects occur as a result of indiscriminate activation of cannabinoid receptors also at sites other than those involved in the transmission of nociceptive stimuli (Carlini, 2004; Ware et al., 2010).

Despite this, almost 10% of patients with chronic pain in the USA are taking cannabinoids for self-medication purposes (Fitzcharles et al., 2012). Clinical trials have shown efficacy in different categories of chronic pain conditions, including neuropathic pain, rheumatoid arthritis, fibromyalgia and mixed chronic pain (Martín-Sánchez et al., 2009; Lynch and Campbell, 2011; Fine and Rosenfeld, 2014). Among the different forms of cannabinoids that have been considered, there were smoked cannabis and different pharmacological cannabinoid preparations. The analgesic effects of cannabinoids were superior to placebo in these different pain conditions, although the therapeutic responses must be balanced with adverse effects mainly on cognition, cardiovascular and motor functions. However, these adverse effects were generally well tolerated (Lynch and Campbell, 2011). Among pharmacological cannabinoid preparations, synthetic forms of THC, like dronabinol (Marinol®) and nabilone (Cesamet®), are commercially available in several countries, and are considered controlled substances. In some particular countries, these preparations have indications for treating cachexia in AIDS patients and as a therapy for intractable nausea

and vomiting during cancer chemotherapy. In a wide range of oral doses, Marinol®, which is chemically identical to the THC extracted from plants, has not demonstrated significant pain relief in several naturally occurring and experimental pain conditions (Buggy et al., 2003; Naef et al., 2003). In contrast, Cesamet®, a synthetic analogue of THC, has demonstrated modest efficacy in fibromyalgia but with dose-limiting adverse effects (Skrabek et al., 2008). Cesamet® use has led to paradoxical increases in pain in the postoperative setting (Beaulieu, 2006).

Cannabidiol is a major constituent of cannabis. It has virtually no psychoactivity compared to THC and has low affinity for both CB1R and CB2R (Mechoulam et al., 2002). The documented ability of cannabidiol to mitigate THC psychotomimetic effects may depend on negative allosteric modulatory activity at CB1R, whereas the anti-inflammatory and antinociceptive properties may be due to the agonist activity on CB2R (Fine and Rosenfeld, 2014). More recently, it has been postulated that cannabidiol may exert its effects via inhibition of anandamide deactivation or otherwise enhancing anandamide signaling, whereas its anxiolytic effects may also be attributed to its agonist effect at the serotonin 1A receptor (Fine and Rosenfeld, 2014). A formulation containing THC and cannabidiol named nabixomol (Sativex®) now exists as an oral spray and is approved in Canada, New Zealand, Israel, and several European countries for the management of spasticity in multiple sclerosis and, in some countries, for the management of central pain. The therapeutic value of THC and Sativex® via oro-mucosal delivery in the treatment of various neuropathic pain conditions showed

promising, albeit, modest results (Fine and Rosenfeld, 2014). This limited efficacy is likely due to the relative low dose of this combination of cannabinoids that depends on the tolerability of THC. In contrast, a clinical trial carried out with Sativex® resulted in significant improvement of pain scores and suppression of disease activity in patients with rheumatoid arthritis without serious adverse events (Blake et al., 2006). However, a recent Phase III clinical trial has revealed that Sativex® did not show significant effects compared to placebo as adjunctive treatment to optimized chronic opioid therapy for pain relief in patients with terminal cancer-related pain (www.gwpharm.com; January 2015).

Besides cannabidiol, phytocannabinoids that have been identified as exerting clinically-useful effects without psychoactivity include tetrahydrocannabivarin, cannabigerol and cannabichromene. Moreover, among the class of terpenes, which share a precursor molecule with phytocannabinoids, and are all flavor and fragrance components common to human diets, β-caryophyllene is effective at reducing neuropathic pain in a CB2R-dependent manner (Fine and Rosenfeld, 2014). Therefore, β-caryophyllene seems an attractive candidate for clinical trials targeting the CB2R in combination therapy (Fine and Rosenfeld, 2014). Beyond these trials, comparative or head-to-head studies evaluating the clinical outcome of individual cannabinoids or various cannabinoid combinations and routes of administration are lacking (Fine and Rosenfeld, 2014). Further clinical trials with larger sample sizes and longer duration are required to evaluate the efficacy and safety of cannabinoid compounds for pain treatment. Moreover, the narrow therapeutic

window of the currently available cannabinoid treatments unveils the need to develop novel compounds for an efficient manipulation of the ECS in specific pain conditions.

2.2.2 ECS in the modulation of cognitive functions

Cognition involves the ability to acquire, store and retrieve information.

Cognitive decline following marijuana consumption has been known since decades in humans and similar cognitive impairment has been revealed in laboratory animals. Cannabis use in humans affects cognitive performance, including attention, working memory, verbal learning, mental flexibility and consolidation of short-term into long-term memory (Ranganathan and D'Souza, 2006; Mechoulam and Parker, 2013). This impairment is related to the dose and the time of consumption, getting worse with increasing years of regular cannabis use, and it is attenuated after long-term abstinence (Mechoulam and Parker, 2013).

Consistent with human literature, reports in animal models suggest that the administration of different CB1R agonists (THC, WIN-55,212 and CP55940, among others) impairs learning and memory leaving largely intact the retrieval of information previously encoded into long-term storage (Mechoulam and Parker, 2013). These effects have been demonstrated in different behavioral tasks that include both emotional learning and memory, as fear conditioning, and non-emotional or neutral memory, such as spatial or working memory and object recognition tasks (Akirav, 2011) (see Table 4 for a description of different learning and memory

paradigms). The memory impairment produced by cannabinoid agonists suggests the possibility that blockade of CB1R may lead to an enhancement of certain memory processes. However, the literature is replete of mixed findings. The CB1R antagonist rimonabant, which has intrinsic activity as inverse agonist, as well as the genetic deletion of CB1R in mice have shown an improvement of certain aspects of memory in rodents, such as olfactory memory, working memory and object recognition memory, whereas an impairment in the extinction of aversive memory has been reported in the fear conditioning test (Terranova et al., 1996; Hampson and Deadwyler, 1998; Lichtman et al., 2002; Maccarrone et al., 2002). Other studies did not reveal any cognitive effect following CB1R blockade (Lichtman et al., 2002). Manipulations that elevate endocannabinoids also produce contradictory results. On one hand, elevating AEA, but not 2-AG, interfered with the consolidation of contextual conditioned fear and object recognition memory (Busquets-Garcia et al., 2011). On the other hand, several studies reported facilitation of spatial and object recognition memory by the elevation of AEA (FAAH inhibitors or FAAH knockout mice) and 2-AG (MAGL knockout mice), respectively (Varvel et al., 2007; Pan et al., 2011; Mechoulam and Parker, 2013).

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. It is used to study spatial learning and memory. There are different cues and strategies to escape the water, including spatial cues around the pool. Animals are trained during several days and the time/path length they take to find the platform is the learning index. There are different alternatives of this task. For example, the platform can be removed and animals are allowed to search for it. In this case, the time that the animal is looking for the platform in the quadrant where it was placed before provides a learning index.
T-maze or Y-maze
T- or Y-shaped maze providing animals with a straightforward choice. It is used to study different spatial memory parameters such as alternation, delayed-alternation, among others. It is often used to study working 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.
Novel-object recognition
Animals are allowed to freely explore two objects in a maze during a training session. In the test session, a novel object replaces one of the objects. It is a non-aversive and non-spatial task to study recognition memory based on the innate animal tendency to explore the novelty. A discrimination index is calculated and the longer the novel object is explored, the higher is the discrimination index indicating good memory.
Social recognition
Similar to the object recognition test, but in this case the objects are replaced with animals (juveniles, from different cages, different strains). Animals have to explore more the new animal in the test session to have a higher discrimination index indicating good social memory.
Fear conditioning
The apparatus has metal grids on the floor that can deliver a footshock. 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. footshock). Conditioned responses that can be active (rearing, diving, locomotion) or passive (freezing) can be used as measures of memory.
Inhibitory avoidance
The apparatus has metal grids on the floor that can deliver a footshock. One part of the grid is covered to provide a safe platform for animals. During training, animals are placed on the safe platform and once they voluntarily step down to the grids they automatically receive a shock. Measuring the time that animals spend on the platform before stepping down assesses memory.
Passive avoidance
Animals learn to inhibit a natural tendency, namely to step into an apparently safer dark compartment that has previously been associated with footshock. The latency to enter this compartment provides a measure of memory.

Table 4. Some of the behavioral models used to study learning and memory. Adapted from (Lee and Silva, 2009)**.**

Despite the large amount of data supporting the involvement of CB1R in the regulation of learning and memory processes, little is known about the role of CB2R in the regulation of cognitive processes. Pharmacological and genetic inactivation of CB2R in mice produced short- and long-term memory impairment in the step-down inhibitory avoidance test (aversive memory), whereas its activation produced an improvement of these responses (García-Gutiérrez et al., 2013). Although the mechanisms involved in these processes are unknown, CB2R seems to be directly involved in the regulation of synaptic plasticity (García-Gutiérrez et al., 2013).

These results suggest that the effects of cannabinoids on memory are complex and depend on several factors, such as the nature of the task (emotional or non-emotional), the memory stage investigated (acquisition, consolidation, retrieval, and extinction), and the experimental model used (Akirav, 2011). The behavioral effects of cannabinoids on memory may also vary as a function of dose, route of administration, and the specific drug used. Moreover, cannabinoid receptor localized in different brain regions would modulate distinct learning and memory processes and this may account for the different effects reported between systemic and localized administration of cannabinoids (Mechoulam and Parker, 2013; Morena and Campolongo, 2014).

2.2.3 ECS in the modulation of emotional responses

Emotionality describes a highly complex behavior in response to various environmental stimuli. Appropriate emotional responses require a fine-tuned neurotransmitter release and functional

neuronal circuits. Therefore, the ECS represents an important endogenous system for the modulation of these responses and the prevention of an imbalanced signaling, especially in stressful situations (Häring et al., 2012).

Many of the psychological effects of cannabis, as well as of THC, are biphasic in humans, depending mainly on the dose and to a certain extent upon the personality of the user. In normal subjects, THC may cause either euphoria, relaxation and stress-relieving effects, or dysphoria and anxiety (Mechoulam and Parker, 2013). The same complex picture applies to studies with cannabinoid agonists or antagonists in animal paradigms used to evaluate the affective state (Table 5) or animal models of psychiatric disorders (Micale et al., 2013). The contradictory results emerged in these studies may be due to the use of different dosages, genetic backgrounds and environmental contexts (Moreira and Wotjak, 2010). It is known that cannabinoid agonists display biphasic effects, eliciting anxiolytic-like responses at low doses, whereas higher doses induce anxiogenic-like effects (Moreira and Wotjak, 2010). One possible explanation for this peculiar feature might be the differences in the initial baseline stress level of the animals, which is controlled by a multitude of genetic, environmental, and experimental factors. The recruitment of other receptors, such as TRPV1 or GPR55, or the differential effects of CB1R activation on distinct neuronal populations are also under discussion (Häring et al., 2012). However, more research is needed to understand the mechanisms of these bimodal effects.

Test/model	Description/measurements
Anxiety-like behavior	
Open field	Avoidance of a novel, brightly illuminated central area of an open field
Elevated plus-maze	Avoidance of open and elevated arms
Elevated zero-maze	Avoidance of open and elevated quadrants
Light/dark box	Avoidance of a brightly illuminated compartment
Active avoidance	Avoidance of a compartment associated with an aversive stimulus (e.g. footshock) by initiating a specific locomotor response (escape)
Passive avoidance	Avoidance of a dark compartment associated with an aversive stimulus (e.g. footshock) that requires the animal to behave contrary to its innate tendency to enter darkened and confining spaces
Depressive-like behavior	
Forced swimming	Immobility (passive coping) in an inescapable cylinder filled with water
Tail suspension	Passive immobility during inescapable upside-down suspension by the tail
Learned helplessness	Escape deficits after repeated exposure to unsignaled inescapable shock
Chronic mild stress	Behavioral and neurochemical alterations developed after the exposure to repeated and unpredictable mild stressors. This model is typically associated with anhedonia that can be measured as a reduced preference for a highly palatable drink solution or food
Olfactory bulbectomy	Behavioral and neurochemical alterations developed after the removal of the olfactory bulbs, which results in a disruption of the limbic hypothalamic axis
Maternal separation	Behavioral and neurochemical alterations developed in adulthood following decreased maternal care during post-natal day 1-14

Table 5. Schematic description of the main models used to study the anxiety-like and depressive-like behaviors in rodents (Micale et al., 2013)**.**

Despite the considerable evidence on the direct effects of the administration of endocannabinoids AEA and 2-AG on anxiety in animals, there are no direct experimental data of their role in human

anxiety. The injection of low endocannabinoid doses into the PFC led to anxiolytic-like reponses in rats (Rubino et al., 2008). Blocking endocannabinoid degradation by using inhibitors of FAAH and MAGL also produced anxiolytic-like effects in rodents (Mechoulam and Parker, 2013). These anxiolitic-like effects seem to be mediated by CB1R in the case of elevated AEA levels, whereas CB2R seems to contribute mainly to the effects of elevated 2-AG (Busquets-Garcia et al., 2011). Presumably, endocannabinoid levels and the levels of their metabolic enzymes are increased in the brain regions involved in the regulation of affective state during anxiety and depression probably to counteract these negative emotional symptoms (Mechoulam and Parker, 2013).

Along with the anxiolytic effects, pharmacological enhancement of endocannabinoid signaling has also been associated with antidepressant effects in animal paradigms used to evaluate depressive-like behavior (Table 5), whereas hypofunctional endocannabinoid signaling contributes to depressive illness (Hill et al., 2008a; Micale et al., 2013). Moreover, several interventions known to produce antidepressant effects in humans have been shown to increase endocannabinoid/CB1R signaling in the brain, including chronic treatment with the antidepressant desipramine (Hill et al., 2008a). However, controversial results have also been obtained in different studies evaluating the role of the ECS in depression (Micale et al., 2013). Indeed, there is also evidence of an association of hyperfunctional ECS and depression (Hill et al., 2008a). This discrepancy can be explained by a differential alteration of the ECS in different cortical and sub-cortical brain

regions that could underlie different forms of depressive illness (Hill et al., 2008a; Micale et al., 2013).

In accordance with the data that involve a decreased CB1R signaling in mood disorders, the CB1R antagonist rimonabant used to treat obesity was withdrawn from the market due to undesirable psychiatric side effects, such as depression, anxiety and suicidal ideation (Mechoulam and Parker, 2013; Micale et al., 2013). Genetic deletion of the CB1R in mice also results in a phenotype that is reminiscent of the symptoms of certain mood disorders, such as increased anxiety-like and depressive-like behaviors (Martin et al., 2002; Micale et al., 2013). It has also been suggested that genetic variations in CB1R gene could facilitate the development of stress related disorders in humans (Lazary et al., 2011).

Recently, CB2R has also been involved in the control of the emotional responses and CB2R polymorphisms seem associated with a certain vulnerability to neuropsychiatric disorders, including alcoholism, eating disorders, autism, schizophrenia, depression and anxiety-related disorders (Onaivi et al., 2012). However, both pharmacological and genetic manipulations of CB2R revealed controversial results in different studies evaluating its role in affective responses. In agreement, CB2R activation could have either protective or deleterious effects in these responses, depending on the experimental conditions (Micale et al., 2013). Therefore, taking into account the controversial results obtained so far, the possible modulation of CB2R, exempt from undesirable psychoactivity compared to CB1R, still lacks sufficient

experimental evidence to justify its potential interest in anxiety-like and depressive-like disorders.

2.2.4 ECS in the modulation of stress-related responses: the interactions with the hypothalamic-pituitary-adrenal axis

Stress is produced by stimuli that represent a challenge to homeostasis, including any actual or potential disturbance of the individual's environment. Persistent stress constitutes a main risk factor for neuropsychiatric diseases, such as anxiety and depression. Therefore, stress models in rodents have been extensively used to study the mechanisms underlying these processes and find potential therapeutic targets. These studies have revealed the important involvement of the ECS in the regulation of the behavioral and neuroendocrine responses to stress. A fundamental physiological response to stress is the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress-induced neuronal stimulation produces the activation of the parvocellular neurosecretory cells in the paraventricular nucleus (PVN) of the hypothalamus, which synthesize and release the corticotropin-releasing hormone (CRH). This hormone is transported to the anterior pituitary leading to the release of the adrenocorticotropic hormone (ACTH) into the bloodstream. Upon reaching the adrenal gland, ACTH stimulates the release of glucocorticoids (primarily corticosterone in rodents and cortisol in humans) from the adrenal cortex, which act on peripheral and neuronal glucocorticoid receptors (GR). These hormonal responses prepare the organism for possible threat by

mobilizing energy stores (Riebe and Wotjak, 2011). In addition, glucocorticoids produce a range of effects on cardiovascular, immune, metabolic, and neural systems that facilitate optimal responses to aversive stimuli, including increase of arousal and focused attention (Hill et al., 2010b). HPA axis is rapidly self-regulated in order to normalize its own activity. Indeed, elevated circulating levels of glucocorticoids rapidly suppress HPA axis activity by a negative feedback mechanism (Figure 16).

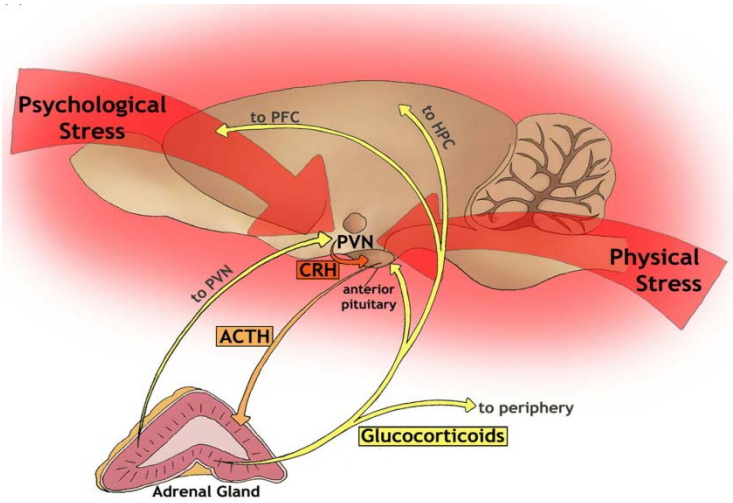

Figure 16. The HPA-axis and its glucocorticoid feedback mechanisms (Riebe and Wotjak, 2011)**.**

Within the PVN, glucocorticoids exhibit both rapid (gene transcription independent) and delayed (transcription dependent) suppression of HPA axis activity (Hill et al., 2010b). In addition, extra-hypothalamic limbic structures that communicate with the PVN exert both positive and negative effects on HPA axis activity (Hill et al., 2010b). However, continued and prolonged stress may

have detrimental effects on HPA axis activity and the adaptive responses may then become maladaptive.

Bidirectional and functional relationships between glucocorticoids and the ECS have been demonstrated. The ECS is widely distributed in cortico-limbic structures that are involved in the regulation of the behavioral responses to stress through HPA axis activity, in agreement with the pronounced effects of cannabis on emotionality and the processing of stressful information (Hill et al., 2010b; Häring et al., 2012) (Figure 17).

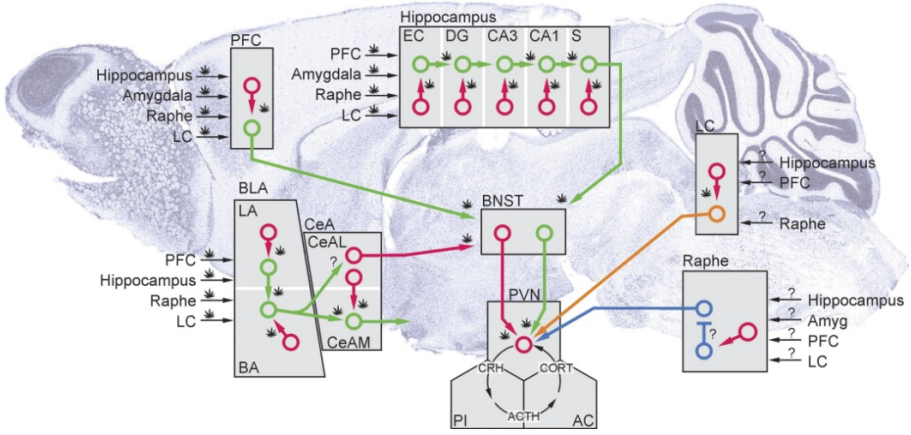

Figure 17. Schematic illustration of CB1R distribution within the main stress circuits. CB1R, indicated as a *Cannabis sativa* leaf, is found at the majority of synaptic connections within and between each major brain region related to the activity of the HPA axis, which is controlled by the PVN of the hypothalamus, the pituitary (PI), and the adrenal cortex (AC). A most dominant distribution of CB1R is found in GABAergic (red) and glutamatergic (green) neurons in limbic regions, such as PFC, amygdala (BLA, CeA), bed nuclei of the stria terminalis (BNST), and hippocampus. Additionally, serotonergic (blue) and noradrenergic (orange) neurons from brainstem nuclei are also involved in the stress response. A projection to a particular brain region is depicted as an

arrow with the specification where the projection originates from. Question marks indicate that the presence of CB1R at a given projection has not yet been clearly proven (Häring et al., 2012).

In addition, CB1R is expressed and both AEA and 2-AG are synthesized in the hypothalamus, including locally within the PVN, as well as in upstream structures involved in hypothalamic function, such as the amygdala, hippocampus, and PFC (Herkenham et al., 1991; Malcher-Lopes et al., 2006; Hill et al., 2010a, 2011b; Tasker and Herman, 2011). Application of cannabinoid drugs directly influences excitatory and inhibitory inputs to PVN neurons, resulting in the modulation of HPA axis activity (Häring et al., 2012). Stressful situations produce rapid changes in the ECS in these stress-responsive brain regions, where endocannabinoids mediate the physiological suppression of HPA-axis activity, and ultimately restore homeostasis. One of the mechanisms involved in these responses is the glucocorticoid-induced release of endocannabinnoids by a fast-feedback mechanism that involves membrane GR (Figure 18) (Tasker and Herman, 2011). Exogenous cannabinoid administration may influence HPA axis activity differently than the endocannabinoids, as they lack both spatial and temporal specificity (Akirav, 2013). Nevertheless, pharmacological enhancement of endocannabinoid signaling attenuates the HPA axis activity by reduction of corticosterone release in animal models of acute stress (Akirav, 2013).

Endocannabinoid signaling is altered in the brain areas involved in these responses after chronic stress exposure (Hill et al., 2009a, 2010a, 2011b; Wamsteeker et al., 2010). These changes may help to

habituate HPA axis activity and protect against the deleterious effects of long-term HPA axis activation and chronically elevated glucocorticoids, which may induce psychological and cognitive disturbances, and severe chronic disease (Riebe and Wotjak, 2011). However, it remains to be determined whether the observed ECS alterations are adaptive or maladaptive in these circumstances.

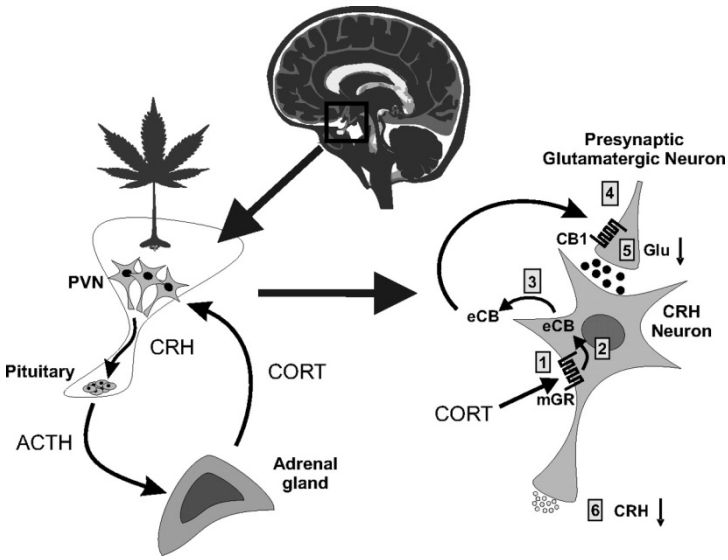

Figure 18. Fast-feedback inhibition of the HPA axis via glucocorticoid-induced endocannabinoid (eCB) release in the hypothalamus. (Left), Stress activation of the HPA axis consists of CRH release from PVN neurons and CRH-evoked ACTH release from the pituitary, which, in turn, stimulates corticosteroid (CORT) release from the adrenal cortex and CORT feedback onto the PVN. (Right), In PVN CRH neurons, CORT binds to a membrane-associated glucocorticoid receptor (mGR), which causes endocannabinoid (eCB) synthesis and retrograde eCB release; eCB binds to pre-synaptic CB1R on glutamate terminals and inhibits glutamate release onto the CRH neurons, suppressing the excitatory synaptic drive and decreasing CRH neuron activity and CRH release, which suppresses HPA axis activation (Hill et al., 2010b).

3 ECS and osteoarthritis

3.1 ECS in the joint tissues

The presence of a functional ECS at the level of nerve fibers and different tissues of the joints, including cartilage, synovium and bone, has been demonstrated in both rodents (Schuelert and McDougall, 2008; Schuelert et al., 2010; Gómez et al., 2014) and humans (Richardson et al., 2008; Gómez et al., 2014). This suggests a possible role for the ECS in the regulation of important processes taking place during osteoarthritis. The local activation of CB1R and CB2R expressed at the level of the joint nerve fibers has revealed their contribution to the regulation of synovial blood flow and joint nociceptor activity (Baker and McDougall, 2004; Anand et al., 2008; McDougall et al., 2008; Schuelert and McDougall, 2008; Schuelert et al., 2010) (see section 3.2). Moreover, the main elements of the ECS, including CB1R and CB2R mRNA and protein, were found in human chondrocytes and synovial tissues deriving from total knee arthroplasty of osteoarthritis and rheumatoid arthritis patients (Richardson et al., 2008; Dunn et al., 2014; Fukuda et al., 2014; Gómez et al., 2014; Gui et al., 2014). The functional relevance of cannabinoid receptors in regulating the metabolism of these tissues has been confirmed by pharmacological studies (Richardson et al., 2008).

The presence of endocannabinoids (AEA and 2-AG) was also reported in the synovial fluid and the synovium of osteoarthritis and rheumatoid arthritis patients, but was not detected in the synovial fluid of healthy volunteers and was negligible in control joints of non arthritic rodents (Richardson et al., 2008; Schuelert and

McDougall, 2008). Therefore, the endocannabinoid tone is locally increased in osteoarthritic joints as a potential protective mechanism, despite the low basal activity of the ECS.

3.2 ECS role in nociceptive responses during osteoarthritis

Recent behavioral and electrophysiological studies have demonstrated that cannabinoids exert antinociceptive effects in rodent models of osteoarthritis (Schuelert and McDougall, 2008; Yao et al., 2008). The administration of the CB1R agonist, arachidonyl-2-chloroethylamide (ACEA), into the knee joint reduced the hypersensitivity of afferent nociceptors in the rat MIA model by a mechanism involving CB1R and TRPV1 (Schuelert and McDougall, 2008). In addition, the intra-articular application of a CB1R antagonist alone increased the activity of afferent nerve fibers in the osteoarthritic joint, but not in the control joint, suggesting a tonic release of endocannabinoids at the joint level during osteoarthritis (Schuelert and McDougall, 2008). Surprisingly, the local administration of a selective CB2R agonist, GW405833, into the knee joint produced a paradoxical sensitizing effect on joint mechanoreceptors and increased hindlimb incapacitance in osteoarthritic rats, but not in control animals (Schuelert et al., 2010). This effect was mediated by the interaction of GW405833 with TRPV1 on C afferent fibers, where it co-localizes with CB2R (Schuelert et al., 2010). In contrast, the systemic administration of the CB2R agonist A-796260 attenuated pain in the rat MIA model (Yao et al., 2008). The distinct results obtained with these CB2R agonists could depend on the route of

administration since the systemic route may produce analgesic effects also through other peripheral mechanisms or spinal and supraspinal mechanisms. The possibility of alleviating pain by targeting the degradation pathways of endocannabinoids was also tested in animal models of osteoarthritis. Systemic or intra-articular administration of the FAAH inhibitor URB597 reduced nociception in spontaneous and chemically induced models of osteoarthritis, an effect blocked by CB1R, but not CB2R antagonism (Schuelert et al., 2011). Importantly, URB597 had no effect on control rat joints, further suggesting a release of articular endocannabinoids only in osteoarthritic animals. URB597 also reduced the spontaneous activity of knee joint afferents in aged osteoarthritic guinea pigs reflecting the abrogation of osteoarthritis pain at rest (Schuelert et al., 2011). Pain at rest is one of the main debilitating and treatment-resistant symptoms of osteoarthritis, and FAAH inhibition could offer an innovative strategy to treat this aspect of pain (Schuelert et al., 2011), although these results have not been confirmed in humans (Huggins et al., 2012) (see section 3.5). Recent evidence suggests that the dual inhibition of FAAH and TRPV1 would represent a new strategy to obtain better analgesic profiles in osteoarthritis (Malek et al., 2015).

Therefore, targeting the endocannabinoid-metabolizing enzymes or CB1R at the peripheral level may represent an important therapeutic approach to alleviate osteoarthritis pain. The potential utility of CB2R agonists deserves critical appraisal, but requires additional investigations.

3.3 Potential role of the ECS in the structural progression of osteoarthritis

Several studies have demonstrated the ability of cannabinoids to exert a direct effect on cartilage, as well as synovium and bone metabolism, revealing an attractive therapeutic potential for these compounds. Synthetic cannabinoids (i.e. WIN55,212-2) showed protective effects toward cytokine-induced extracellular matrix degradation in cartilage through the inhibition of the synthesizing enzymes of inflammatory mediators, such as prostaglandin E2 and NO, and the inhibition of MMPs (MMP-3 and MMP-13) expression (Mbvundula et al., 2005, 2006; Dunn et al., 2014). Excessive prostaglandin and NO production, together with increased MMP activity, are involved in the aetiopathogenesis of osteoarthritis (Henrotin et al., 2003; Martel-Pelletier et al., 2003). Therefore, cannabinoids could have potential modulatory effects on the early stages and the progression of osteoarthritis.

Cannabinoids have also shown several effects on fibroblast-like synovial cells, the stromal cells of the joint capsule that during arthritic disorders express an hyperplasic, inflammatory, cartilage- and bone-destructive phenotype, which includes secretion of cytokines and MMPs (Aupperle et al., 1998). In agreement, different cannabinoid agonists, such as the non selective CP55,940 and WIN55,212-2, and the CB2R selective agonists JWH133 and HU-308, reduced the levels of IL-6, IL-8 and MMPs released by cytokine-stimulated fibroblast-like synoviocytes obtained from patients with rheumatoid arthritis (Selvi et al.; Gui et al., 2014). Moreover, the ability of ajulemic acid, a synthetic derivative of

THC, to reduce the severity of adjuvant-induced arthritis (Zurier et al., 1998, 2003) seems to be partially mediated by its inhibitory effect on the production of MMPs and by promoting an anti-inflammatory prostaglandin profile in these cells (Johnson et al., 2007). However, the mechanisms by which cannabinoids exert these effects do not seem to be completely mediated by CB1R or CB2R expressed in synovial cells.

The ECS has also been recently implicated in the control of bone metabolism by regulating bone mass, bone loss and bone cell functions, although the mechanisms involved are still not fully understood (Idris and Ralston, 2012). CB1R and CB2R are expressed by osteoblasts, osteoclasts, and osteocytes, with higher expression levels of CB2R than CB1R (Idris and Ralston, 2012). Thus, the ECS could also participate in regulating the excessive turnover of the subchondral bone observed in patients and animal models of osteoarthritis (Kwan Tat et al., 2010).

All together, these *in vitro* studies reveal promising data about a potential role of the ECS in the modulation of the mechanisms underlying structural pathology during osteoarthritis. However, further studies exploring the overall *in vivo* effects of these agents in preclinical models are necessary to support the potential interest of cannabinoids for halting the progression of this disease.

3.4 Pharmacological modulation of the osteoarthritis inflammatory processes by the ECS

The inflammatory component in osteoarthritis is more variable than in other non-degenerative forms of arthritis, such as rheumatoid

arthritis. However, it would be useful to exploit the possible immunosuppressive and anti-inflammatory actions of cannabinoids for osteoarthritis treatment. The mechanisms by which cannabinoids exert these actions include effects on apoptosis, inflammatory cell proliferation and trafficking, cytokine production and regulatory T-cells (Rieder et al., 2010). In agreement, the non-selective cannabinoid agonists, cannabidiol and HU-320, as well as the CB2R selective agonist JWH133, showed immunosuppressive, anti-inflammatory and anti-arthritic effects in the murine collagen-induced arthritis (Malfait et al., 2000; Sumariwalla et al., 2004; Fukuda et al., 2014). In addition, ajulemic acid reduced the severity of adjuvant-induced arthritis (Zurier et al., 1998, 2003). Although these cannabinoid compounds displayed anti-arthritic effects in these inflammatory arthritis models, their potential effects on structural pathology in specific models of osteoarthritis have not been yet evaluated. Thus, the well known antinociceptive and anti-inflammatory properties of cannabinoids, together with their potential role in disease modification, strongly support their promising therapeutic potential for osteoarthritis.

3.5 Clinical implications for cannabinoid use in osteoarthritis

Limited clinical evidence is available to fully support the medical use of cannabinoids in osteoarthritis. The only major clinical trial targeting the ECS for the symptomatic relief of osteoarthritis pain was conducted with the irreversible FAAH1 inhibitor, PF-04457845 (Huggins et al., 2012). PF-04457845 showed an excellent tolerability profile (Li et al., 2012) and strongly elevated the plasma

levels of AEA and other N-acylethanolamines, but failed to elicit effective analgesia in knee osteoarthritis patients (Huggins et al., 2012), despite the analgesic properties of FAAH inhibition in rodent models of osteoarthritis pain (Ahn et al., 2011; Schuelert et al., 2011). The lack of analgesic effects in this clinical trial was disappointing and suggested translational questions regarding animal models. Therefore, it is possible that FAAH inhibitors may affect downstream targets in different manners between species (rodent vs. humans). Thus, the inhibition of FAAH activity might have unmasked alternative pathways of elevated AEA and N-acylethanolamines in humans, such as the activation of TRPV1, which is associated with inflammatory processes and has pronociceptive effects (Ross, 2003; Burston and Woodhams, 2013). Another possible explanation could be an alternative breakdown of these molecules via COX-2 that would be up-regulated during this chronic pain state (Di Marzo, 2012). Other inhibitors need to be considered in future studies. Thus, it has been suggested that dual FAAH/COX-2 inhibitors or dual FAAH inhibitor/TRPV1 antagonist would produce better analgesic profiles (Di Marzo, 2012; Burston and Woodhams, 2013; Malek et al., 2015).

None of the clinical studies evaluating the effects of cannabis-based pharmacological preparations (i.e. Sativex®) involved patients with osteoarthritis. However, the present evidence of cannabinoid-induced analgesia in different clinical pain states (see 2.2.1.5 section), including inflammatory arthritis, offers opportunities for cannabinoid therapeutic use in osteoarthritis pain. The substantial differences in the mechanisms underlying osteoarthritis and other

chronic pain conditions limit the direct extrapolation of the data from these studies and reveal the need for detailed clinical trials with cannabinoids in specific osteoarthritis patient populations.

OBJECTIVES

Objective 1

To investigate the role of CB1R and CB2R in the nociceptive, histological and neurochemical alterations associated with osteoarthritis pain in mice.

Article #1

Role of CB1 and CB2 cannabinoid receptors in the development of joint pain induced by monosodium iodoacetate

Carmen La Porta*, Simona Andreea Bura*, Auxiliadora Aracil-Fernández, Jorge Manzanares, Rafael Maldonado

Pain, 154:160–174 (2013)

* Equal contribution

Objective 2

To study the specific involvement of the ECS in the nociceptive, emotional and cognitive manifestations of osteoarthritis pain in mice and humans.

Article #2

Role of the endocannabinoid system in the emotional manifestations of osteoarthritis pain

Carmen La Porta, Andreea S. Bura, Jone Llorente-Onaindia, Antoni Pastor, Francisco Navarrete, María Salud García-Gutiérrez, Rafael De la Torre, Jorge Manzanares, Jordi Monfort, Rafael Maldonado

Pain, In Press

Objective 3

To analyze the potential alterations of glutamate receptor expression and structural plasticity in the mouse medial PFC associated with

the affective and cognitive alterations promoted by osteoarthritis pain.

Supplementary results

Osteoarthritis pain decreases glutamate receptor expression and induces structural plasticity alterations in the mouse medial prefrontal cortex

Carmen La Porta and Rafael Maldonado

Objective 4

To validate different behavioral outcomes to measure emotional and cognitive alterations promoted by neuropathic pain in mice at different time points and the effects of the repeated administration of pregabalin on these manifestations.

Article #3

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

Carmen La Porta*, Itzel Lara Mayorga*, Roger Negrete, Rafael Maldonado.

Submitted (2015)

* Equal contribution

❖ Main articles # 4 and #5 from the Annex are reviews related to our work.

RESULTS

ARTICLE #1

Role of CB1 and CB2 cannabinoid receptors in the development of joint pain induced by monosodium iodoacetate

Carmen La Porta*, Simona Andreea Bura*, Auxiliadora Aracil-Fernández, Jorge Manzanares, Rafael Maldonado

Pain, 154:160–174 (2013)

* Equal contribution

La Porta C, Bura SA, Aracil-Fernández A, Manzanares J, Maldonado R. Role of CB1 and CB2 cannabinoid receptors in the development of joint pain induced by monosodium iodoacetate. Pain. 2013 Jan;154(1):160-74. doi: 10.1016/j.pain.2012.10.009.

ARTICLE #2

Role of the endocannabinoid system in the emotional manifestations of osteoarthritis pain

Carmen La Porta, Andreea S. Bura, Jone Llorente-Onaindia, Antoni Pastor, Francisco Navarrete, María Salud García-Gutiérrez, Rafael De la Torre, Jorge Manzanares, Jordi Monfort, Rafael Maldonado

Pain, In Press

La Porta C, Bura SA, Llorente-Onaindia J, Pastor A, Navarrete F, García-Gutiérrez MS, De la Torre R, Manzanares J, Monfort J, Maldonado R. Role of the endocannabinoid system in the emotional manifestations of osteoarthritis pain. Pain. 2015 Oct;156(10):2001-12. doi: 10.1097/j.pain.0000000000000260

SUPPLEMENTARY RESULTS

Osteoarthritis pain decreases glutamate receptor expression and induces structural plasticity alterations in the mouse medial prefrontal cortex

Carmen La Porta and Rafael Maldonado

Introduction

Osteoarthritis is a chronic degenerative joint disease characterized by pain and physical disability (Smith et al., 2014). The current treatment of this disease is mainly symptomatic with limited efficacy and significant side effects. One of the reasons for this unmet clinical need is the insufficient knowledge of the exact mechanisms involved in the generation and maintenance of osteoarthritis pain and the existence of pain-related comorbidities such as affective (anxiety, depression) and cognitive disorders (Liu and Chen, 2014). These comorbid disorders can negatively affect the life quality of patients, and further aggravate the sensory abnormalities of chronic pain. It is therefore an important challenge to treat not only the sensory symptoms, but also the comorbidities accompanying chronic pain. Most of the basic research efforts have been focused primarily on the peripheral and spinal mechanisms of osteoarthritis pain (Malfait et al., 2013), whereas much less attention has been directed at the mechanisms involving the cortico-limbic circuits outside the traditional pain pathways. In agreement, structural and functional alterations in osteoarthritis patients have also been described in different cortico-limbic brain areas related to the control of the affective and cognitive processes (Malfait and Schnitzer, 2013; Baliki et al., 2014; Lluch et al., 2014). Therefore, the knowledge of the changes promoted by persistent pain in these brain areas would be essential to understand the mechanisms underlying chronic pain and to develop new therapeutic targets.

The medial PFC (mPFC) is an important brain area involved in pain processing (Lorenz et al., 2002) and emotional and cognitive

functions, including attention, decision making, goal-directed behavior and working memory (Gusnard et al., 2001; Phelps et al., 2004). Dysfunctions within the circuitries that connect the mPFC to other cortical and limbic structures account for the disturbances in emotional, cognitive and neuroendocrine responses associated with psychiatric disorders (Drevets et al., 2008). Functional and structural abnormalities in this area have also been demonstrated during chronic pain (Apkarian, 2004; Metz et al., 2009) and are accompanied by impairment in mPFC-dependent tasks in both humans (Apkarian et al., 2004) and rodents (Pais-Vieira et al., 2009; Ji et al., 2010).

Glutamate receptor-mediated synaptic transmission is a crucial neuronal substrate for mPFC functions, including pain modulation (Goldman-Rakic, 1995; Lisman et al., 1998; Millecamps et al., 2007; Metz et al., 2009). In agreement, local mPFC activation of glutamate receptors exerts analgesic effects in a rodent model of neuropathic pain (Millecamps et al., 2007). Moreover, alterations in glutamatergic signaling and structural plasticity in mPFC participate in the affective and cognitive dysfunctions produced by chronic stress (Drevets et al., 1997; Radley et al., 2006; Kang et al., 2012; Yuen et al., 2012; Ota et al., 2014). So far, no evidence for functional or morphological changes at the synapses of mPFC neurons under osteoarthritis pain has been provided. However, we have previously found an increased endocannabinoid tone in the mPFC of osteoarthritic mice (see *Article #2*) that could modify synaptic transmission at this level.

In this study, we investigated the disruption of cognitive and affective behaviors that require proper mPFC functions promoted by osteoarthritis chronic pain in mice. In parallel, we evaluated potential alterations involving glutamate receptor expression and structural reorganization in the mPFC of these mice. Finally, we have also analyzed the expression of key ECS elements in this neocortical region considering the involvement of the ECS in the adaptive changes occurring during osteoarthritis (*Article #2*).

Materials and methods

Animal experimental conditions

Swiss albino male mice (Charles River, Lyon, France) were used in all the experiments. Mice were 8-12 weeks old (25 to 30 g) at the beginning of the experiments and were housed in groups of 3 to 4 with free access to water and food. The housing conditions were maintained at 21 ± 1ºC and 55 ± 10% relative humidity in a controlled light/dark cycle (light on between 8:00 AM and 8:00 PM). All experimental procedures and animal husbandry were conducted according to standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and were approved by the local ethical committee (Comité Etico Experimental Animal, Instituto Municipal de Asistencia Sanitaria/Universitat Pompeu Fabra). All the experiments were performed under blind conditions.

Osteoarthritis pain induction

Osteoarthritis pain was induced in mice briefly anaesthetized with isoflurane by the intra-articular injection of MIA (Sigma) into the knee joint. The knee joint was shaved and flexed at a 90˚ angle. Five µL of 5 mg/mL MIA in sterile saline (0.9%) were injected through the infrapatellar ligament into the joint space of the right (ipsilateral) knee with a 30-gauge needle. This concentration of MIA has been previously demonstrated to precipitate histological changes in the cartilage (van der Kraan et al., 1989; van Osch et al., 1994) and to induce joint pain in mice (Harvey and Dickenson, 2009). Control mice received an intra-articular injection of vehicle (5 µL of sterile saline, 0.9%).

Behavioral alterations associated with osteoarthritis pain

Spontaneous alternation behavior (spatial working memory)

Spontaneous alternation was evaluated as an indicator of working memory in the Y-maze 2 weeks after MIA or saline intra-articular injection in mice (Coutellier et al., 2011). The apparatus consists of a black, non-reflective plastic maze formed of three arms (A, B and C) placed as to form a Y shape. Each mouse was placed in an arm facing the centre and was allowed to freely explore the maze during 5 min. This test is based on a strong tendency in rodents to alternate arm choices, explained by their natural propensity to explore a novel environment over a recently explored one. The series of arm entries (e.g. ACBCABCBCA) was scored simultaneously from a video camera. A correct alternation occurred when the animal moved to the other two arms without retracing its steps (i.e. arms A

to B to C). Each alternation was defined as successive entry into the three different arms, on overlapping triplet sets (e.g. in the sequence ACBCABCBCA, five alternations were recorded). The percentage of spontaneous alternations was calculated as the ratio of actual alternations to possible alternations (defined as the total number of arm entries minus 2), multiplied by 100. The total number of arm entries was used as an indicator of locomotor activity.

Depressive like behavior

The tail suspension test (TST) was performed 2 weeks after MIA or saline intra-articular injection, as previously reported (Aso et al., 2008). Mice were suspended 50 cm above a solid surface by the use of adhesive tape applied to the tail (3/4 of the distance from the base of mouse tail). The total time of immobility was recorded during a 6 min interval. Long periods of immobility are characteristic of a depressive-like state. An alternative test is the forced swimming test (FST) (Porsolt et al., 1977), which was also used at a different time point (3 weeks after MIA or saline intra-articular injection). In the FST, mice were gently lowered into a plastic cylinder containing water (23°-25°C), deep enough to prevent touching the bottom of the cylinder and forcing the mouse to swim during 6 min. The mouse was considered immobile when it floated in an upright position and made only small movements to keep its head above water. The duration of immobility was quantified over the last 4 min of the 6 min test since little immobility is usually observed during the first 2 min.

Immunoblot analysis

At the end of the behavioral experiments, mPFC tissues (from Bregma 1.98 mm to Bregma 1.70 mm) (Paxinos and Franklin, 2001) were dissected on ice, frozen on dry ice and stored at −80°C until use. Samples from both MIA and saline groups were processed in parallel to minimize variations, as previously described (Ozaita et al., 2007). Frozen brain areas were dounce-homogenized in 30 volumes of lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptine, 1 μg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, and 40 mmol/L beta-glycerolphosphate) plus 1% Triton X-100. After 10 min incubation at 4°C, samples were centrifuged at 16,000 *g* for 30 min to remove insoluble debris. Protein content from each sample was determined by DC-micro plate assay (Bio-Rad), following manufacturer's instructions. Samples with equal amounts of total protein (10 μg per lane) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel before electrophoretic transfer onto nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 h at 21 ± 1°C in Tris-buffered saline (TBS) (100 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) with 0.1% Tween-20 (TBS-T) and 5% non-fat milk. Afterwards, membranes were incubated for 2 h with the primary antibodies. For immunoblotting, the following antibodies were used: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mouse, 1:5000; Santa Cruz Biotechnology); anti-PSD95 (rabbit, 1:500; Cell Signaling); anti-

synaptophysin (mouse, 1:500; Sigma); anti-CB1R (rabbit, 1:1000; Frontier Science); anti-diacylglycerol lipase α (DGLα) (guinea pig, 1:800; Frontier Institute); anti-monoacylglycerol lipase (MAGL) (rabbit, 1:200; Abcam); anti-cyclooxygenase-2 (COX-2) (rabbit, 1:500; Cayman Chemical Company); anti-NR1(mouse, 1:500; Novus Biological); anti-NR2A (rabbit, 1:500; Millipore); anti-NR2B (rabbit, 1:500; Millipore); anti-GluR1 (rabbit, 1:500; Abcam); anti-GluR3 (mouse, 1:500; Millipore); anti-GABA-A receptor α1 (GABA_ARα1) (goat, 1:500; Santa Cruz Biotechnology); anti-GABA-A receptor γ2 (GABA_ARγ2) (rabbit, 1:500; Synaptic System). Bound antibodies were detected with horseradish peroxidase-conjugated antibody against mouse (1:2500; Thermo Fisher Scientific), rabbit (1:10000; Cell Signaling Technologies), goat (1:1000; Sigma), or protein A for the detection of anti-DGLα primary antibody (1:5000; Zymed Laboratories Inc.). The secondary antibodies were visualized by enhanced chemiluminescence detection (SuperSignal West Femto; Thermo Fisher Scientific). When necessary, Immobilon-P membranes (Millipore) were stripped in buffer containing 100 mmol/L glycine, pH 2.5, 200 mmol/L NaCl, 0.1% (v/v) Tween 20 and 0.1% (v/v) beta-mercaptoethanol, for 45 min at 21 ± 1°C, followed by extensive washing in TBS-T, before reblocking and reprobing. The optical density of the relevant immunoreactive bands was quantified after acquisition on a ChemiDoc XRS system (Bio-Rad) controlled by Quantity One software v4.6.3 (Bio-Rad). For quantitative purposes, the optical density values for the proteins of interest were normalized to the detection of the housekeeping control GAPDH in

the same samples and expressed as a percentage of the control (saline) treatment.

Structural plasticity analysis

Ballistic labeling with the fluorescent dye DiI

At the end of the behavioral experiments, mice were deeply anesthetized by intraperitoneal injection (0.2 mL/10 g body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) prior to rapid intra-cardiac perfusion, delivered with a peristaltic pump at 20 mL/min, with 10 mL of Na_2HPO_4/NaH_2PO_4/NaCl buffer (PBS) 0.1 M, pH 7.5, and followed by perfusion with 40 mL of 4 % paraformaldehyde (PFA) in PBS 0.1 M, pH 7.5. Brains were quickly removed from the skull and post-fixed in 4 % PFA for 10 min. Brain coronal sections (100 µm) containing the mPFC (from bregma 1.98 mm to bregma 1.70 mm) (Paxinos and Franklin, 2001) were obtained by using a vibratome (Leica VT 1000 S, Nussloch, Germany) and kept in PBS 0.1 M until they were processed for fluorescent labeling. Brain slices were labeled by ballistic delivery of fluorescent dye DiI (Molecular Probes, Eugene, OR, USA) using a gene gun apparatus (Helios Gene Gun System, Bio-Rad, Deutschland), as described previously (Grutzendler et al., 2003), and post-fixed with PFA for 4 h at room temperature to further preserve structures and to allow the diffusion of the dye DiI. Sections were mounted on microscope gelatine-coated slides and cover-slipped with mounting medium (Mowiol).

Image acquisition and analysis

In order to analyze dendritic spine density and morphology, images of dendrites were acquired with confocal microscope (Leica TCS SP5 II CW-STED, Germany) with a glycerol immersion lens (63x), and an additional 3x objective zoom. Individual pyramidal neurons of the mPFC were chosen for spine analysis based on several criteria, as described previously (Lee et al., 2006): (i) minimal or no overlap with other labeled cells to ensure that processes from different cells would not be confused, (ii) at least three primary dendrites needed to be visible for cells to be used for analysis and (iii) distal dendrites from secondary dendrites to terminal dendrites were examined. Dendrites of pyramidal neurons taken predominantly from the prelimbic and infralimbic areas of the mPFC (from Bregma 1.98 mm to 1.70 mm) were analyzed. All images of dendrites were taken at different z levels (0.25 μm depth intervals). Blind deconvolution with Huygens essentials software (Scientific Volume Imaging, Hilversum, The Netherlands) was applied to raw three-dimensional images. Dendrite length tracing to calculate spine density and the morphological classification of dendritic spines were made using Imaris analysis software (Bitplane, Zurich, Switzerland). Protrusions from dendrites were classified into four types based on their morphology and on previously established criteria (Lee et al., 2006): stubby protuberances were 0.5 μm in length, lacked a large spine head, and did not appear to have a neck; mushroom-shaped spines were between 0.5 and 1.25 μm in length and were characterized by a short neck and large spine head; thin spines ranged between 1.25

and 3.0 μm and had elongated spine necks with small heads; filopodia extensions, were long filamentous protrusions that lacked a discernible spine head. The morphological classification of dendritic spines was performed manually under blind conditions.

Statistical analysis

Data from behavioral, biochemical and imaging studies were analyzed by Student's *t* tests for two group comparisons (MIA vs. saline), performed with Statistica (StatSoft, Inc., OK, USA). The differences were considered statistically significant when the *P* value was below 0.05.

Results

Cognitive and affective alterations associated with osteoarthritis pain in mice

Two weeks after receiving MIA intra-articular injection, mice presented a reduction in the percentage of spontaneous alternation evaluated in the Y-maze compared to mice receiving saline ($P < 0.05$, Student's *t* test) (Figure 19A). Moreover, an increase in the immobility time was observed in both the TST and the FST in MIA mice compared to saline mice, at 2 and 3 weeks post-injection, respectively ($P < 0.01$ and $P < 0.05$, respectively, Student's *t* test) (Figure 19B). Therefore, osteoarthritis pain induced by MIA intra-articular injection (Harvey and Dickenson, 2009) was associated with an impairment of working memory functions and an increase of the depressive-like behavior.

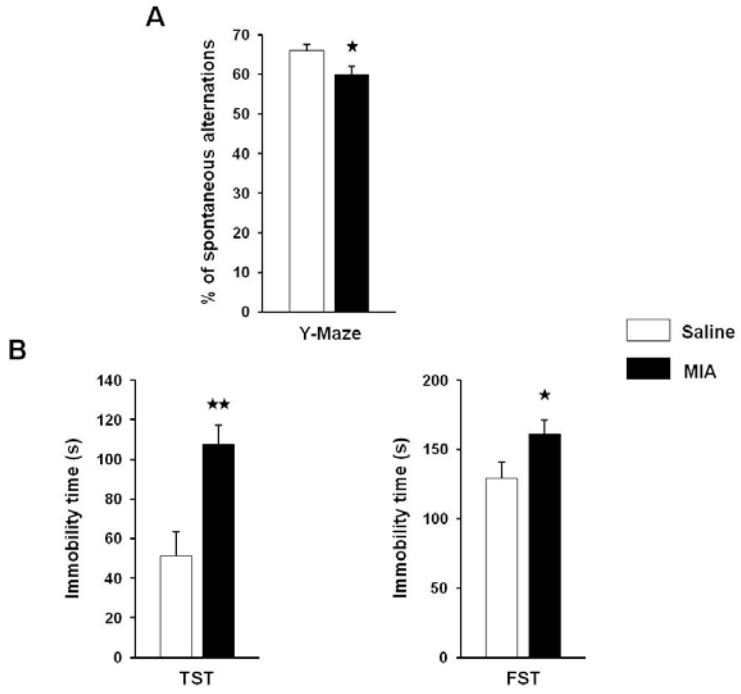

Figure 19. Cognitive and affective behaviors associated with osteoarthritis in mice. The percentage of spontaneous alternation in the Y-maze (A) and the immobility time in the TST (B) were evaluated 2 weeks after the intra-articular injection of MIA or saline. The immobility time in the FST (C) was evaluated at 3 weeks post-injection. Data are expressed as mean ± SEM (n= 14 per group). ★ $P < 0.05$, ★★ $P < 0.01$, vs. saline injection (Student's *t* test).

Osteoarthritis decreases protein levels of AMPAR and NMDAR subunits in mouse mPFC

Immunoblot analysis was performed in total homogenized of mouse mPFC tissues extracted at the end of the behavioral experiments (3 weeks after MIA or saline injection). Protein expression of the subunits composing ionotropic glutamate (NMDAR and AMPAR) and GABA (GABA_AR) receptors were evaluated in this brain area to investigate if the behavioral alterations observed in osteoarthritic mice could be associated with modifications in glutamatergic or GABAergic synaptic transmission at this level. This study revealed a significant decrease of NR1 and NR2A subunits of NMDAR, and GluR1 subunit of AMPAR in the mPFC of MIA mice compared to saline ($P < 0.01$, Student's *t* test) (Figure 20A, B). However, protein expressions of other glutamate receptor subunits (NR2B and GluR3) and GABA receptor subunits (GABA_ARα1 and GABA_ARγ2) were not significantly affected in MIA mice (Figure 20A, B, C). Therefore, the protein expression of specific subunits of ionotropic glutamate receptors, but not GABA receptors, is reduced in the mPFC of osteoarthritic mice. These results suggest that an alteration in the glutamatergic signaling at the mPFC level (e.g. decreased signaling) could participate in the affective and cognitive alterations occurring during osteoarthritis.

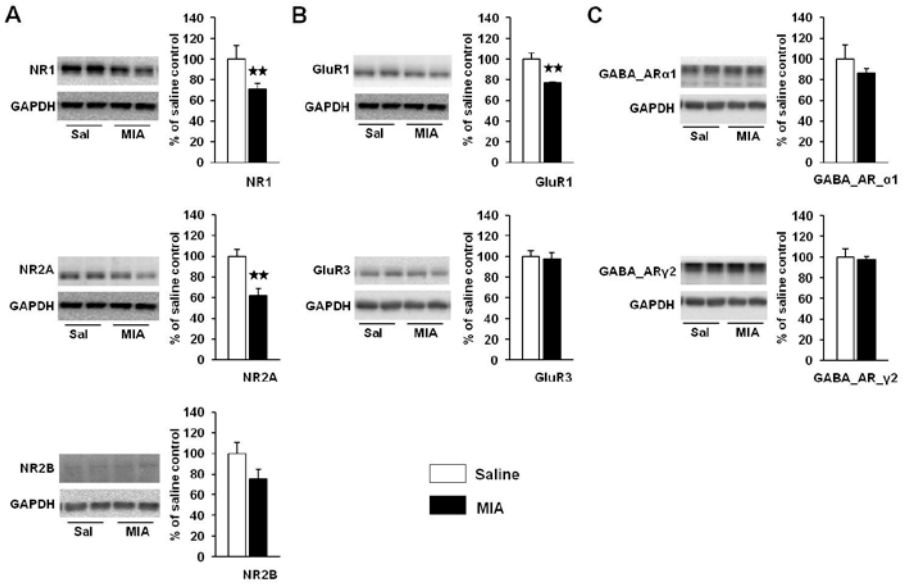

Figure 20. The presence of osteoarthritis decreases total AMPAR and NMDAR protein subunits in mouse mPFC. mPFC tissue lysates from MIA or saline injected mice were used for immunoblot analysis (n=6 per group). Antibodies directed against (A) NMDAR subunits (NR1, NR2A, NR2B), (B) AMPAR subunits (GluR1, GluR3) and (C) GABA_AR subunits (GABA-A receptor γ2, GABA-A receptor α1) were used. The antibody directed against GAPDH was used as loading control for densitometric analysis. The value of the protein of interest was normalized to the amount of GAPDH of the same sample and expressed as a percentage of control (saline intra-articular injection). Each lane represents an individual mouse of a representative experiment (2 representative mice per group are included in each immunoblot illustration). Data are expressed as mean ± SEM. ★★ $P < 0.01$, vs. saline injection (Student's *t* test).

The alterations involving glutamate receptors were not associated with major modifications of ECS proteins in the mPFC of osteoarthritic mice

The presence of osteoarthritis pain was previously found to be associated with an increase of the 2-AG levels in the mPFC that could contribute to the regulation of synaptic transmission at this level (*Article #2*). Therefore, the expression of the principal elements involved in the metabolism and signaling of this endocannabinoid were evaluated. Immunoblot studies did not reveal any significant difference between MIA and saline mice in protein expression of CB1R, DGLα, MAGL, and COX-2 in mPFC (Figure 21). Non significant trends to decrease CB1R expression and to increase DGLα expression were found in MIA mice compared to controls. Thus, the behavioral and biochemical alterations observed in osteoarthritic mice were not associated with major changes in the main ECS proteins involved in 2-AG signaling.

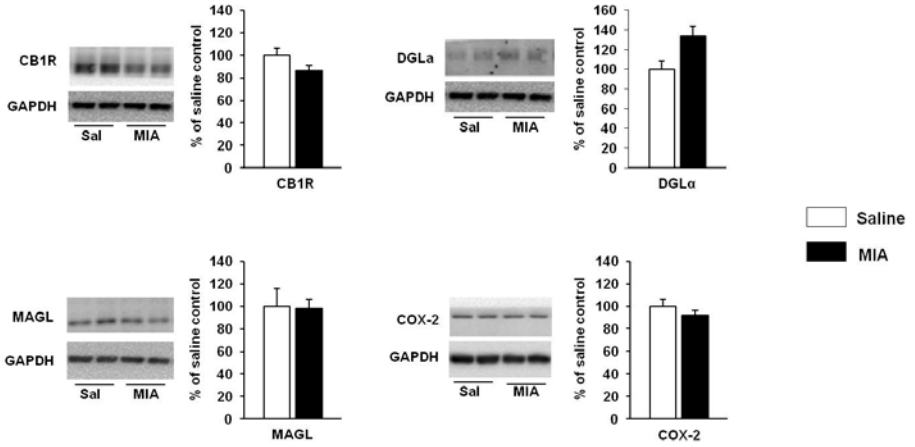

Figure 21. Osteoarthritis did not induce significant changes in the expression of proteins involved in 2-AG metabolism and signaling. mPFC tissue lysates from MIA or saline injected mice were used for immunoblot analysis (n=6 per group). Antibodies directed against CB1R, DGLα, MAGL and COX-2 were used. The antibody directed against GAPDH was used as loading control for densitometric analysis. The value of the protein of interest was normalized to the amount of GAPDH of the same sample and expressed as a percentage of control (saline intra-articular injection). Each lane represents an individual mouse of a representative experiment (2 representative mice per group are included in each immunoblot illustration). Data are expressed as mean ± SEM.

Structural plasticity changes in the mPFC of osteoarthritic mice

Structural plasticity was evaluated in the mPFC to investigate if the behavioral and biochemical alterations observed in osteoarthritic mice could be associated with possible structural changes affecting synaptic transmission. The mPFC pyramidal neurons contained in mouse coronal brain sections were stained with the fluorescent Dye DiI by ballistic delivery and the structural analysis of dendritic spines was performed (Figure 22). No significant differences were revealed in the total dendritic spine density (number of spines per 10 μm of dendrite) between osteoarthritic (MIA) and control (saline) mice (Figure 22A, B). However, the morphological analysis that allowed the classification of dendritic spines in four principal subtypes (Lee et al., 2006) revealed a significant reduction in the density of the mushroom type spines in MIA mice compared to saline ($P< 0.01$, Student's *t* test) (Figure 22C), whereas no significant differences were revealed in the density of stubby, filopodia and thin types. Moreover, immunoblot analysis showed a reduction in the expression of the post-synaptic protein PSD95, a marker of mature spines, in mPFC tissues of MIA mice in comparison with controls ($P< 0.05$, Student's *t* test), without significant changes in the expression of the pre-synaptic protein synaptophysin (Figure 23).

Therefore, a reduction in the proportion of the mature-like mushroom type spines was revealed in the dendrites of the mPFC pyramidal neurons of osteoarthritic mice, although the total spine density was not altered. This morphological alteration in the mPFC

was associated with a concomitant reduction of PSD95 in these mice.

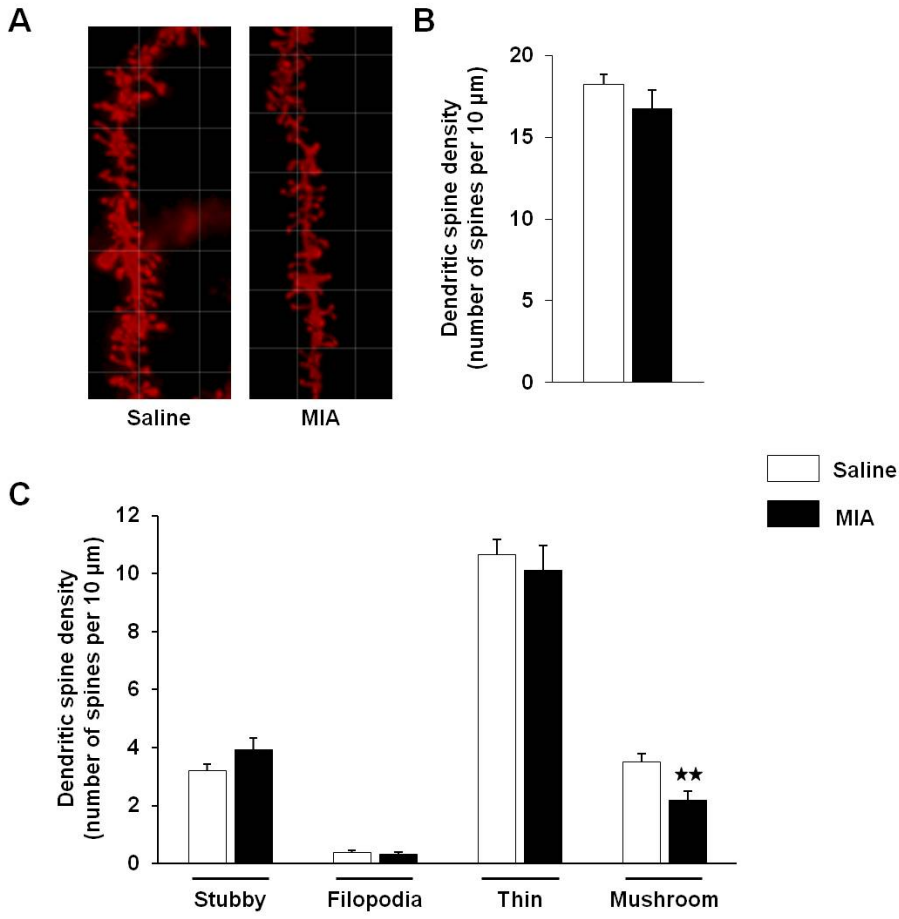

Figure 22. Morphological changes induced by osteoarthritis in the dendrites of mPFC pyramidal neurons. (A) Representative images from high-magnification z stack projections of segments of mPFC pyramidal neuron dendrites of MIA and saline injected mice. (B) Total dendritic spine density and (C) dendritic spine density for each type of spine based on morphological analysis after MIA or saline injection. Data are represented as mean ± SEM (n= 7-10 dendrites per mouse; 7-8 mice per group). ★★ $P < 0.01$, vs. saline injection (Student's *t* test).

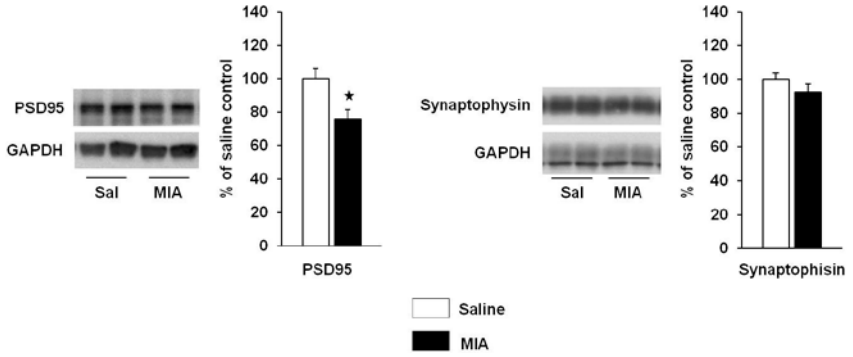

Figure 23. Osteoarthritis decreases the expression of the post-synaptic protein PSD95. mPFC tissue lysates from MIA or saline injected mice were used for immunoblot analysis (n=6 per group). Antibodies directed against PSD95 and synaptophysin were used. The antibody directed against GAPDH was used as loading control for densitometric analysis. The value of the protein of interest was normalized to the amount of GAPDH of the same sample and expressed as a percentage of control (saline intra-articular injection). Each lane represents an individual mouse of a representative experiment (2 representative mice per group are included in each immunoblot illustration). Data are expressed as mean ± SEM. ★ $P < 0.05$, vs. saline injection (Student's *t* test).

All together, these results suggest that the presence of osteoarthritis chronic pain may negatively influence PFC-mediated cognitive and affective processes by disrupting glutamatergic signaling and reducing the proportion of mature dendritic spines with potential deleterious effects on synaptic functions.

ARTICLE #3

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

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Submitted (2015)

* Equal contribution

This article will be also presented in the PhD Thesis of Itzel Lara Mayorga

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DISCUSSION

INVOLVEMENT OF THE ECS IN THE NOCICEPTIVE, EMOTIONAL AND COGNITIVE MANIFESTATIONS OF OSTEOARTHRITIS

Emerging evidence from recent studies has suggested the interest of the ECS as a new potential therapeutic target for osteoarthritis. The ECS regulates a wide range of physiopathological processes including articular metabolism, pain, emotions and cognitive functions, and a therapeutic intervention on this system could offer the potential advantage to treat multiple aspects of this disease. Therefore, investigating the role of specific components of the ECS in the different nociceptive, emotional and cognitive manifestations of osteoarthritis would be helpful to find an appropriate therapeutic target for this disease. We have used both genetic and pharmacological approaches to study the involvement of the ECS in different osteoarthritis pain-related alterations in mice, and explored the potential usefulness of ECS components as biomarkers for human osteoarthritis.

Role of the ECS in the physiopathology of osteoarthritis pain

On the basis of previous studies demonstrating the antinociceptive effects of CB1R and CB2R agonists in rodent osteoarthritis models (Schuelert and McDougall, 2008; Yao et al., 2008), we used genetically modified mice to clarify the contribution of these cannabinoid receptors in the nociceptive, histological and neurochemical alterations associated with osteoarthritis in the MIA model. Our results revealed a crucial role of CB2R in the development of the nociceptive responses induced by the intra-

articular injection of MIA. Indeed, mechanical allodynia was enhanced in CB2KO, as revealed by a mirror image of pain in the contralateral hind paw of these mice. In the absence of CB2R, central sensitization mechanisms could promote changes in the contralateral spinal cord that would facilitate the contralateral mechanical responses, as previously reported in a model of neuropathic pain (Racz et al., 2008b). The important role of CB2R expressed at central level in the control of these manifestations was in agreement with the attenuation of the nociceptive responses observed in CB2xP mice that over-express CB2R in brain and spinal cord. These nociceptive manifestations were not modified in mice lacking CB1R, suggesting that this receptor does not play a major role in the physiopathological processes leading to pain responses during osteoarthritis.

The potential involvement of the ECS in the physiopathology of osteoarthritis has been recently proposed. In agreement, the presence of a functional ECS at the level of nerve fibers and different tissues of the joints, including cartilage, synovium and bone, has been demonstrated in both rodents (Schuelert and McDougall, 2008; Schuelert et al., 2010; Gómez et al., 2014) and humans (Richardson et al., 2008; Gómez et al., 2014). A tonic release of the two main endocannabinoids, AEA and 2-AG, was reported locally in human and rodent osteoarthritic joints presumably to counteract peripheral sensitization and nociception (Richardson et al., 2008; Schuelert and McDougall, 2008; Schuelert et al., 2011). Spinal cord levels of AEA and 2-AG, as well as their synthesizing enzymes, were also increased in the rat MIA model

and modulated the excitability of spinal neurons under this chronic pain state (Sagar et al., 2010). Our data suggest that these high endocannabinoid levels during osteoarthritis would produce a tonic activation of CB2R expressed in the CNS contributing to pain modulation. A potential increase of the endocannabinoid tone in the ipsilateral spinal cord would also be responsible for the down-regulation observed in both CB1R and CB2R gene expression in the ipsilateral spinal cord after MIA injection. A similar down-regulation was revealed in CB1KO and CB2KO in the remaining cannabinoid receptor. The recent report that ipsilateral spinal CB2R gene expression and protein are increased at earlier time-points (week 4) in the rat MIA model, as opposed to the decrease that we found at later stages (week 6), indicates that there are temporal changes involving CB2R during osteoarthritis progression (Burston et al., 2013). Therefore, increased spinal CB2R expression early during the development of osteoarthritis may act to counter nociceptive signaling (Burston et al., 2013), whereas later reductions in CB2R gene expression may represent a failure of these homeostatic mechanisms and contribute to the progression of central sensitization and pain chronification. Interestingly, CB2R gene expression in the spinal cord, but not CB1R, negatively correlated with joint chondropathy scores in osteoarthritis patients (Burston et al., 2013), further reflecting our observations of decreased CB2R gene expression at later stages, since joint damage increases progressively during the course of the disease. The enhanced pain manifestations induced by MIA in CB2KO were not associated with a greater extent of histological alterations in the

mouse knee joints, further supporting a centrally mediated control of pain by CB2R. The ability of spinally administered CB2R agonists or antagonists to inhibit or facilitate, respectively, the evoked neuronal responses in MIA rats provided the first functional evidence for a spinal cord-related role of CB2R in this osteoarthritis model (Sagar et al., 2010b; Burston et al., 2013). In contrast, the peripheral administration of a selective CB2R agonist, GW405833, into the knee joint of osteoarthritic rats produced a paradoxical increase in the mechanosensitivity of joint afferent fibers and increased the hindlimb incapacitance by a mechanism involving the activation of TRPV1 on C afferent fibers, where it co-localizes with CB2R (Schuelert et al., 2010).

The precise mechanisms by which CB2R exerts this control on the nociceptive responses during osteoarthritis remain still unknown. CB2R seems to be expressed in neurons and microglia cells, but not in astrocytes (Racz et al., 2008b; Romero-Sandoval et al., 2008; Burston et al., 2013). Although CB2R presence and functional role in neurons is still a controversial issue, its expression in the spinal cord is associated with the appearance of activated microglia cells in models of chronic pain, including osteoarthritis, where it regulates the neuroinflammatory processes involved in these chronic pain states (Zhang et al., 2003; Racz et al., 2008a, 2008b; Burston et al., 2013).

Beside the ECS changes previously found in the affected joint and spinal cord, we have revealed an increase in the plasmatic levels of 2-AG, but not AEA, in both the MIA mouse model and knee osteoarthritis patients. Interestingly, 2-AG plasmatic levels

positively correlated with knee pain scores in patients. This suggests that the endocannabinoid tone would be increased at both local and systemic levels as a potential protective mechanism. However, if these changes are adaptive or maladaptive responses still need to be determined. A potential contribution of elevated 2-AG levels to osteoarthritis pain cannot be excluded as it is also a major source of arachidonic acid in numerous tissues and is, therefore, also a precursor of pro-inflammatory and pro-algesic eicosanoids (Nomura et al., 2011). This is in accordance with a very recent study demonstrating that both peripheral (synovial fluid) and central (cerebrospinal fluid) 2-AG levels were significantly elevated in patients developing severe post-operative pain after total knee arthroplasty (Azim et al., 2015).

Although the source of circulating endocannabinoids remains unknown, immune cells could critically contribute to their release in the blood (Randall, 2007; Centonze et al., 2008). We have also observed an up-regulation of CB1R and CB2R gene expression in peripheral blood lymphocytes of osteoarthritis patients suggesting a generalized adaptive response of the ECS in the immune system during osteoarthritis. In agreement, alterations in circulating levels of pro- and anti-inflammatory cytokines, which can modulate pain through peripheral and central mechanisms, have been described in clinical osteoarthritis and the rat MIA model (Sellam and Berenbaum, 2010; Sohn et al., 2012; Burston et al., 2013). However, we have shown that only CB2R gene expression levels, but not CB1R, in lymphocytes correlated with knee pain scores in

patients, providing further evidence of the crucial role of CB2R in the control of pain during osteoarthritis.

Antinociceptive effects of CB1R and CB2R agonists on osteoarthritis pain

Our results support the idea that CB2R activation could reduce pain during osteoarthritis. The use of CB2R selective agonists as potential analgesic drugs for osteoarthritis would also represent an advantage with respect to CB1R agonists since CB2R activation would circumvent the psychoactive side effects classically attributed to CB1R activation in the CNS (Malan et al., 2003). Thus, we have demonstrated that the systemic administration of the selective CB2R agonist, JWH133, produced antinociceptive effects in osteoarthritic mice, in agreement with a recent study in rats (Burston et al., 2013). No evidence of tolerance to the effects of repeated systemic administration of JWH133 was recently observed (Burston et al., 2013). The demonstration that this CB2R agonist can also attenuate evoked spinal neuronal responses in the rat MIA model (Burston et al., 2013) is consistent with the reduced pain phenotype observed in MIA mice over-expressing CB2R in the CNS, and with previous reports of a functional role of spinal CB2R in modulating neuropathic pain via modulation of microglia and astrocytic pro- and anti-inflammatory responses (Guindon and Hohmann, 2008b; Racz et al., 2008a, 2008b; Yamamoto et al., 2008; Luongo et al., 2010; Sagar et al., 2010a). It is established that reactive gliosis plays a crucial role in the maintenance of central sensitization in chronic pain states, including osteoarthritis pain

(Racz et al., 2008b; Romero-Sandoval et al., 2008; Burston et al., 2013). Importantly, the systemic administration of JWH133 prevented the MIA-induced increase in spinal astrocyte reactivity (Burston et al., 2013), which is considered an important feature in the transition from acute to chronic pain (Gao and Ji, 2010). This effect is associated with JWH133 ability to reduce spinal MMP-2 and MMP-9 activity that has been implicated in astrocytic activation (Kawasaki et al., 2008). Moreover, JWH133 also reversed the changes in circulating pro- and anti-inflammatory cytokines in the rat MIA model, and showed immunosuppressive, anti-inflammatory and anti-arthritic effects in the murine collagen-induced arthritis model (Malfait et al., 2000; Sumariwalla et al., 2004; Burston et al., 2013; Fukuda et al., 2014). Immunomodulatory and anti-inflammatory effects of CB2R agonists were also observed *in vitro* on cell cultures derived from different joint tissues, in agreement with CB2R expression on chondrocytes, synovium and bone cells (Dunn et al., 2014; Fukuda et al., 2014; Gómez et al., 2014; Gui et al., 2014). Thus, the antinociceptive and anti-inflammatory properties of CB2R agonists, together with their potential role in disease modification, strongly support their promising therapeutic potential for osteoarthritis.

Although CB1R does not have a major role in the physiopathological processes of osteoarthritis pain, we have demonstrated that its activation by the systemic administration of the selective agonist ACEA produced antinociception in MIA mice. The antinociceptive effects of ACEA were previously described in a model of neuropathic pain at a dose similar to that used in our

study, which was devoid of central side effects (Vera et al., 2013). The local administration of this selective CB1R agonist into the knee joint also reduced the hypersensitivity of afferent nociceptive fibers in the rat MIA model by a mechanism involving both CB1R and TRPV1 (Schuelert and McDougall, 2008). Therefore, an indirect blockade of TRPV1 following CB1R activation would contribute to the antinociceptive effects of ACEA at peripheral level, which would be in part due to its ability to inhibit NGF-induced sensitization of TRPV1 in afferent neurons (Wang et al., 2014).

The neuropathic component of osteoarthritis pain: possible involvement of the ECS

Our results in the MIA model of osteoarthritis correlate with the findings reported in a model of neuropathic pain using the same lines of genetically modified mice that revealed a crucial role of CB2R, but not CB1R, in the development of neuropathic pain (Castañé et al., 2006; Racz et al., 2008b). However, the present results demonstrate an earlier development of mechanical allodynia in the contralateral paw of CB2KO, compared with neuropathic pain conditions (Racz et al., 2008b), suggesting the presence of earlier central adaptive changes involving CB2R during osteoarthritis pain. Moreover, a previous study providing a behavioral and electrophysiological characterization of both neuropathic and MIA-induced pain in mice revealed that these two nociceptive manifestations are distinct diseases, with different behavioral and neuronal responses (Harvey and Dickenson, 2009).

A neuropathic component of osteoarthritis pain has been previously demonstrated in the rodent MIA model by the expression of a biomarker of nerve damage/neuropathy, the ATF-3 protein, in DRG cells, the reduction of nerve fiber density in plantar hind paw skin, and ipsilateral spinal cord microgliosis (Ivanavicius et al., 2007; Orita et al., 2011). Importantly, studies using the PainDETECT questionnaire, which gives indication of the presence of neuropathic characteristics, have also estimated 5–50% prevalence of neuropathic pain in osteoarthritis patients, depending on the osteoarthritis population considered in each study (Thakur et al., 2014). Simultaneous loss of innervation in synovial lining together with increased innervation of cartilage and osteochondral junctions in the joints of osteoarthritis patients demonstrate that plasticity occurs in intra-articular somatosensory system and further support the presence of a neuropathic component in osteoarthritis pain (Thakur et al., 2014).

These findings further justify the potential use of cannabinoid compounds in osteoarthritis patients presenting neuropathic pain characteristics since improving effects of these compounds on pain have been already demonstrated in neuropathic pain patients (Fine and Rosenfeld, 2014). However, no significant correlations were observed in our study between endocannabinoid levels or CB1R and CB2R gene expression levels and PainDETECT scores in osteoarthritis patients, despite the correlations found with knee pain scores. This suggests a possible differential regulation of the nociceptive and neuropathic components of osteoarthritis pain by the ECS.

The interaction between the ECS and endogenous opioid system in the control of osteoarthritis pain

The ECS has close relationships with the endogenous opioid system in the control of several physiological responses, including pain (Maldonado and Valverde, 2003). Plastic changes in the opioid system have been revealed in animal models of inflammatory and neuropathic pain, mainly in primary afferents and spinal cord (Cahill et al., 2003; Pol et al., 2006; Puehler et al., 2006; Obara et al., 2009), and to a lesser extent at supraspinal levels (Millan et al., 1987; Neto et al., 2008). Endogenous opioids and their receptors are also present in bone and joint tissues, revealing their potential involvement in the regulation of osteoarthritis processes (Spetea, 2013). A down-regulation of opioid receptor expression has been reported at the peripheral level in arthritic joint tissues and afferent fibers (Li et al., 2005; Shen et al., 2005). However, little is known about the changes involving the opioid system in the CNS during osteoarthritis. We have demonstrated that MIA-induced osteoarthritis promoted a decrease of MOR and a concomitant increase of DOR and KOR in the ipsilateral spinal cord of wild-type mice. The reduced MOR expression in the spinal cord could potentially contribute to facilitate pain responses by reducing the ability of its endogenous ligands to inhibit nociceptive transmission at pre-synaptic and post-synaptic levels (Yoshimura and North, 1983; Glaum et al., 1994; Kohno et al., 1999). Similarly, a peripheral down-regulation of MOR seems responsible for the loss of opioid-induced analgesia in a model of chronic inflammatory joint disease (Li et al., 2005). The enhanced DOR gene expression

in the spinal cord during osteoarhritis pain is in accordance with previous studies indicating that DOR function and expression increase and account for the enhanced antinociceptive effects of DOR agonists under chronic pain states (Bie and Pan, 2007; Cahill et al., 2007). The increased KOR gene expression could represent a complementary change potentially associated with enhanced spinal dynorphin levels, as previously reported in inflammatory and nerve injury models (Dubner and Ruda, 1992; Maekawa et al., 1996; Malan et al., 2000; Laughlin et al., 2001), which may exert pronociceptive actions by non-opioid mechanisms (Vanderah et al., 1996; Obara et al., 2003; Ossipov et al., 2003) or by inhibiting pre-synaptic GABA release through KOR expressed on GABAergic neurons (Li et al., 2003; Xu et al., 2004).

Our findings have also revealed functional interactions between the ECS and opioid system in the control of osteoarthritis pain. First, adaptive changes in the expression of different opioid receptors were revealed by the genetic manipulation of the ECS. We have observed a basal decrease in the gene expression of DOR and KOR, but not MOR, in the spinal cord of CB1KO. DOR and KOR changes would be potentially associated with increased levels of enkephalin and dynorphin, as previously reported in the brain of CB1KO, suggesting a role for CB1R in the tonic regulation of these peptides (Zimmer et al., 1999). Moreover, a decrease of MOR gene expression was revealed in the spinal cord of CB2KO under basal conditions. This suggests that CB2R would be involved in the control of this receptor function, in agreement with a recent study demonstrating that CB2R blockade reduced the expression and

activation of MOR in the mouse brainstem (Páldy et al., 2008). We have also observed a basal increase of KOR gene expressions in the spinal cord of CB2KO, which is in accordance with the opposed decrease in this receptor expression found in CB2xP. The differential basal changes observed for MOR and KOR in CB2KO suggest an opposite modulation of these two opioid receptors by CB2R activity.

The adaptive changes promoted by MIA on MOR were facilitated in both CB1KO and CB2xP, suggesting an opposite regulation of MOR by CB1R and CB2R during osteoarthritis. MOR and CB1R share a variety of functions and can be reciprocally regulated (Maldonado and Valverde, 2003; Bushlin et al., 2010), which would also occur in the spinal cord during osteoarthritis pain. However, the lack of CB1R and the concomitant further decrease of spinal MOR expression induced by MIA were not accompanied by increased nociceptive manifestations in CB1KO. In CB2xP, the increased CB2R activity could induce a concomitant enhancement of endogenous opioid levels acting on MOR, which would lead to a further down-regulation of this receptor. In agreement, previous studies have revealed elevated opioid levels after cannabinoid receptor activation (Mason et al., 1999; Su et al., 2011). However, the over-expression of CB2R would be sufficient to alleviate pain, despite MOR down-regulation. In the absence of CB2R activity, no changes would be induced in endogenous opioid levels and MOR expression would not be further modulated in CB2KO during osteoarthritis. The lack of CB2R could be enough to exacerbate pain manifestations without further adaptive responses in MOR

expression. Similar to MOR, the changes observed in KOR were comparable in both CB1KO and CB2xP, further suggesting an opposite role of these two cannabinoid receptors in modulating opioid activity. The reduced KOR gene expression already observed under basal conditions in both lines of mice was not modified after MIA injection. In contrast, the enhanced DOR gene expression promoted by MIA was not observed when CB1R and CB2R activity was altered in these genetically modified mice. These results suggest that both CB1R and CB2R could have a parallel role in the modulation of DOR activity under osteoarthritis pain state.

The mechanisms by which the ECS regulates the expression and, potentially, the activity of the opioid system during osteoarthritis pain have not been elucidated. However, the ability of cannabinoid activation to stimulate endogenous opioid release, as well as physical (heteromers) and functional (intracellular pathways) interactions between cannabinoid and opioid receptors could be involved (Welch, 2009; Pertwee et al., 2010; Al-Hasani and Bruchas, 2011).

Role of the ECS in the emotional and cognitive alterations associated with osteoarthritis pain

Our results have revealed that osteoarthritis pain induced in mice by MIA intra-articular injection was associated with increased anxiety-like (EPM) and depressive-like (TST, FST) behaviors, and reduced memory functions (ORM, Y-maze), as previously reported in other chronic pain models (Liu and Chen, 2014). In agreement with these findings in mice, we have also observed that pain in osteoarthritis

patients was associated with mood, cognitive and psychosocial alterations, as previously reported (Karp et al., 2006; Axford et al., 2010; Goldenberg, 2010). We have revealed significant correlations between knee pain scores and scores for anxiety, depressive state and self-perceived quality of life in these subjects, confirming previous studies (Axford et al., 2010; Goldenberg, 2010). In agreement, spontaneous osteoarthritis pain has strong affective components in patients and is associated with high activity in cortico-limbic brain areas related to emotions and attention (Kulkarni et al., 2007; Goldenberg, 2010; Parks et al., 2011). Interestingly, significant positive correlations between 2-AG plasmatic levels and depressive state scores, as well as significant negative correlations with memory performances and health-related quality of life scores were observed in these subjects. It has been proposed that chronic pain is a form of stress producing maladaptive changes in brain circuits leading to emotional and cognitive dysfunctions (Blackburn-Munro and Blackburn-Munro, 2001). Endocannabinoid contents are regulated by a variety of stressful stimuli in limbic and hindbrain regions (Hill et al., 2010b), and the peripheral ECS response to stress has also been recently demonstrated (Hill et al., 2008b, 2009b). In agreement, elevated 2-AG, but not AEA, serum content was found in individuals suffering from major depression and healthy individuals after stress exposure (Hill et al., 2009b). While the unequivocal source of circulating endocannabinoids is unknown, as discussed above, immune and endothelial cells, adipocytes, and visceral organs, such as liver and intestines, all possess the ability to synthesize and release

endocannabinoids into the blood (Matias et al., 2006; Matias and Di Marzo, 2007; Randall, 2007). Therefore, the observed increase in peripheral 2-AG in both osteoarthritic mice and patients could be associated with increased endocannabinoid activity in these peripheral organs, where they regulate immune, metabolic and cardiovascular processes. In agreement, these are all physiological processes that could be affected by stress (Hill et al., 2009b) and, potentially, by osteoarthritis. Indeed, beside the alterations in immune response, mood and cognitive functions, the reduced physical activity in the daily life of osteoarthritis patients increases the risk of developing other medical co-morbidities, such as cardiovascular disease and diabetes (Smith et al., 2014). The protective increase of endocannabinoid activity, would be specifically limited to 2-AG, as high systemic AEA levels could be potentially associated with a detrimental effect on emotional and cognitive responses, as demonstrated in preclinical animal models (Akirav, 2011; Busquets-Garcia et al., 2011; Riebe and Wotjak, 2011). This is in contrast with the increased levels of both endocannabinoids previously found in the synovial fluid of osteoarthritis patients (Richardson et al., 2008), indicating that both AEA and 2-AG would be involved in the regulation of pain and local structural alterations, although 2-AG would be predominantly involved in the emotional and cognitive osteoarthritis symptoms.

We have demonstrated that the alterations in the anxiety-like behavior induced by osteoarthritis in mice appeared more pronounced in CB1KO and were absent in CB2KO, revealing an opposite role of CB1R and CB2R in the control of these

manifestations. The role of CB1R in these emotional responses resembled that observed in different models of chronic stress. Thus, CB1KO displayed an increased sensitivity to develop anxiety and depressive-like states following repetitive stress procedures (Martin et al., 2002; Hill et al., 2011a), in agreement with the expression of CB1R in cortico-limbic circuits related to stress responses (Herkenham et al., 1991; Malcher-Lopes et al., 2006; Hill et al., 2010a, 2011b; Tasker and Herman, 2011). CB1R is highly expressed in GABAergic neurons and at moderate to low levels in glutamatergic terminals (Häring et al., 2012). In our study, we do not have investigated which of the two neuronal populations contributes to the anxiogenic-like responses observed in CB1KO, although it seems that CB1R on glutamatergic neurons mediates the anxiolytic, and on GABAergic neurons the anxiogenic responses, respectively (Häring et al., 2012). In our experimental conditions, both CB1KO and wild-type mice developed high levels of anxiety three weeks after MIA injection. Therefore, a further enhancement of this behavior in CB1KO was unlikely to be detected due to a possible floor effect in this behavioral model, which could have masked significant differences between the two genotypes at this later time point.

Despite the protective role of CB2R in osteoarthritis pain modulation, an opposite regulation by this receptor was observed in the anxiety-like behavior induced under this chronic pain state. CB2R has been mainly found in glutamatergic neurons (i.e. pyramidal cells of the hippocampus and cerebral cortex) and it has been proposed to participate in emotional responses, despite the

controversy about its functional role in neurons (García-Gutiérrez et al., 2010; Onaivi et al., 2012). In line with our findings, chronic CB2R blockade produced anxiolysis and antidepressant-like effects following stress, although the mechanisms have not been elucidated (García-Gutiérrez et al., 2010, 2012).

The different correlations of CB1R and CB2R gene expression in peripheral lymphocytes observed with depression and pain scores, respectively, provide further evidence of a differential role of these two receptors in the control of these osteoarthritis-related symptoms. Recent findings suggest that peripheral ECS changes could mirror similar alterations at central level and correlate with emotional and cognitive dysfunctions in neuropsychiatric disorders, including depression and schizophrenia (Hill et al., 2008b; Ferretjans et al., 2014).

In contrast, the memory impairment induced by osteoarthritis was not modified in mice lacking CB1R or CB2R, suggesting that these receptors do not participate in the physiopathology of these cognitive manifestations. This is also in line with the absence of significant correlations between the expression levels of cannabinoid receptors in lymphocytes and the memory scores of osteoarthritis patients involved in our study. It is worth mentioning that we have evaluated memory functions in mice through a non aversive and neutral cognitive task (ORM) and the responses obtained after genetic or pharmacological manipulation of cannabinoid receptors highly depend on the nature of the task (Akirav, 2011). Thus, fear learning was enhanced in CB1KO in an active avoidance task following chronic mild stress exposure

(Martin et al., 2002). This is in agreement with the pivotal role of CB1R in extinction of aversive memories, but not in the extinction of learned responses in appetitive-motivated tasks (Niyuhire et al., 2007; Riebe and Wotjak, 2011). Recently, it has also been proposed that CB2R is involved in the consolidation of aversive memory (García-Gutiérrez et al., 2013).

Role of the ECS in the adaptation of HPA-axis responses during osteoarthritis

Chronic pain could be considered a stressor producing similar effects to those observed in other stress-related disorders (Blackburn-Munro and Blackburn-Munro, 2001). However, the precise contribution of CRH and other components of the HPA axis in chronic pain manifestations remains unclear. In our study, we have found a down-regulation of CRH gene expression in the PVN of osteoarthritic mice that may represent an adaptive modification to limit HPA axis activity under this chronic pain state and may underlie the absence of HPA neuroendocrine alterations in osteoarthritis patients (Khoromi et al., 2006). Similarly, no alterations in corticosterone and ACTH levels were found in neuropathic rodents under basal conditions or after restraint stress, despite the changes found in CRH expression in limbic brain areas (Bomholt et al., 2005; Ulrich-Lai et al., 2006; Yalcin et al., 2011). In agreement with our results, CRH signaling in the limbic system seems to be involved in the nociceptive, affective and cognitive alterations in different rodent chronic pain models (Ulrich-Lai et al., 2006; Ji et al., 2010).

Our results suggest an important role of the mPFC in the control of these responses under osteoarthritis pain state. mPFC is an important brain area involved in pain, cognitive and emotional processing (Miller, 1999) that constitutes one of the primary targets of HPA axis hormones (McLaughlin et al., 2014). Interestingly, osteoarthritis pain in mice was associated with increased levels of 2-AG in the mPFC, but not in other brain areas involved in these processes (i.e. amygdala, hippocampus), suggesting a regional specificity of the effects of this chronic pain state. This specific 2-AG increase in mPFC may represent a compensatory mechanism to maintain proper neuroendocrine and behavioral functions in response to persistent pain. This is in accordance with the inhibitory role of endocannabinoids in the regulation of the HPA axis activity and termination of stress responses (Riebe and Wotjak, 2011). Moreover, these central changes were also reflected at periphery by the increase of 2-AG plasmatic levels observed in osteoarthritic mice, suggesting that this endocannabinoid would be an integral component of the adaptive responses occurring at both central and peripheral levels under stressful conditions due to chronic pain. An increase in mPFC 2-AG levels with similar functional consequences has also been demonstrated in chronic stress animal models (Hill et al., 2010a; McLaughlin et al., 2014). Indeed, the stress-induced increase of endocannabinoid signaling through CB1R in the mPFC mediates a long-loop negative feedback mechanism to suppress HPA axis activity (Hill et al., 2011b). Immunohistochemical and electron microscopy data indicate that CB1R is expressed almost entirely by GABAergic terminals that make synapses with

pyramidal neurons in layer V of the prelimbic region of the mPFC (Hill et al., 2011b). Therefore, increased 2-AG/CB1R signaling would inhibit GABA release onto layer V pyramidal neurons, thus, disinhibiting the excitatory output of these neurons on inhibitory relays within sub-cortical structures that, in turn, regulate CRH secretion in PVN (Hill et al., 2011b). The same mechanisms may be responsible for the CRH down-regulation found in the PVN of osteoarthritic mice. Notably, this CB1R-dependent disinhibition of mPFC pyramidal neurons would modulate also the cognitive impairment observed in a model of inflammatory arthritis pain (Ji et al., 2010; Kiritoshi et al., 2013).

We have tested the hypothesis that the opposite role of CB1R and CB2R in the control of the anxiety-like behavior during osteoarthritis pain could be related to a different role of these two receptors in the regulation of HPA axis components. Thus, we have revealed that MIA-induced CRH down-regulation in the PVN was not modified in CB1KO and was fully reversed in CB2KO. A similar opposite regulation in the absence of these cannabinoid receptors was observed for the expression of GR gene in the PVN of osteoarthritic mice. The lack of CB1R and the concomitant deregulation of GR gene expression in the PVN may interfere with the ability of endocannabinoids to exert the glucocorticoid-dependent control of HPA axis within the PVN (Hill et al., 2010b), contributing to the altered affective responses observed in CB1KO. However, the absence of further modifications in CRH gene expression in the PVN of MIA CB1KO suggests the possibility that other neurotransmitter inputs to PVN may compensate the absence

of CB1R and maintain unaltered the CRH down-regulation in osteoarthritic mice. The lack of CB2R together with the basal CRH and GR gene expression modifications in the PVN and mPFC may facilitate adaptive responses during osteoarthritis pain to prevent the affective alterations in CB2KO. The mPFC seems to be particularly involved in the modulation of these responses in CB2KO. Thus, the higher basal expression of GR in the mPFC of CB2KO could promote the down-regulation of CRH gene expression in this brain area by a glucocorticoid-mediated mechanism (Meng et al., 2011). This would limit the excitatory influence of cortical CRH on HPA axis and the anxiety-like behavior in CB2KO (Jaferi and Bhatnagar, 2007). However, MIA CB2KO showed increased gene expression of CRH and GR in the PVN, suggesting that a more efficient regulation of HPA axis activity occurs in the absence of CB2R. In agreement, a failure to increase CRH gene expression in the PVN after 30 min restraint stress was found in CB2xP in a previous study (García-Gutiérrez and Manzanares, 2011). However, our behavioral results in CB2KO are apparently in contrast with the decreased vulnerability of CB2xP to anxiogenic-like stimuli (García-Gutiérrez and Manzanares, 2011), revealing the great complexity of brain CB2R-mediated signaling in the control of these behavioral responses highly depending on the specific experimental conditions. Therefore, the endocannabinoid signaling through CB1R and CB2R seems to be crucial for the emotional responses produced by osteoarthritis pain, although the exact mechanisms involved remain to be elucidated.

mPFC alterations may contribute to osteoarthritis symptoms

The protein expression levels of specific subunits of ionotropic glutamate receptors, but not GABA receptors, were reduced in the mPFC of osteoarthritic mice. This result suggests an alteration in the glutamatergic signaling at the mPFC level (e.g. decreased signaling) that could underlie the affective and cognitive alterations occurring during osteoarthritis. It has been proposed that glutamate receptor-mediated synaptic transmission is a crucial neuronal substrate for mPFC functions, including pain modulation (Goldman-Rakic, 1995; Lisman et al., 1998; Millecamps et al., 2007; Metz et al., 2009). In agreement, the local mPFC activation of glutamate receptors exerts analgesic effects in a rodent model of neuropathic pain (Millecamps et al., 2007). More than 90% of the excitatory synaptic connections occur upon dendritic spines, which vary in shape and size in an activity-dependent manner (Kirov et al., 1999). Dendritic spine remodeling could participate in maladaptive mechanisms in different pathological conditions, including pain (Tan and Waxman, 2014). However, the alterations observed in synaptic and structural plasticity during chronic pain seem to depend on the specific spinal and supraspinal region evaluated and/or the specific pain model (Luo et al., 2014; Tan and Waxman, 2014). Thus, we revealed a reduction in the proportion of mushroom type dendritic spines in the mPFC pyramidal neurons of osteoarthritic mice, although the total spine density was not altered. Mushroom type spines are mature or “memory” spines, which have been associated with increased magnitude and faster latency of evoked post-synaptic potentials that correlate with improved

synaptic efficacy following memory formation (Lüscher et al., 2000; Calabrese et al., 2006). These mature spines maintain activity-dependent synaptic strength and present increased function and membrane expression of ionotropic glutamate receptors (Tan and Waxman, 2012). Therefore, the reduced expression of AMPAR and NMDAR subunits observed in our model would be related to the reduced proportion of mushroom dendritic spines in the mPFC. Interestingly, the specific glutamate receptor subunits that were altered in our experimental conditions (NR1, NR2A, GluR1) seem to be highly associated with mature synapses and with the expression of LTP (Tan and Waxman, 2012; Sanz-Clemente et al., 2013; Luo et al., 2014). These neurochemical and morphological alterations in the mPFC were also associated with a concomitant reduction of the post-synaptic protein PSD95, a marker of mature spines associated with glutamate receptors (El-Husseini et al., 2000; Chetkovich et al., 2002), but not the pre-synaptic protein synaptophysin present in contact sites and considered a marker for synaptogenesis (Masliah et al., 1991). Therefore, the presence of osteoarthritis pain seems to have specific effects on post-synaptic components and function in the absence of major pre-synaptic alterations. Similar changes in glutamatergic signaling and structural plasticity in the mPFC provide the molecular basis for the affective and cognitive dysfunctions produced by chronic stress (Drevets et al., 1997; Radley et al., 2006; Kang et al., 2012; Yuen et al., 2012; Ota et al., 2014). These alterations may also account for the decreased output of mPFC pyramidal cells demonstrated in a rat model of arthritis, which would contribute to the impairment in

mPFC-dependent tasks (e.g. decision-making) observed in both chronic pain rodent models (Pais-Vieira et al., 2009; Ji et al., 2010) and patients (Apkarian et al., 2004). However, an enhancement of mPFC spine density that was functionally coupled with an increase of evoked glutamatergic currents was observed in a model of neuropathic pain one week after surgery (Metz et al., 2009). This is in apparent contrast with our data obtained in the osteoarthritis model at the end of the third week post-MIA injection. Both results together suggest the possibility that the initial adaptive changes that would facilitate synaptic strength could be potentially reversed at later stages in this brain area. In addition, our data are more consistent with the reduced gray matter, reduced activation and reduced levels of excitatory neurotransmitters reported in the PFC of chronic pain patients (Grachev et al., 2001; Apkarian, 2004; Neugebauer et al., 2009). Opposed changes were also found at spinal level and in the primary somatosensory cortex in different models of chronic pain (Tan and Waxman, 2014). In a model of neuropathic pain, increased dendritic spine density in dorsal horn neurons of the spinal cord was associated with an increase in the proportion of mushroom type spines and increased PSD95, NR1, NR2 and GluR1 expression, which contribute to abnormally strengthened synaptic connections in the nociceptive pathways (Tan and Waxman, 2012). Similarly, synaptic remodeling that includes an increase of both synaptogenesis and synapse elimination, and an enhanced strength of persisting synapses occurred in primary somatosensory cortex within few days after peripheral nerve injury

and was associated with neuropathic pain development (Kim et al., 2012).

The alterations that we observed in the mPFC would also be in agreement with the increased 2-AG tone found in mPFC of osteoarthritic mice, which would increase the signaling on CB1R mainly expressed on GABAergic neurons to compensate the inhibition of glutamatergic (pyramidal) neurons. However, the opposite situation cannot be excluded since endocannabinoid signaling in layer V neurons also directly inhibits excitatory transmission *in vivo* (Fortin and Levine, 2007). In agreement, electron microscopy analyses have revealed that pre-synaptic CB1R directly face mGluR5 in layer V/VI of the mouse prelimbic cortex and DGLα is also expressed in dendrites containing mGluR5 (Lafourcade et al., 2007), although CB1R is prominently expressed in GABAergic neurons. In spite of the increase of 2-AG levels in the mPFC, CB1R expression was not significantly modified in this brain area of osteoarthritic mice, which would contribute to maximize the binding sites for endocannabonoid signaling.

All together, these results suggest that osteoarthritis chronic pain may negatively influence mPFC-mediated cognitive and affective processes by disrupting glutamatergic signaling and reducing the proportion of mature dendritic spines with potential deleterious effects on synaptic functions. Therefore, ECS signaling would be specifically enhanced in this brain area as a protective adaptive mechanism.

Effects of CB1R and CB2R activation on anxiety-like behavior and memory alterations associated with osteoarthritis pain

We have demonstrated that CB1R and CB2R pharmacological activation by the acute administration of ACEA and JWH133, respectively, improved nociceptive and anxiety-like behaviors associated with osteoarthritis in mice, whereas only ACEA improved the memory impairment. A treatment designed to improve these emotional and cognitive alterations would be essential for an effective management of osteoarthritis. The improvement of the affective and cognitive alterations observed in the MIA model could be a direct consequence of pain relief produced by these cannabinoid agonists. However, the lack of effects of JWH133 in the memory task, despite its antinociceptive and anxiolytic effects, suggests that the amelioration of these symptoms is more likely to depend on a direct effect in emotional and cognitive processes. Accordingly, ACEA and JWH133 produce anxiolysis (Moreira and Wotjak, 2010; Busquets-Garcia et al., 2011), whereas CB1R activation usually impairs memory performance (Akirav, 2011). In agreement with our results, the emotional responses induced by CB1R agonists, including ACEA, after systemic or local injection in brain areas involved in anxiety (i.e. PFC) are biphasic, being anxiolytic at low doses and ineffective or anxiogenic at higher doses (Moreira and Wotjak, 2010; Casarotto et al., 2012; Fogaça et al., 2012). The recruitment of other receptors, such as TRPV1 or GPR55, or the differential effects of CB1R activation on distinct neuronal populations depending on the dose, the brain circuits activated and other different experimental factors

could underlie these biphasic effects of cannabinoid agonists (Häring et al., 2012). Moreover, the lowest ACEA dose (1 mg/kg) used in our study was effective in reversing the impairment of the anxiety-like behavior in osteoarthritic mice without affecting the responses in control mice, which reflects differences in the initial baseline stress/anxiety levels and/or a differential regulation of the ECS activity in pathological vs. physiological conditions. This "bidirectional" neuromodulatory role of the ECS in the emotional responses is more complicated in the case of CB2R agonists. While the CB2R agonist JWH015 induced anxiogenic effects in the light-dark box test and no effects in the anhedonia paradigm following chronic mild stress (Onaivi et al., 2008), JWH133 had no effects in the light-dark box test after acute treatment, but elicited an anxiogenic response after chronic treatment in mice (García-Gutiérrez et al., 2012). In agreement, a CB2R antagonist, AM630, induced anxiogenic effects after acute administration, and anxiolytic effects after chronic treatment (García-Gutiérrez et al., 2012). These data may in part correlate with the different emotional responses observed in our osteoarthritis model in CB2KO (a condition resembling a chronic CB2R blockade) and in wild-type mice after acute CB2R agonist administration (JWH133). In accordance with our findings in anxiety-like behavior, the acute administration of another CB2R selective agonist, GW405833, reversed the increased depressive-like behavior (FST) in a rat model of neuropathic pain, without affecting the responses in sham rats (Hu et al., 2009).

The effects of cannabinoid agonists on memory also depend on different factors, including the nature of the task (emotional or non-

emotional), the memory stage investigated (acquisition, consolidation, retrieval, extinction), and the experimental model used (Akirav, 2011). We evaluated the effect of the acute administration of CB1R and CB2R agonists on memory retrieval in the ORM that is a non-emotional task. CB1R agonists dose-dependently affect all components of short- and long-term recognition memory, including retrieval (Galanopoulos et al., 2014). However, conflicting results are also reported in the ORM task after pharmacological modulation of the ECS, depending on the specific drug, dose, route of administration, intervals between training and test phases, previous habituation to the apparatus (Morena and Campolongo, 2014), or the specific endocannabinoid involved in the memory process evaluated (Busquets-Garcia et al., 2011). In our experimental conditions, ACEA did not affect memory performance in control mice, but improved the memory deficit in osteoarthritic mice. Chronic treatment with this drug also improved the memory deficit in an Alzheimer's disease mouse model at a similar dose used in our study, which did not produce amnesic-like effects in control mice in the ORM (Aso et al., 2012). Moreover, systemic ACEA administration prior to trace fear conditioning or memory-recall test reduced freezing behavior in rats exposed to chronic stress procedures (Reich et al., 2013). This suggests that CB1R activation would restore the potential neurotransmission alterations occurring in the different brain regions involved in memory processes under pathological conditions. The ameliorating effects of ACEA on memory in osteoarthritic mice could be related to its ability to decrease the

inhibitory transmission in the mPFC through CB1R activation on GABAergic terminals (Kiritoshi et al., 2013), restoring mPFC functioning that is important for proper cognitive performances in the ORM task (Abush and Akirav, 2013). A suppression of the GABAergic inhibitory signaling was also observed after the application of JWH133 to entorhinal cortex slices (Morgan et al., 2009), demonstrating a functional role of neuronal CB2R. In agreement, the acute injection of JWH133 enhanced the consolidation of aversive memory in mice (García-Gutiérrez et al., 2013), and the chronic treatment with another CB2R agonist, MDA7, reversed the deficit in memory retrieval in the Morris water maze induced in a rat Alzheimer's disease model (Wu et al., 2013). The lack of effects of JWH133 on memory retrieval in our experiments suggests that the responses on memory after CB2R activation highly depend on the specific experimental model and the treatment protocol used.

General considerations

In summary, the results in the MIA model have demonstrated that the nociceptive and emotional manifestations of osteoarthritis pain were differentially regulated by CB1R and CB2R. In contrast, the acute activation of both receptors improved the nociceptive and emotional manifestations, whereas only the CB1R activation improved the memory impairment. The interest of CB2R as a potential therapeutic target for osteoarthritis is supported by the antinociceptive and anti-inflammatory properties of CB2R selective agonists, together with their potential role in disease modification,

and the lack of the psychoactive side effects classically attributed to CB1R activation in the CNS. Our results underline that the effects of chronic treatment with CB2R agonists on pain, emotional and cognitive symptoms should also be investigated in future experiments. In agreement, registered trials demonstrate that the clinical research on cannabinoids in the next future will also include selective CB2R agonists as single analgesics or in combination with standard analgesics (Davis, 2014).

Moreover, we have revealed for the first time a specific increase of 2-AG, but not AEA systemic levels during osteoarthritis. This result could explain the disappointing findings obtained in the clinical trial with the selective FAAH inhibitor, PF-04457845, that elevated plasmatic AEA levels, but failed to elicit effective analgesia in knee osteoarthritis patients (Huggins et al., 2012), despite the antinociceptive properties of FAAH inhibitors in rodent osteoarthritis models (Ahn et al., 2011; Schuelert et al., 2011). Changes in AEA biotransformation in aged patient populations, as those usually suffering from osteoarthritis, may also contribute to the lack of analgesia following FAAH inhibition. In agreement, a recent report described greater susceptibility to chronic pain and decreased AEA-mediated antinociceptive effects in aged animals (Bishay et al., 2013; Burston and Woodhams, 2013). The increased 2-AG levels in osteoarthritis patients as a potential protective mechanism under conditions of chronic pain further suggests that the use of MAGL inhibitors would represent a novel promising strategy to obtain analgesia in these patients. One concern of the use of MAGL inhibitors is the potential desensitization of CB1R

associated with analgesic tolerance when administered at moderate to high doses (Schlosburg et al., 2010). However, preclinical studies with the recent developed MAGL inhibitors, such as JZL184 and KML 29 (Long et al., 2009; Chang et al., 2012), suggest that low doses of MAGL inhibitors are devoid of analgesic tolerance (Busquets-Garcia et al., 2011; Kinsey et al., 2013). These low doses of MAGL inhibitors also decrease arachidonic acid pools that are required for the generation of pronociceptive molecules such as prostaglandin E2 (Nomura et al., 2011). This approach delivers a dual analgesic mechanism elevating 2-AG and reducing pro-inflammatory prostaglandin levels, and has been recently reported to induce anxiolytic effects, without producing the memory impairment associated with chronic FAAH inhibition (Busquets-Garcia et al., 2011). The antinociceptive effects of MAGL inhibition would be mediated by CB1R and, possibly, by CB2R, whereas the anxiolytic effects seem to be exclusively mediated by CB2R (Schlosburg et al., 2010; Busquets-Garcia et al., 2011; Kinsey et al., 2013).

Therefore, a combination of selective CB2R agonists with low doses of MAGL inhibitors could represent an alternative strategy for the improvement of pain, anxiety and, possibly, the memory impairment associated with osteoarthritis. This approach would offer the potential advantage to target important components of the ECS that are already altered in osteoarthritis and seem to be involved in the physiopathological mechanisms of this disease.

NOCICEPTIVE, EMOTIONAL AND COGNITIVE MANIFESTATIONS OF CHRONIC PAIN

Our results provide further insights in the field of preclinical pain research to facilitate the development of behavioral animal models that could be comparable in several aspects to the complex human pain experience. Thus, the behavioral paradigms used in our studies included the analysis of the affective-motivational and cognitive dimensions of chronic pain in parallel with the classic tests widely used in the past decades to evaluate the sensory component. These are essential pain aspects to take into consideration to improve the validity of preclinical studies.

Chronic pain was associated with increased anxiety- and depressive-like behaviors, and reduced memory functions in both the osteoarthritis and peripheral neuropathy mouse models. The increased anxiety-like behavior and memory deficits already appeared within the first week after pain induction, indicating that the early presence of pain is sufficient for the development of these affective and cognitive manifestations. In contrast, increased depressive-like behavior became evident only at later stages, suggesting that the depressive symptoms are manifested only once pain persists along time. The EPM and the FST were the more sensitive tests in detecting increased anxiety-like and depressive-like responses, respectively, in both pain experimental models. In contrast, the TST detected increased depressive-like behavior only in the osteoarthritis, but not in the neuropathic pain model (data not shown), as previously reported (Hasnie et al., 2007; Benbouzid et al., 2008; Yalcin et al., 2011; Liu and Chen, 2014). Therefore, these

behavioral responses promoted by chronic pain seem to be highly dependent on the time point of measurements, the specific behavioral paradigm and pain model used, which can also explain the contradictory results often obtained in previous studies (Liu and Chen, 2014).

Pain and reward are opponent responses that interact and influence each other (Becker et al., 2012). Indeed, rewarding stimuli decrease pain sensitivity (Leknes and Tracey, 2008) and pain impairs reward processing, as demonstrated by the association of chronic pain with anhedonia (i.e. the inability to feel pleasure) (Marbach et al., 1983; Bura et al., 2013) and altered reward responsiveness (Becker et al., 2012; Elvemo et al., 2015). Neuropathic pain in mice was associated with the development of an anhedonic-like state as revealed by a reduced sucrose preference, which could potentially occur also during osteoarthritis. The reduced consumption and preference for highly palatable sweet solutions have been previously used to reveal anhedonia in different chronic pain models (Andersen et al., 2009; Shi et al., 2010; Bura et al., 2013). Nutritional intake is mediated by taste-derived enjoyment and post-ingestion satisfactory experiences and their absence can reflect the development of anhedonia (Andersen et al., 2009). Therefore, the anhedonic-like state observed in neuropathic pain mice seems to be associated with reduced reward responsiveness. We have further investigated if chronic neuropathic pain could alter reward responsiveness and motivation by using an operant paradigm. We compared the responses to obtain standard or highly palatable food in neuropathic pain and sham mice under different operant

schedules of reinforcement. Mice exposed to neuropathic pain showed a reduction in the operant responding only when the efforts required to obtain highly palatable food were increased under the FR5 schedule, whereas the responses to seek for standard or highly palatable pellets under FR1 were not affected. This result, together with the data of the anhedonia model, suggests that chronic neuropathic pain exposure leads to the loss of gratified responses. The results obtained under FR5 were also mirrored by the reduced motivation to work for reward revealed in neuropathic pain mice under the PR schedule requiring progressively more effort to earn each subsequent food pellet. This reduced motivation did not depend on the type of reinforcement since a similar decrease in the breaking point was observed with standard and highly palatable pellets. In agreement with our results, the responding maintained by standard food as evaluated in the PR schedule was also reduced in another neuropathic pain model (Schwartz et al., 2014). The altered responses found in these operant difficult tasks may also be partially attributed to a learning impairment since operant responding highly depends on proper cognitive functions. Thus, the anhedonia model and the operant tasks allowed modeling in mice the decreased reward responsiveness and motivation to initiate and complete goal-directed actions, which represent important features of symptoms, such as central fatigue and depression, often reported in patients with chronic pain (Turk et al., 2010; Schwartz et al., 2014). Large overlaps exist in the anatomical and neurochemical substrates of pain and reward, which could explain the mutual influence between these processes (Leknes and Tracey, 2008; Becker et al., 2012).

Notably, changes in the opioid and dopamine systems accompanied by alterations in neuronal activity and connectivity in the nucleus accumbens have been proposed as potential mechanisms of this altered reward processing during chronic pain in patients and animal models (Baliki et al., 2012; Becker et al., 2012; Chang et al., 2014; Schwartz et al., 2014; Elvemo et al., 2015).

We have also demonstrated that the antinociceptive effects of a drug are not necessarily accompanied by an improvement of the emotional and cognitive pain-related symptoms. Indeed, pregabalin, a well validated analgesic drug for neuropathic pain treatment (Micó and Prieto, 2012; Verma et al., 2014), improved pain, anxiety, anhedonia, memory and operant responding, but did not modify depressive-like symptoms in the neuropathic pain model. Higher doses of pregabalin could not be evaluated since they induce important locomotor changes in rodents (Vartanian et al., 2006; Yokoyama et al., 2007), which would bias the behavioral responses evaluated (Liu and Chen, 2014). The anxiolytic effects of pregabalin have been widely reported in animals and humans (Micó and Prieto, 2012; Navarrete et al., 2012) and improving effects on the altered cognitive functions produced by long-term benzodiazepine treatment have been recently described (Oulis et al., 2014). In agreement with our results, chronic pregabalin treatment did not affect the depressive-like behavior in a model of chronic inflammatory pain despite its analgesic effects (Maciel et al., 2013). Taken together these results suggest that depressive-like symptoms do not necessarily resolve when pain is treated, possibly implying that these behavioral alterations, once established, could not require

ongoing nociceptive signaling. Therefore, a potential treatment exerting a direct effect on the different symptoms would be essential for an effective management of chronic pain states. This conclusion is further supported by a study showing that mechanical allodynia produced by placing a plastic cuff around the sciatic nerve in mice completely resolved when the cuff was removed, whereas the affective and cognitive manifestations persisted (Dimitrov et al., 2014). In contrast, the anhedonic-like state associated with neuropathic pain in our study completely disappeared after chronic pregabalin treatment, when allodynia and hyperalgesia were only partially reversed. Pregabalin treatment also completely abolished the impairment induced by neuropathic pain in the food-maintained operant responses under the FR5 schedule. The improvement of anhedonic and operant responses by pregabalin in neuropathic pain mice could be an indirect consequence of pain relief, although a direct effect of this drug on the reward processing cannot be excluded. Repeated pregabalin treatment (10 and 30 mg/kg) reduced operant responding to obtain alcohol and cocaine, whereas the responses to obtain food pellets where not affected (Stopponi et al., 2012; de Guglielmo et al., 2013; Spencer et al., 2014) accordingly with the results obtained in sham mice in the present study. However, we have also observed a decrease in motivation to seek for food under PR schedule in sham mice that was comparable with that of mice exposed to neuropathic pain after pregabalin treatment. These similar responses found in sham and neuropathic pain groups make difficult the interpretation of the results obtained after pregabalin treatment in neuropathic pain mice. The

mechanisms by which pregabalin could produce this impairing effect on motivation have not been explored. Similarly to its structural analogue gabapentin, pregabalin would decrease the release of several neurotransmitters, including glutamate and dopamine in brain areas regulating reward processing (Reimann, 1983; Spencer et al., 2014). This effect in brain areas controlling goal-directed behaviors could potentially contribute to the impaired responses observed in sham mice after chronic pregabalin treatment.

In conclusion, we have validated behavioral models to evaluate multiple responses associated with chronic pain. These responses include the nociceptive, affective-motivational and cognitive dimensions, which closely reflect human experience and potentially increase the predictive value of preclinical drug discovery. Our results also reveal the need to further investigate in future studies the emotional and cognitive alterations associated with chronic pain to optimize the future therapeutic approaches and minimize side-effects.

CONCLUSIONS

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

1) CB2R plays a crucial role in the control of the nociceptive manifestations of osteoarthritis induced in mice by the intra-articular injection of MIA, whereas CB1R is not critically involved in the control of these responses.
2) CB1R and CB2R do not participate in the development of the joint histological alterations promoted by osteoarthritis in mice.
3) CB1R and CB2R gene expression is down-regulated in the spinal cord of osteoarthritic mice as a potential compensatory mechanism in response to an increased spinal endocannabinoid signaling under this chronic pain state.
4) CB1R and CB2R are involved in the regulation of opioid receptor gene expression in the spinal cord of mice during osteoarthritis, suggesting a bidirectional interaction between the cannabinoid and opioid systems in the modulation of this chronic pain.
5) CB1R and CB2R have an opposite role in the development of the emotional alterations associated with osteoarthritis pain, as the alterations promoted by osteoarthritis in the anxiety-like behavior are enhanced in CB1KO and absent in CB2KO.
6) CB1R and CB2R do not participate in the development of the cognitive manifestations of osteoarthritis pain since the memory impairment induced by osteoarthritis is not modified in mice lacking CB1R or CB2R.

7) CB1R and CB2R have an opposite role in the regulation of CRH and GR gene expression in the PVN and mPFC of osteoarthritic mice. Therefore, the distinct role of these cannabinoid receptors in the control of the anxiety-like behavior could be related to a different regulation of HPA axis activity in response to chronic pain conditions.
8) The acute activation of CB1R and CB2R with the selective agonists ACEA and JWH133, respectively, reduces the nociceptive manifestations and the alterations in anxiety-like behavior associated with osteoarthritis in mice, whereas only CB1R activation improves the memory impairment.
9) The presence of osteoarthritis pain in mice is associated with a reduced density of mature-like dendritic spines and decreased protein expression of PSD95, NMDAR and AMPAR subunits in the mPFC. These changes in structural plasticity and glutamate receptor expression could have deleterious effects on synaptic functions in the mPFC and potentially contribute to the emotional and cognitive alterations observed during this pain state.
10) Osteoarthritis pain in mice is associated with increased 2-AG levels in the mPFC as a possible compensatory mechanism to maintain proper synaptic functions.
11) 2-AG plasmatic levels are increased in osteoarthritic mice and knee osteoarthritis patients. 2-AG plasmatic levels positively correlate with knee pain and depression scores, and negatively correlate with memory and health-related quality of life scores in humans.

12) CB1R and CB2R gene expression is increased in peripheral blood lymphocytes of osteoarthritis patients and positively correlates with depression and pain scores, respectively. This result provides further evidence of the differential role of CB1R and CB2R in the control of the emotional and pain osteoarthritis symptoms.
13) Similarly to the osteoarthritis pain model, the presence of allodynia and hyperalgesia in the mouse neuropathic pain model is associated with increased anxiety- and depressive-like behaviors, reduced memory functions, development of an anhedonic-like state and impaired responses in highly demanding operant tasks.
14) The chronic administration of pregabalin in mice exposed to neuropathic pain improves the nociceptive responses, anxiety and anhedonic-like states, memory deficits and the performance in the FR5 operant responding, but not the depressive-like symptoms.
15) The evaluation of different nociceptive, affective-motivational and cognitive manifestations of chronic pain is essential to increase the validity of preclinical pain research.

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ANNEX

ARTICLE #4

Involvement of the endocannabinoid system in osteoarthritis pain

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La Porta C, Bura SA, Negrete R, Maldonado R. Involvement of the endocannabinoid system in osteoarthritis pain. Eur J Neurosci. 2014 Feb;39(3):485-500. doi: 10.1111/ejn.12468

ARTICLE #5

Involvement of the opioid and cannabinoid systems in pain control: new insights from knockout studies

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