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

Roger Negrete ^a, María Salud García Gutiérrez ^{b,c}, Jorge Manzanares ^{b,c}, Rafael
Maldonado ^a

^a Laboratory of Neuropharmacology, Department of Experimental and Health Science,
Pompeu Fabra University (CEXS-UPF), Barcelona, Spain.

^b Instituto de Neurociencias, Universidad Miguel Hernández-CSIC, Alicante, Spain.

^c Red Temática de Investigación Cooperativa en Salud (RETICS), Red de Trastornos
Adictivos, Instituto de Salud Carlos III, MICINN and FEDER, Madrid, Spain.

Correspondence should be addressed to Rafael Maldonado:

Laboratori de Neurofarmacologia, Universitat Pompeu Fabra, Parc de Recerca
Biomedica de Barcelona (PRBB), C/ Dr. Aiguader, 88, 08003, Barcelona, Spain

Phone: +34933160824

Fax: +34933160901

E-mail address: rafael.maldonado@upf.edu

Abstract

Joint pain is a major clinical problem mainly associated to osteoarthritis, and characterized by articular cartilage degradation resulting in a complex chronic pain state that includes nociceptive, emotional and cognitive manifestations. Memory impairment, depressive- and anxiety-like symptoms have been reported to be associated with chronic pain, leading to a decrease of life quality. In this study, we evaluated the involvement of the endogenous dynorphin/kappa opioid receptor (KOR) system on the nociceptive, emotional, cognitive, neurochemical and epigenetic manifestations of joint pain. The murine model of monosodium iodoacetate (MIA) was used to induce joint pain in knockout mice for KOR (KOR-KO), prodynorphin (PDYN-KO) and their wild-type (WT) littermates. KOR-KO and PDYN-KO mice developed mechanical allodynia after intra-articular injection of MIA. This allodynia was significantly increased in both KOR-KO and PDYN-KO when compared to WT mice. Accordingly, both mutants showed increased microglial activation on the lumbar section of the spinal cord after MIA. The emotional responses were evaluated by measuring anxiety-like behaviour in the elevated plus maze and anhedonia as depressive-like behaviour, and cognitive alterations in the object recognition paradigm. Emotional and cognitive impairments after joint pain were differently modified in KOR-KO and PDYN-KO mice. Alterations of corticotropin-releasing factor (CRF) on the amygdala and hippocampus and down regulation of histone 3 acetylation on the amygdala suggest a possible mechanism to explain these emotional and cognitive manifestations. Our results reveal a specific involvement of the dynorphin/KOR system on joint pain manifestations that are usually associated to osteoarthritis.

1. Introduction

Joint pain is mainly related to osteoarthritis associated with articular cartilage degradation, structural and functional deterioration of synovial and presence of inflammation leading to a complex chronic pain disease (Thakur et al., 2014; Voscopoulos and Lema, 2010). Patients with osteoarthritis often experience emotional symptoms, such as anhedonia (Apkarian et al., 2004; Bushnell et al., 2013; Leknes and Tracey, 2008) or anxiety (Leknes and Tracey, 2008; Maldonado and Valverde, 2003) and memory dysfunctions (La Porta et al., 2014; Moriarty and Finn, 2014) producing an impairment of the quality of life (World Health Organization technical report series, 2003). Therapies to treat osteoarthritis are limited, having modest efficacy and significant side effects (Burnham and Dickenson, 2013; Malfait and Schnitzer, 2013; Rahman et al., 2009). Several findings support the interest of specific components of the endogenous opioid system (EOS) to develop novel therapeutic approaches for osteoarthritis (Milligan and Watkins, 2009; Ozaita et al., 2007). The EOS consists of three opioid receptors μ (MOR), δ (DOR) and κ (KOR), and three families of opioid peptides derivatives of proopiomelanocortin, proenkephalin and prodynorphin (PDYN) (Bodnar, 2015). The EOS is involved on pain transmission at peripheral and central level (Mantyh et al., 2002; Nadal et al., 2013; Ossipov et al., 2010; Przewłocki and Przewłocka, 2001). Tissue injury produced a constitutive activation of opioid receptors that repressed the nociceptive signalling (Corder et al., 2013). The EOS also plays an important role in the modulation of osteoarthritis. Indeed, an induction of the endogenous peripheral opioid system in a surgical model of osteoarthritis may delay the onset of pain in mice (Inglis et al., 2008). The presence of the EOS in bone and joint provides additional findings to explore novel pharmacological strategies to manage joint pain (La Porta et al., 2013; Spetea, 2013). The dynorphin/KOR system has recently

raised a particular interest in chronic and osteoarthritis pain. Indeed, a down-regulation of KOR has been revealed in fibroblast-like synoviocytes from patients with osteoarthritis, while KOR activation produces analgesia in other chronic pain manifestations, such as neuropathic pain in rodents (Fernihough et al., 2004; Shen et al., 2005). The dynorphin/KOR system has also been widely implicated in emotion and cognition (Bilkei-Gorzo et al., 2014; Carey et al., 2009; Carlezon et al., 2009). The activation of this system produces dysphoric effects (Hang et al., 2015; Negus et al., 2012), whereas the decrease of its activity leads to antidepressive- and anxiolytic-like effects (Lalanne et al., 2014; Van't Veer and Carlezon, 2013). Moreover, pharmacological blockade of KOR prevented impairments in memory performance, whereas its activation induced cognitive deficits in mice (Bilkei-Gorzo et al., 2014; Carey et al., 2009). The hippocampus (HIP) and amygdala (AMY) are closely related to nociceptive inputs (Mutso et al., 2012; Neugebauer and Li, 2003; Neugebauer, 2015; Veinante et al., 2013) and seem to participate in processing the emotional and cognitive manifestations of chronic pain. Corticotropin-releasing factor (CRF) is a neuropeptide that plays a crucial function in the control of emotional and cognitive responses in these brain structures (Holsboer, 2000; Ji et al., 2013; Reul and Holsboer, 2002)]. CRF is expressed in hippocampal neurons involved in neuroplasticity (Regev and Baram, 2014), and CRF-1 activation in the AMY contributes to synaptic transmission and pain responses (Ji et al., 2013). Changes in gene expression have been reported to be involved in the neurobiological substrate underlying chronic pain. Recent studies have identified a crucial role of epigenetic modulation of histones in these gene expression alterations involved in the nociceptive, emotional and cognitive manifestations of chronic pain (Descalzi et al., 2015; Liang et al., 2015; Nestler et al., 2015; Tran et al., 2015).

The aim of the present study was to evaluate the involvement of the dynorphin/KOR system in the behavioural, histological, emotional, cognitive, neurochemical and epigenetic manifestations induced of joint pain in mice. For this purpose, we have used a well-established model of joint pain (Guingamp et al., 1997) by the intra-articular injection of monosodium iodoacetate (MIA) that produces histological alterations similar to those found in clinical histopathology (Harvey and Dickenson, 2009; Kobayashi et al., 2003).

We have evaluated the expression of glial cells on the spinal cord, changes of CRF gene in the AMY and HIP, epigenetic modulation by histone 3 acetylation levels in AMY and the different joint pain manifestations in mice genetically deficient for the main components of the dynorphin / KOR system.

2. Materials and methods

2.1. Animal experimental conditions

KOR and PDYN constitutive knockout with C57Bl/6 genetic background and their WT littermates were used. Mice weighed 20-22 g at the beginning of the experiments. They were housed in groups of 2-4 with free access to water and food. The housing conditions were maintained at $22 \pm 1^\circ\text{C}$ and $55 \pm 10\%$ relative humidity in a controlled light/dark inverted cycle (light on between 8:00_{PM} and 8:00_{AM}). The experiments were performed during the initial 6 h of the dark period. 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 behavioural, histological, and neurochemical experiments were performed under blinded conditions.

2.2. Intra-articular administration of MIA

Osteoarthritis pain was induced in mice briefly anaesthetized with isoflurane by the intra-articular injection of MIA (Sigma, UK) into the knee joint. The knee joint was shaved and flexed at 90° angle. Ten μl of 10 mg/ml MIA in sterile saline (0.9%) were administered through the infrapatellar ligament into the joint space of the right (ipsilateral) knee with a 30-gauge needle. Control mice received an intra-articular injection of saline (10 μl of sterile saline, 0.9%).

2.3. Nociceptive behavioural test

Allodynia to mechanical stimulus was used as outcome measure of joint pain by measuring the hind paw withdrawal response to von Frey filaments stimulation (Barrot, 2012; Chaplan et al., 1994). Briefly, animals were placed in Plexiglas boxes (20 cm high, 9 cm diameter) positioned on a grid surface through which the von Frey filaments (North Coast Medical, USA) were applied by using the up-down paradigm, as previously reported (Chaplan et al., 1994; Hargreaves et al., 1988). The mechanical threshold of response was then calculated by the up-down Excel program generously provided by Dr A. Basbaum (University of California, San Francisco, USA). Animals were habituated for 1 h before testing to allow an appropriate behavioural immobility. Clear paw withdrawal, shaking, or licking was considered as nociceptive response. Both ipsilateral and contralateral hind paws were tested.

2.4. Depressive-like behaviour

Anhedonic state associated with osteoarthritis chronic pain was evaluated using a new food and drink monitoring system recently validated in our laboratory (PHECOMP cages) (Bura et al., 2010), in order to determine the preference for a palatable drink with an extremely high sensitivity (less than 0.02 g for both food and drink). In this study, two different kinds of drinking solutions were used: water and 2 % sucrose solution. The anhedonic state related to a depressive-like behaviour was evaluated by measuring the preference for the sucrose solution.

2.5. Anxiety-like behaviour

The elevated plus maze (EPM) was used to evaluate anxiety-like behaviour. A black Plexiglas apparatus with four arms (29 cm long x 5 cm wide), with two open and two closed set in cross from a neutral central square (5 cm x 5 cm) elevated 40 cm above the floor. Test sessions of 5 min were performed, and the time spent in the open arms compared to time spent in the closed arms provides an estimation of the anxiety-like behaviour (Busquets-Garcia et al., 2011).

2.6. Cognitive-like behaviour

Object recognition memory (ORM) was assessed in a V-maze, consisting in a black Plexiglas maze with two corridors (30 cm long $\times$ 4.5 cm wide) set in V with a 90° angle, and 15 cm high walls (Puighermanal et al., 2009). On day 1, mice were habituated for 9 min to the maze. On the second day, mice were placed back in the maze for 9 min where two identical objects were presented. The time that the animals spent exploring each object was recorded. 24 h later, mice were placed again in the maze for 9 min. Here, one of the familiar objects was changed for a novel object, and the total exploration time spent for each object (novel and familiar) was measured. Exploration of the object was defined as the orientation of the nose to the object at a distance < 2 cm. A discrimination index was calculated as the difference between times spent exploring the novel and familiar object divided by the total exploration time of the two objects. High discrimination index is considered to reflect greater memory retention for the familiar object. The total exploration time was also recorded as an indicator of the locomotor activity.

2.7. Experimental protocol

Mice were habituated for 1 h to the environment of the nociceptive test during 7 days. For the anhedonic experiment, mice were trained to a 24 h session every second day for 1 week in the monitoring boxes in order to be familiarized to the new environment. After each training session, mice were replaced again to their home cages. After this habituation period, baseline values for von Frey filaments and drink intake were measured. One day after the baseline responses, mice were administered with MIA or saline, followed by 24 h sessions in the monitoring boxes every 5 days, during 30 days for the anhedonia experiment. Mice were tested in the von Frey paradigm on days 1, 3, 9, 13, 18, 22 and 27 after MIA or saline administration. A different set of mice were tested in the ORM and EPM test on day 30 after MIA or saline administration. Animals were sacrificed at day 30 after osteoarthritis induction to perform the histological and neurochemical experiments (Fig. 1).

2.8. Histology

2.8.1. Knee joint isolation

At day 30 after osteoarthritis induction, both MIA and saline control mice were deeply anesthetized with ketamine/xylazine (50/10 mg/kg) and intracardially perfused with 4% paraformaldehyde. The ipsilateral and contralateral knee joints were removed, postfixed overnight in 4% paraformaldehyde, and then cryopreserved in a 30% sucrose solution at 4°C.

2.8.2. Histological preparation

The fixed knee joints were decalcified in Osteomoll (Merck, Germany) for 6 to 7 h and left overnight in 30% sucrose solution. The joints were subsequently embedded in

gelatine (7.5%) and frozen in cold 2-methyl-butane. Coronal 16- to 18- μ m sections were cut in a cryostat from the frontal plane toward the back of each joint and mounted on gelatinized slides (6 to 7 slides with 10 sections each). All the serial sections were stained with Safranin O–Fast Green staining protocol. Briefly, after hydrating sections with decreasing concentrations of ethanol, they were stained with haematoxylin (Merck, Germany) and subsequently with 0.002% Fast Green (Sigma, Spain) and 0.2% Safranin O (Merck, Germany) solutions. The sections were finally dehydrated and cleared with increasing ethanol concentrations and xylene, then mounted with Eukitt (O. Kindler, Germany) and a covering glass. All the stained sections were viewed at 10X objective with a Leica DMR microscope equipped with a Leica DFC 300 FX digital camera.

2.8.3. Histological scoring

A semiquantitative scoring system for murine histopathology, the OARSI score (Glasson et al., 2010) was applied and adapted to our experimental conditions. All 4 quadrants of the knee joint were evaluated: medial tibial plateau, medial femoral condyle, lateral tibial plateau, and lateral femoral condyle. The scores were expressed as the summed histological score. The summed score represents the additive scores for each quadrant of the joint on each section through the joint of each animal. The average summed score for each experimental group was calculated. The same observer scored all the histological changes and was blinded to the specimen samples.

2.9. Immunoblot analysis

Four weeks after osteoarthritis induction, both MIA and saline control mice were killed by cervical dislocation. For the analysis of glial cells expression, the lumbar (L5-L6) region of the spinal cord was isolated and separated into ipsilateral and contralateral

parts with respect the side of MIA or saline injection. For the epigenetic study, the histone 3 (H3) acetylation levels were measured in the AMY. Tissues were fresh frozen and stored rapidly at -80°C until use. Then, frozen tissues were processed as previously described (Puighermanal et al., 2009). For western blot, the following antibodies were used: anti GAPDH (mouse, 1:5000) from Santa Cruz Biotechnology, anti-IBA1 (rabbit, 1:500) from Wako Pure Chemical Industries; anti-glial fibrillary acidic protein (GFAP) (rabbit, 1:500) from Cell Signal, and anti-Acetyl-Histone H3 Lys9 (H3K9ac) (rabbit, 1:1000) from Abcam. Blots containing equal amounts of tissue protein samples (40 µg) were probed with different primary antibodies. Both antibodies were visualized by enhanced chemiluminescence detection (SuperSignal West Femto; Thermo Fisher Scientific). 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.

2.10. CRF gene expression analyses by real-time PCR

Gene expression of CRF in HIP and AMY was assessed by real-time PCR in PDYN-KO and KOR-KO mice and their WT littermates treated with MIA or saline. Total RNA was isolated from frozen (-80 °C) HIP and AMY regions with Tri Reagent (Ambion) and subsequently retrotranscribed to cDNA. Quantitative analysis of gene expression was measured with the “Mm01293920_s1” TaqMan® Gene Expression assay for CRF (Life Technologies, Madrid, Spain). Real time PCR experiments were performed on the StepOne Plus system (Life Technologies, Madrid, Spain) and the reference gene used was Rn18S rRNA, detected with TaqMan® ribosomal RNA control reagent

“Mm03928990_g1”. Data for target gene was normalized to the endogenous reference gene, and the fold change in target gene mRNA abundance was determined by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.11. Statistical analysis

Behavioural, histological and neurochemical data obtained from KOR-KO, PDYN-KO and their WT littermates were compared by two-way analysis of variance (ANOVA) between groups (injection and genotype as factors of variance), followed by post hoc analysis (Fisher's least significant difference LSD test) when appropriate. Nociceptive data were analysed separately for each experimental day for the ipsilateral and contralateral hind paws. Data passed the Shapiro–Wilk test for normality, and parametric statistics were consequently applied. The statistical analyses were performed using the STATISTICA data analysis software system (StatSoft, Tulsa, OK, USA, 2001). The differences between means were considered statistically significant when the P value was below .05.

3. Results

3.1. Development of mechanical allodynia after MIA in KOR-KO mice

Mechanical allodynia after MIA administration was evaluated using the von Frey stimulation paradigm. Baselines values were similar in all the groups before the intra-articular injection of MIA or saline in both ipsilateral and contralateral paw. In the saline control groups, no differences on the nociceptive responses were observed although significant differences ($P < 0.05$; Fisher's LSD test) were shown at day 1 after saline administration, probably due to the intra-articular injection procedure. After induction of osteoarthritis by MIA injection, a significant decrease of the withdrawal threshold was revealed in the ipsilateral paw of both genotypes, but not in the contralateral side. Mechanical allodynia was shown from the first day after MIA administration ($P < 0.001$; Fisher's LSD test vs saline injection) and was maintained during the entire experimental period (day 1, $P < 0.001$; day 3, $P < 0.001$; day 9, $P < 0.001$; day 13, $P < 0.001$; day 18, $P < 0.001$; day 22, $P < 0.001$; day 27, $P < 0.001$) (Fisher's LSD test vs saline injection). Interestingly, KOR-KO mice developed an enhanced mechanical allodynia compared to the WT mice at different time points of the experiment (day 3, $P < 0.01$; day 13, $P < 0.01$; day 22, $P < 0.05$) (Fisher's LSD test vs WT MIA) (Fig. 2A and C).

3.2. Development of mechanical allodynia after MIA in PDYN-KO mice

Baselines values obtained by von Frey stimulation were similar in all the groups before the MIA or saline administration in both ipsilateral and contralateral paw. In the saline groups, no differences on the nociceptive responses were observed although significant differences ($P < 0.05$; Fisher's LSD test) were shown after 1 day of saline administration, probably due to the intra-articular injection procedure. MIA injection significantly

decreased the threshold for evoking hind paw withdrawal on the ipsilateral paw of both genotypes, but not in the contralateral side. Mechanical allodynia appeared from the first day after MIA administration ($P < 0.001$; Fisher's LSD test vs saline injection) and was sustained during the whole experimental period (day 1, $P < 0.001$; day 3, $P < 0.001$; day 9, $P < 0.001$; day 13, $P < 0.001$; day 18, $P < 0.001$; day 22, $P < 0.001$; day 27, $P < 0.001$) (Fisher's LSD test vs saline injection). PDYN-KO mice developed an enhanced mechanical allodynia compared to the WT mice during the whole experiment (day 1, $P < 0.001$; day 3, $P < 0.001$; day 9, $P < 0.001$; day 13, $P < 0.001$; day 18, $P < 0.01$; day 22, $P < 0.001$; day 27, $P < 0.001$) (Fisher's LSD test vs WT MIA) (Fig. 2B and D).

3.3. Histopathological changes after MIA in KOR-KO and PDYN-KO mice

Serial images representing the histological sections of the knee joints of KOR-KO and PDYN-KO mice and their WT littermates stained with Safranin O-Fast Green are shown in Fig. 3A. The histopathological changes were determined by the OARSI score (Glasson et al., 2010) that was analysed for all the quadrants of each knee joint and was expressed as summed score combined for the entire knee joint (Fig. 3B). This scoring system evaluates the cartilage alterations excluding other osteoarthritis consequences, such as bone and synovial alterations. A clear loss of proteoglycans and chondrocytes degeneration was shown in this model of osteoarthritis in the ipsilateral side, as revealed by regions with no living cells in the cartilage, without major changes in subchondral bone, Accordingly with previous histopathological studies with this model (La Porta et al., 2014, 2013). No histopathological alterations were produced after saline administration in WT, KOR-KO and PDYN-KO mice. However, the intra-articular administration of MIA induced significant histological changes in the ipsilateral knee joint of KOR-KO and PDYN-KO, and their WT littermates ($P < 0.001$). No significant

differences were observed when compared WT and the different groups of KO mice after MIA. No changes were observed in the contralateral side of WT, KOR-KO and PDYN-KO mice after the induction of osteoarthritis pain.

3.4. Spinal glial cell expression after MIA in KOR-KO and PDYN-KO mice

According to pain behaviour, the development of osteoarthritis by MIA administration induced an increase of microglia expression on the ipsilateral side of the lumbar spinal cord in WT, KOR-KO and PDYN-KO mice. Indeed, the western blot performed at day 30 after osteoarthritis induction revealed an increased expression of Iba-1 on the ipsilateral side of the lumbar spinal cord of KOR-KO ($P < 0.01$; Fisher's LSD test vs saline injection) and WT littermates when compared to the saline treated mice ($P < 0.05$; Fisher's LSD test vs saline injection) (Fig. 4A). Similarly, an increased expression of Iba-1 on the ipsilateral side of the lumbar spinal cord of PDYN-KO mice ($P < 0.05$; Fisher's LSD test vs saline injection) and WT littermates ($P < 0.05$; Fisher's LSD test vs saline injection) was revealed when compared to the saline treated mice (Fig. 4B). No differences between genotypes were observed in any of the experimental conditions revealing a similar increased expression of microglia after MIA induction in all the genotypes. In contrast, no significant changes were observed in the astrocyte expression under these experimental conditions. Indeed, western blot assay for the astroglial expression performed at day 30 after MIA administration did not show significant differences on the GFAP expression on the lumbar section of the spinal cord on KOR-KO, PDYN-KO and their WT littermates, when compared with the saline treated mice. (Fig. 4C and D).

3.5. Anhedonic-like state associated to osteoarthritis pain in KOR-KO and PDYN-KO mice

Anhedonia after the induction of osteoarthritis was evaluated by measuring the preference for a sucrose solution in PHECOMP cages (Bura et al., 2010). For the KOR-KO mice, two-way ANOVA for the percentage of sucrose preference revealed a significant effect of genotype ($F_{(8,12)}=3.441$; $P<0.05$), treatment ($F_{(8,12)}=8.750$; $P<0.01$) and interaction between these factors ($F_{(8,12)}=3.921$; $P<0.05$). Subsequent *post hoc* analysis indicated that the basal sucrose preference before MIA injection was similar in all groups. Saline administration did not modify the preference for sucrose. However, osteoarthritis pain developed an anhedonic-like state in KOR-KO and their WT littermates, at different time points (day 1, $P<0.01$; day 5, $P<0.05$; day 10, $P<0.01$; day 15, $P<0.01$; day 20, $P<0.05$; day 25, $P<0.01$; day 30, $P<0.01$). (Fisher's LSD test vs saline), as revealed by a significant decrease in sucrose preference (Fig 5A). This anhedonic-like state induced by MIA administration was increased in KOR-KO, when compared to their WT mice at different experimental days (day 1, $P<0.05$; day 10, $P<0.05$; day 15, $P<0.05$). (Fisher's LSD test vs WT).

For the PDYN-KO, two-way ANOVA revealed a significant effect of treatment on the percentage of sucrose preference ($F_{(8,24)}=3.878$; $P<0.01$). Subsequent *post hoc* analysis indicated that the basal sucrose preference before MIA was similar in all groups. Saline administration did not modify the preference for sucrose. However, osteoarthritis pain developed an anhedonic-like state in PDYN-KO and their WT littermates, at different time points (day 1, $P<0.05$; day 5, $P<0.01$; day 10, $P<0.05$; day 20, $P<0.05$; day 30, $P<0.05$). (Fisher's LSD test vs saline), as revealed by a significant decrease in the preference for sucrose (Fig 5B). Moreover, PDYN-KO developed a similar anhedonic-like state than their WT littermates.

3.6. Cognitive manifestations associated to osteoarthritis pain in KOR-KO and PDYN-KO mice

For the KOR-KO mice, two-way ANOVA revealed a significant effect of MIA ($F_{(1,26)}=15.53$; $P<0.01$), genotype ($F_{(1,26)}=2.53$; $P<0.05$) and interaction between these factors ($F_{(1,26)}=1.27$; $P<0.05$) in the ORM discrimination index four weeks after osteoarthritis (Fig. 6A). Subsequent post hoc analysis indicated that MIA significantly impaired memory, as revealed by a reduction in the ORM discrimination index of KOR-KO mice ($P<0.05$; Fisher's LSD test vs saline injection) and WT littermates ($P<0.01$; Fisher's LSD test vs saline injection). Interestingly, a significant difference was revealed between genotypes after MIA administration ($P<0.05$; Fisher's LSD test vs WT), suggesting a protective role of KOR blockade on these cognitive manifestations.

For PDYN-KO mice, two-way ANOVA showed a significant effect of MIA in the discrimination index four weeks after induction of osteoarthritis ($F_{(1,20)}=26.61$; $P<0.001$) and genotype ($F_{(1,20)}=1.42$; $P<0.05$) (Fig. 6C). Subsequent post hoc analysis indicated that osteoarthritis significantly impaired memory, as revealed by a reduction in the ORM discrimination index of PDYN-KO mice receiving MIA ($P<0.01$; Fisher's LSD test vs saline injection) and WT littermates ($P<0.001$; Fisher's LSD test vs saline injection).

MIA injection did not modify locomotor activity in any of the experimental groups, as no differences were shown by two-way ANOVA in the total exploration time in the ORM test (Fig. 6B and D).

3.7. Anxiety-like behaviour associated to osteoarthritis pain in KOR-KO and PDYN-KO mice

For KOR-KO mice, two-way ANOVA revealed a significant effect of MIA ($F_{(1,34)}=94.61$; $P<0.001$), genotype ($F_{(1,34)}=14.50$; $P<0.01$), and interaction between both factors ($F_{(1,34)}=4.39$; $P<0.05$) on the percentage of time spent in the open arms four weeks after osteoarthritis induction. Subsequent post hoc analysis indicated that osteoarthritis significantly increases anxiety, as revealed by a reduction in the percentage of time in the open arms in all the experimental groups receiving MIA compared to saline injected mice ($P<0.001$; Fisher's LSD test vs saline injection). Interestingly, a significant difference was also revealed between genotypes after MIA administration ($P<0.01$; Fisher's LSD test vs WT) (Fig. 7A).

Two-way ANOVA for the PDYN-KO mice also revealed a significant effect of MIA administration ($F_{(1,28)}=106.05$; $P<0.001$), genotype ($F_{(1,28)}=6.75$; $P<0.05$) and interaction between both factors ($F_{(1,28)}=2.34$; $P<0.05$). Subsequent post hoc analysis revealed a significant anxiety after osteoarthritis when compared with their WT littermates in both experimental groups ($P<0.001$; Fisher's LSD test vs saline). Interestingly, a significant difference between genotypes was also revealed after MIA administration ($P<0.05$; Fisher's LSD test vs WT) (Fig. 7C). These results suggest that the blockade of the dynorphin/KOR system is reducing the anxiogenic-like responses associated to osteoarthritis pain.

MIA injection did not modify locomotor activity in any of the experimental groups, as no differences were shown by two-way ANOVA in the total number of entries shown in the EPM (Fig. 7B and D).

3.8. CRF gene expression in the AMY and HIP after MIA in KOR-KO and PDYN-KO mice

In the HIP, two-way ANOVA with repeated measures revealed a significant effect of genotype ($F_{(1,29)}=2.646$; $P<0.05$), MIA ($F_{(1,29)}=8.421$; $P<0.01$) and interaction between these factors ($F_{(1,29)}=10.529$; $P<0.01$) (Fig. 8A and C). In the AMY, two-way ANOVA with repeated measures revealed a significant effect on genotype ($F_{(1,26)}=5.851$; $P<0.05$), MIA ($F_{(1,26)}=7.699$; $P<0.05$) and interaction between both factors ($F_{(1,26)}=7.365$; $P<0.05$) (Fig 8B and D). Subsequent post hoc analysis ($P<0.05$; Fisher's LSD test vs saline injection) revealed that control PDYN-KO and KOR-KO mice receiving intra-articular saline injection showed a reduced CRF gene expression in the HIP and AMY compared with their control WT littermates. Interestingly, MIA failed to produce alterations in the CRF gene expression of PDYN-KO and KOR-KO at any of the regions analysed. In contrast, MIA significantly reduced CRF gene expression in the HIP and AMY of WT mice.

3.9. Histone 3 (Lys 9) acetylation expression in the AMY in KOR-KO and PDYN-KO mice

For KOR-KO mice, two-way ANOVA revealed a significant effect of genotype ($F_{(1,18)}=2.871$; $P<0.05$) and MIA ($F_{(1,18)}=8.935$; $P<0.01$). Subsequent post hoc analysis revealed a reduction in H3K9ac in WT mice after MIA administration when compared to saline controls ($P<0.01$; Fisher's LSD test vs saline injection). However, mice lacking KOR did not show a significant reduction of H3K9 acetylating levels after MIA administration compared to saline treated mice. A significant difference between genotypes was also revealed after MIA administration ($P<0.05$; Fisher's LSD test vs WT) (Fig. 9A).

For the PDYN-KO mice, two-way ANOVA revealed a significant effect of MIA ($F_{(1,8)}=14.848$; $P<0.05$) in the H3K9ac expression. Subsequent post hoc analysis revealed a significant decrease of H3K9 acetylation levels after MIA administration compared to saline injected mice in both genotypes ($P < 0.05$; Fisher's LSD test vs saline) (Fig. 9B).

4. Discussion

In this study, we used genetically modified mice to elucidate the involvement of the dynorphin/KOR system in the behavioural, histopathological and neurochemical alterations associated with osteoarthritis pain in mice. Mechanical allodynia produced by MIA was enhanced in mice lacking KOR or PDYN gene. In contrast with the nociceptive manifestations induced by osteoarthritis, the increased microglial expression on the lumbar section of the spinal cord after MIA administration was similar in KOR-KO, PDYN-KO and WT littermates. Moreover, anhedonic- and anxiolytic-like states were revealed after MIA administration. These emotional alterations were associated with alterations in the CRF gene expression in both AMY and HIP. Mice deficient in dynorphin/KOR exhibited an attenuation of these anxiogenic manifestations of osteoarthritis chronic pain. In addition, osteoarthritis impaired memory in WT mice and these cognitive responses were not modified in PDYN-KO mice, whereas this cognitive deficit was attenuated in KOR-KO mice.

Several studies have reported the involvement of the dynorphin/KOR system in the development of other chronic pain conditions. Thus, inhibiting KOR, either through KOR antagonists or by KOR-KO mice, enhanced mechanical allodynia in different models of neuropathic pain (Cahill et al., 2014; Obara et al., 2003), and a KOR down regulation has been reported in fibroblast-like synoviocytes of patients with osteoarthritis (Shen et al., 2005).

Our results correlate with previous findings reporting a differential activation of spinal glial cells in rodents in this MIA model of osteoarthritis (Ogbonna et al., 2013; Sagar et al., 2011) and other neuropathic and inflammatory chronic pain models (Mika et al., 2011; Milligan and Watkins, 2009; Obara et al., 2009). Accordingly, an increase in the microglial expression was found in the lumbar section of the spinal cord four weeks

after the induction of osteoarthritis chronic pain, whereas no significant changes in the spinal astrocytes activation were revealed, as previously shown in other chronic pain models in rodents (Mika et al., 2009; Sagar et al., 2011). However, no significant differences were revealed in the microglia expression promoted by osteoarthritis pain in the different genotypes, in spite of the increased nociceptive manifestations revealed in both KOR-KO and PDYN-KO mice. These results suggest that the dynorphin/KOR system is not necessary for the microglial activation promoted by osteoarthritis in this experimental model.

The dynorphin/KOR system is implicated in the development of emotional dysfunctions, such as anxiety and anhedonia (Bruchas et al., 2010; Carlezon et al., 2009; Lalanne et al., 2014; Van't Veer and Carlezon, 2013). Anhedonia, defined as the inability to feel pleasure, is a core symptom of depression (Bura et al., 2010). In our study, KOR-KO, PDYN-KO and their WT littermates developed an anhedonic-like state from the first day after MIA administration, and this emotional response was maintained during the whole experimental period. These decreases in sucrose preference were selectively due to the reduced sucrose intake, and not to changes in water intake, suggesting an anhedonic-like state and not an impairment of general fluid intake. Our results show a more pronounced decrease in the preference for sucrose during osteoarthritis in KOR-KO mice when compared to WT littermates. This increase in the anhedonic-like state in mice lacking dynorphin/KOR system correlates with the increased mechanical allodynia in these mutant mice. Accordingly, a similar anhedonic-like state has been reported after neuropathic pain in mice that disappeared after the partial reversion of nociceptive manifestations by chronic pregabalin treatment (Dickenson and Ghandehari, 2007; La Porta et al., 2016). Therefore, the facilitation of the nociceptive manifestation in the absence of these dynorphin/KOR system

components could produce the enhancement of these emotional chronic pain symptoms. Human and rodent studies have reported an involvement of the dynorphin/KOR system in mood control, although its specific role has not been yet fully clarified (Bilkei-Gorzo et al., 2014; Carey et al., 2009; Lutz and Kieffer, 2013). Antidepressive-like effects have been reported in different animal models of depression after KOR pharmacological blockade by nor-binaltorphimine (Aldrich and McLaughlin, 2009; Knoll and Carlezon, 2010; Lalanne et al., 2014) or activation by Salvinorin A (Carlezon et al., 2006; Harden et al., 2012). In contrast, prodepressive-like responses have also been found after KOR activation in the mouse forced swimming test (Bruchas et al., 2010).

Osteoarthritis pain was also associated to anxiogenic-like responses in the WT mice. In contrast with the anhedonic responses, the genetic suppression of KOR or PDYN decreased this anxiety-like responses promoted by osteoarthritis. A downregulation of the CRF gene was found under basal conditions in both AMY and HIP in these mice lacking KOR and PDYN gene, suggesting the crucial role of these genes in the control of anxiety-like responses. Interestingly, osteoarthritis induced by MIA reduced CRF gene expression in both AMY and HIP in WT mice, which might represent an adaptive modulation of the hypothalamic-pituitary-adrenal (HPA) axis to limit the stress responses after chronic pain. MIA administration did not alter the levels of CRF expression in both KOR-KO and PDYN-KO mice compared with saline treated mice, correlating with the important role of this system in the control of anxiolytic-like responses. The activation of other areas, such as prefrontal cortex (PFC) and nucleus accumbens (NAc), also produces antinociceptive effects, and may regulate the emotional manifestations of pain. In agreement, recent human studies indicate altered connections between PFC and NAc in chronic pain that affected the corticostriatal circuit (Lee et al., 2015).

A recent work from our laboratory (La Porta et al., 2015) also showed a downregulation of CRF expression in the paraventricular nucleus (PVN) of hypothalamus and the PFC of mice with osteoarthritis pain. These alterations may represent an adaptive modification to limit HPA axis activity under chronic pain and may underlie the absence of HPA neuroendocrine alterations previously reported (Khoromi et al., 2006). Furthermore, CRF signaling in the limbic system seems to contribute to nociceptive, affective, and cognitive alterations in chronic pain in rodents that were not associated with HPA axis dysfunctions (Ji et al., 2013; Ulrich-Lai et al., 2006). Therefore, the reduced anxiety in PDYN- and KOR-KO mice exposed to joint pain revealed in our study may produce a compensatory suppression of HPA activity leading to a CRF downregulation in HIP and AMY, in agreement with other previous studies (La Porta et al., 2015). Moreover, anxiolytic-like effects have been reported after pharmacological blockade of the dynorphin/KOR system in different animal models (Aldrich and McLaughlin, 2009; Hang et al., 2015).

Osteoarthritis pain was associated to impaired memory function, as previously reported (La Porta et al., 2014). This cognitive impairment was significantly reduced in mice lacking KOR, suggesting that the blockade of the dynorphin/KOR system plays a protective role in the cognitive manifestations associated to osteoarthritis pain. Accordingly, previous studies have reported that the cognitive impairments produced by stress were prevented by pharmacological antagonism of KOR or prodynorphin gene disruption (Carey et al., 2009).

A growing number of studies implicate modifications in gene expression in the pathophysiological substrate underlying the development of chronic pain. Accordingly, epigenetic alterations that are important for the control of gene expression have been

reported after exposure to chronic pain (Buchheit et al., 2012; Descalzi et al., 2015; Liang et al., 2015). Thus, the expression of histone deacetylase 4 (HDAC4) was revealed in the osteoarthritis cartilage, whereas it was barely detected in the normal cartilage, and a reduction of histone H3 acetylation in the spinal cord, and an upregulation of histone deacetylase 1 (HDAC1) expression were shown after spinal nerve ligation in rats (Schroeder et al., 2007; Tran et al., 2015). Epigenetic modulations also contribute to long-term regulation of CRF expression (Tran et al., 2015). In our study, a downregulation on H3K9ac was found in the AMY four weeks after MIA administration. These modifications induced by chronic pain, as well as those previously reported (Descalzi et al., 2015), lead to a more condensed chromatin structure, which prevent gene transcription that finally could contribute to nociceptive, emotional and cognitive manifestations of chronic pain (Bagot et al., 2014; Liang et al., 2015). These modifications in H3 acetylation may lead to adaptations that prevent the inhibitory pain control by MOR, the main pharmacological target for opioids in chronic pain (Descalzi et al., 2015). The ablation of PDYN did not significantly modify H3K9 acetylation levels in the AMY after osteoarthritis compared to WT mice. However, these changes in H3K9 acetylation were significantly attenuated in KOR-KO mice after MIA administration. These results provide a novel epigenetic mechanism that could be involved in the modulation of the emotional and cognitive manifestations of chronic joint pain by KOR.

Our study reveals that the inactivation of the dynorphin/KOR system modifies the nociceptive, emotional and cognitive manifestations of chronic joint pain. The present study provides important correlations of behavioural, histological and neurochemical mechanisms demonstrating for the first time the potential involvement of the

dynorphin/KOR system as an interesting pharmacological target for the management of the different manifestations of chronic joint pain.

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Author contributions

R.N. participated in experimental design, performed the experiments and contributed to write the article. M.S.G.G. performed a part of the experiments. R.M. conceived the study, participated in experimental design, supervised and wrote the article. All authors discussed the results, commented and approved the final version of the article.

Conflicts of interest

The authors report no conflicts of interest.

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Figure legends

Fig. 1. Experimental protocol to evaluate the nociceptive, emotional and cognitive manifestations of joint pain in mice. MIA, monosodium iodoacetate. VF, von Frey test. ORM, object recognition memory. EPM, elevated plus maze. Hab, habituation. Tr, Training.

Fig. 2. Development of mechanical allodynia in KOR-KO and PDYN-KO mice exposed to joint pain. Mice were tested in the ipsilateral (A, B) and contralateral (C, D) paws to evaluate mechanical allodynia under basal conditions and on days 1, 3, 9, 13, 18, 22 and 27 after MIA or saline administration. Mechanical allodynia evaluated by the von Frey model on the ipsilateral (A) and contralateral paw of KOR-KO mice (C). Mechanical allodynia evaluated by the von Frey model on the ipsilateral (B) and contralateral (D) paw of PDYN-KO mice. The mechanical threshold (g) under von Frey filament stimulation are expressed as mean $\pm$ SEM. (n=12 per group). ★ $P<0.05$, ★★ $P<0.01$, ★★★ $P<0.001$ vs. saline treatment (Fisher's LSD test). ☆ $P<0.05$, ☆☆ $P<0.01$, ☆☆☆ $P<0.001$, vs. WT (Fisher's LSD test).

Fig. 3. Histopathological changes in KOR-KO and PDYN-KO after MIA intra-articular injection. (A) Representative histological knee joint sections (medial side) stained with Safranin O and Fast Green. Both ipsilateral and contralateral joints of KOR-KO and PDYN-KO and their WT littermates 30 days after the intra-articular injection of saline or MIA are represented. (B) Quantification of articular cartilage alterations using the OARSI scoring system for KOR-KO, PDYN-KO and their WT littermates. Data are expressed as the mean of the summed score for each knee joint $\pm$ SEM (n=5 animals per group). ★★★ $P<0.001$ vs. saline treatment (Fisher's LSD test).

Fig. 4. Spinal glial expression in KOR-KO and PDYN-KO mice during joint pain.

Immunoblot detection and quantification of Iba-1 for the microglial expression in the ipsilateral lumbar section of spinal cord of KOR-KO and PDYN-KO mice 4 weeks after MIA or saline intra-articular injection (A). Immunoblot detection and quantification of GFAP for the astroglial expression in the ipsilateral lumbar section of spinal cord of KOR-KO and PDYN-KO mice 4 weeks after MIA or saline intra-articular injection (B). Data are expressed as mean $\pm$ SEM. (n=5-6 per group). ★ $P<0.05$, ★★ $P<0.01$ vs saline injection (Fisher's LSD test).

Fig. 5. Development of anhedonic-like state in KOR-KO and PDYN-KO mice exposed to joint pain.

The percentage of sucrose preference during 24 hour sessions was evaluated in the monitoring system (Phecomp boxes) before and after MIA or saline administration every fifth day during 30 days. (A) Anhedonic state evaluated in KOR-KO mice and their WT littermates (B) Anhedonic state evaluated in PDYN-KO mice and their WT littermates. Data are expressed as mean $\pm$ SEM (n=12 per group). ★ $P<0.05$, ★★ $P<0.01$ vs. saline treated mice (Fisher's LSD test). ☆ $P<0.05$ vs. WT (Fisher's LSD test).

Fig. 6. Evaluation of the cognitive performance in KOR-KO and PDYN-KO mice exposed to joint pain.

The ORM discrimination index (A, C) was evaluated after four weeks of MIA or saline injection. The total exploration time (B, D) was also evaluated as a control for locomotor activity. Data are expressed as mean $\pm$ SEM (n=6-8 per group). ★ $P<0.05$, ★★ $P<0.01$, ★★★ $P<0.001$ vs. saline treatment (Fisher's LSD test). ☆ $P<0.05$, vs. WT (Fisher's LSD test).

Fig. 7. Evaluation of the anxiety-like behaviour in KOR-KO and PDYN-KO mice exposed to joint pain. The percentage of entries in the EPM open arms (A,C) was evaluated four weeks after MIA or saline injection. The total number of entries (B, D) was also evaluated as a control for locomotor activity. Data are expressed as mean $\pm$ SEM (n=8 per group). ★★ ★ P<0.001 vs. saline treatment (Fisher's LSD test). ☆ P<0.05, ☆ ☆ P<0.01, vs. WT (Fisher's LSD test).

Fig. 8. Relative corticotropin-releasing factor gene expression in KOR-KO and PDYN-KO mice exposed to osteoarthritis pain. CRF gene expression analysis in the amygdala and hippocampus of WT, KOR-KO and PDYN-KO mice four weeks after MIA or saline administration. Data are expressed as mean $\pm$ SEM. (n=5-6 per group). ★ P<0.05, ★ ★ P<0.01, ★ ★ ★ P<0.001 vs. saline treatment (Fisher's LSD test). ☆ P<0.05, ☆ ☆ P<0.01, ☆ ☆ ☆ P<0.001, vs. WT (Fisher's LSD test). AMY, amygdala. HIP, hippocampus.

Fig. 9. Histone 3 (Lys9) acetylation levels in KOR-KO and PDYN-KO mice during joint pain. Immunoblot detection and quantification of H3K9ac expression in the amygdala of WT and KOR-KO mice after MIA or saline intra-articular injection (A). Immunoblot detection and quantification of H3K9ac levels in the amygdala of WT and PDYN-KO mice after MIA or saline intra-articular injection (B). Data are expressed as mean $\pm$ SEM. (n=5-6 per group). ★ P<0.05, ★ ★ P<0.01 vs saline treatment (Fisher's LSD test). ☆ P<0.05 vs. WT (Fisher's LSD test).

Table 1. Cognitive manifestations in KOR-KO and PDYN-KO mice after four weeks of MIA administration

Two-way ANOVA				
	KOR-KO		PDYN-KO	
	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Genotype	$F_{(1,26)} = 2.53$	$P < 0.05$	$F_{(1,20)} = 1.42$	$P < 0.05$
Lesion	$F_{(1,26)} = 15.53$	$P < 0.01$	$F_{(1,20)} = 26.61$	$P < 0.001$
Genotype x Lesion	$F_{(1,26)} = 1.27$	$P < 0.05$	$F_{(1,20)} = 0.53$	<i>n.s.</i>

Two-way ANOVA with group as between-subjects factor. *n.s.*: non-significant

Table 2. Anxiety-like manifestations in KOR-KO and PDYN-KO mice after four weeks of MIA administration

Two-way ANOVA				
	KOR-KO		PDYN-KO	
	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Genotype	$F_{(1,34)} = 14.50$	$P < 0.01$	$F_{(1,28)} = 6.75$	$P < 0.001$
Lesion	$F_{(1,34)} = 94.61$	$P < 0.001$	$F_{(1,28)} = 106.05$	$P < 0.001$
Genotype x Lesion	$F_{(1,34)} = 4.39$	$P < 0.05$	$F_{(1,28)} = 2.34$	$P < 0.05$

Two-way ANOVA with group as between-subjects factor. *n.s.*: non-significant

Table 3. Relative corticotropin-releasing factor gene expression in KOR-KO and PDYN-KO mice after MIA administration

	Two-way ANOVA							
	KOR-KO				PDYN-KO			
	Amygdala		Hippocampus		Amygdala		Hippocampus	
	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Genotype	$F_{(1,20)} = 11.174$	$P < 0.01$	$F_{(1,29)} = 2.646$	$P < 0.05$	$F_{(1,26)} = 5.851$	$P < 0.01$	$F_{(1,27)} = 18.549$	$P < 0.001$
Lesion	$F_{(1,20)} = 15.025$	$P = 0.001$	$F_{(1,29)} = 8.421$	$P < 0.01$	$F_{(1,26)} = 7.699$	$P < 0.05$	$F_{(1,27)} = 4.024$	$P < 0.05$
Genotype x Lesion	$F_{(1,20)} = 5.851$	$P < 0.05$	$F_{(1,29)} = 10.529$	$P < 0.01$	$F_{(1,26)} = 7.365$	$P < 0.05$	$F_{(1,27)} = 5.087$	$P < 0.05$

Two-way ANOVA with group as between-subjects factor. See materials and methods for details.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Highlights

- Blockade of dynorphin/KOR enhanced mechanical allodynia during osteoarthritis.
- Deficient dynorphin/KOR mice attenuated anxiogenic manifestations of osteoarthritis.
- KOR-KO mice decreased cognitive impairment and enhanced anhedonia in osteoarthritis.
- Epigenetic levels of H3K9 acetylation was attenuated in osteoarthritis KOR-KO mice.