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

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Abstract

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INTRODUCTION

Osteoarthritis is the most prevalent joint disorder with a social cost of up to 0.5% of the gross domestic product in developed countries [48]. Osteoarthritis is characterized by pain and physical disability, problems that are often associated with anxiety, depression [3,15] and alterations of certain cognitive functions including mental flexibility and memory [28,42], all of which have a negative impact on the quality of life. A treatment designed to improve these emotional and cognitive alterations would be essential for an effective management of the disease. The endocannabinoid system (ECS) has recently emerged as a possible therapeutic target for osteoarthritis [32,33]. The ECS is composed of at least two cannabinoid receptors, CB1R and CB2R, their endogenous ligands (endocannabinoids), mainly AEA and 2-AG, and the enzymes responsible for endocannabinoid biosynthesis and inactivation. The ECS regulates several pathophysiological processes, including articular metabolism, pain, emotions and memory functions [33,39], and a therapeutic intervention on this system could offer the advantage to target multiple aspects of osteoarthritis. The ECS participates in the nociceptive manifestations of osteoarthritis [32,33], although the specific involvement in the emotional and memory alterations has not been yet investigated. The ECS is widely distributed in cortico-limbic structures, including the paraventricular nucleus of hypothalamus (PVN), prefrontal cortex (PFC), amygdala and hippocampus [17,19,21,36,52], that are involved in the regulation of the behavioral responses to stress through hypothalamic-pituitary-adrenal (HPA) axis activity [24]. Endocannabinoid signaling is altered in these brain areas after stress exposure [19–21,56]. Chronic pain is a form of stress [4] that may produce maladaptive changes within these limbic circuits leading to affective and memory dysfunctions [27,43]. Osteoarthritis has been reported to modify ECS activity in the affected joint and
spinal cord [5,32,50,51], although possible alterations of this system in limbic and other supra-spinal areas remain still unknown.

In this study, we evaluated the specific involvement of the ECS in anxiety and memory alterations associated with knee osteoarthritis in mice. We also analyzed the possible changes induced by osteoarthritis in endocannabinoid levels in mouse cortico-limbic areas and plasma, and explored the potential usefulness of endocannabinoids as biomarkers for human osteoarthritis.

METHODS AND MATERIALS:

Animal experimental conditions
Swiss albino (Charles River, Lyon, France), CB1R and CB2R constitutive knockout (CB1KO and CB2KO, respectively) and wild-type (WT) mice, all on a CD1 genetic background, were used. The generation of mice lacking CB1R and CB2R was previously described [32,34]. Mice were 2–3 months old at the beginning of the experiments and were housed in groups of 3 to 4 with ad libitum access to water and food. The housing conditions were maintained at 21 ± 1°C and 55 ± 10% relative humidity in a controlled light/dark cycle (light on between 8:00 A.M. and 8:00 P.M.). All experimental procedures and animal husbandry were conducted according to standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and approved by the local ethical committee (Comité Etico Experimental Animal–Instituto Municipal de Asistencia Sanitaria/Universitat Pompeu Fabra). Only male mice were used and all experiments were performed under blind conditions with treatments randomized between groups.

Drugs and treatments
The selective CB1R agonist, ACEA (Sigma-Aldrich, Madrid, Spain), and CB2R agonist, JWH-133 (Tocris BioScience, Bristol, UK), were diluted in a vehicle composed of 5% ethanol: 5% Cremophor-EL (Sigma-Aldrich): 90% saline and administered intraperitoneally (ip) in a volume of 10 mL/kg. Both ACEA and JWH133 were administered at doses of 1 and 5 mg/kg based on the antinociceptive effects found for these drugs in previous studies [5,54].

**Intra-articular injection of monosodium iodoacetate (MIA)**

Osteoarthritis pain was induced in mice briefly anaesthetized with isoflurane by the intra-articular injection of monosodium iodoacetate (MIA, Sigma-Aldrich) (5 μl of 5 mg/ml MIA in sterile saline, 0.9%) into the knee joint, as previously described [32]. Control mice received the intra-articular injection of vehicle (sterile saline, 0.9%).

**Nociceptive behavior**

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation. Briefly, the von Frey calibrated filaments (North Coast Medical, USA) were applied by using the up–down paradigm, as previously reported [7]. The threshold of response was then calculated by the up–down Excel program provided by Dr A. Basbaum (University of California, San Francisco, USA). Both ipsilateral and contralateral hind paws were tested.

**Affective behavior**

The elevated plus maze test (EPM) was used to evaluate anxiety and performed in a black Plexiglas apparatus with four arms (29 cm long x 5 cm wide), two open and two closed, set in cross from a neutral central square (5 cm x 5 cm) elevated 40 cm above the floor. Five-minute test sessions were performed and the percentage of entries and time spent in the open arms was determined. The total entries in the open and closed arms of the EPM were measured as a control for locomotor activity [8].
Cognitive behavior

Object recognition memory (ORM) was performed in the V-maze (Panlab) to measure cognitive performance, as previously described [47]. This task consists of three sessions (habituation, training and test). On day 1, mice were habituated for 9 min to the V-maze. On the second day, mice were put back in the maze for 9 min, two identical objects were presented and the time that mice spent exploring each object was recorded. Mice were again placed in the maze 24 h later for 9 min, one of the familiar objects was replaced with a novel object and the time spent exploring each of the two objects (novel and familiar) was computed, and a discrimination index was calculated [47]. The total time of exploration of the two objects was used as a measure of locomotor activity [47].

Behavioral protocol in mice

The affective (EPM) and cognitive (ORM) manifestations induced by osteoarthritis were evaluated in CB1KO, CB2KO and WT at two different time points: one week and three weeks after the intra-articular injection of MIA or saline (supplemental Methods).

In a second experiment, the effects of CB1R (ACEA) and CB2R (JWH133) agonists or vehicle were evaluated in nociceptive (von Frey model), affective (EPM) and cognitive (ORM) behaviors (supplemental Methods). Briefly, after the establishment of nociceptive baseline responses with the von Frey paradigm, osteoarthritis was induced as described above and osteoarthritis symptoms evaluated one and three weeks later. At both time points, mice were first habituated and trained in the ORM. On the following day, mice received the acute injection of ACEA (1 or 5 mg/kg), JWH133 (1 or 5 mg/kg) or vehicle, and tested in the following sequence: ORM, EPM and von Frey model, at 30, 45 and 60 min post-administration, respectively.
Behavioral evaluation at one week and three weeks after MIA or saline injection was performed on independent sets of animals to reduce mice adaptation to the different paradigms.

**Human subjects**

A total of 16 knee osteoarthritis patients from the Osteoarthritis Unit of Rheumatology Department (Hospital del Mar, Barcelona) and 14 healthy volunteers participated in the study (see supplemental Methods). Demographic and clinical characteristics of these subjects are summarized in Table 1. X-ray radiographies were used to determine Kellgren-Lawrence radiological grade in osteoarthritis patients [29]. The study was approved by the local ethical committee (Clinical Research Ethical Committee of the Parc de Salut Mar, CEIC-Parc de Salut Mar, Barcelona), in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). A written informed consent was obtained from participants.

**Clinical assessments**

The Huskisson scale [25], a self-administered visual analogue pain rating scale, was used to record subjective estimates of pain intensity. The scale conventionally consists of a straight line 10 cm long that is marked at each end with labels indicating the range being considered: “pain as bad as it could be” at one end and “no pain” at the other.

The PainDETECT questionnaire [9] was used to screen the presence of neuropathic pain components. This questionnaire assesses pain characteristics (i.e. the presence of burning, tingling, prickling or numbness sensations and pain in response to light touching, pressure, cold or heat), and pain behavior patterns along time. The score range is between -1 and 38. Higher scores indicate higher presence of neuropathic pain components.

The mood state was evaluated by the Hospital Anxiety-Depression (HAD) scale [57].
The HAD scale is a 14-item self-report scale containing two subscales, one for anxiety (HAD_Anxiety) and one for depression (HAD_Depression), each with 7 items, designed to screen for non-psychiatric mood disorders in general. It focuses on subjective disturbances of mood rather than physical signs, and aims at distinguishing depression from anxiety. Higher scores obtained with the two subscales indicate a higher level of subjective mood disturbances.

Health-related quality of life was evaluated by the SF36 questionnaire [55], consisting of a multi-purpose and short-form health survey with 36 questions. It yields an 8-scale profile to measure functional health and well-being scores as well as psychometrically-based physical and mental health summary measures. This questionnaire represents a generic measure, as opposed to those targeting a specific age, disease, or treatment group. Lower scores indicate worst self-perceived health-related quality of life.

Visual memory abilities were explored through the Rey-Osterrieth Complex Figure [45]. The test consists of a copy trial followed by a recall trial of a complex figure. The measures include a copy score (which reflects the accuracy of the original copy and is a measure of visual–spatial constructional ability), the time required to copy the figure, and 30-min delayed-recall scores. The figure is divided into 18 scored elements. Between 0.5 and 2 points are awarded for each element depending on the accuracy, distortion, and location of its reproduction. The maximum score is 36 which indicates perfect accuracy.

All neuropsychological measures were corrected for age and school education [45]. Once the data were collected, blood samples were obtained from each participant for quantification of 2-AG and AEA plasmatic levels, and CB1R and CB2R gene expression in peripheral blood lymphocytes.
Endocannabinoid analysis

Mice were killed by decapitation at the end of the third week (day 26) after receiving the intra-articular injection of MIA or saline to collect trunk blood and extract the brain. The endocannabinoid profile in mouse brain tissues (PFC, amygdala and hippocampus) and mouse or human plasma was determined using liquid chromatography–mass spectrometry method (supplemental Methods).

Gene expression analysis by real-time PCR

At the end of the experiments, corticotropin releasing hormone (CRH) and glucocorticoid receptor (GR) gene expression were evaluated in PVN and PFC of CB1KO, CB2KO and WT. CB1R and CB2R gene expression were evaluated in lymphocytes of osteoarthritis patients and healthy controls (supplemental Methods).

Statistical analysis

Data from behavioral and gene expression studies in mice were compared by using two-way ANOVA between groups (intra-articular injection and genotype or dose as factors of variance), followed by post-hoc analysis (Fisher’s LSD test) when appropriate. Data from human subjects and endocannabinoid quantification were analyzed by Student’s t test. Correlations were determined with Pearson’s correlation analysis. STATISTICA 6.0 software was used. The differences were considered statistically significant when the P value was below 0.05.

RESULTS

Role of CB1R and CB2R in anxiety and memory alterations associated with osteoarthritis pain in mice

Osteoarthritis pain induced by MIA intra-articular injection [32] increased the anxiety-like behavior in WT and CB1KO. At one week post-injection, two-way ANOVA
revealed a significant effect of intra-articular injection \( F(1,59) = 13.405; P < 0.001 \) and genotype \( F(1,59) = 16.139; P < 0.001 \) in the percentage of entries in the open arms, and a significant effect of intra-articular injection \( F(1,59) = 5.571; P < 0.05 \) and genotype \( F(1,59) = 19.938; P < 0.001 \) in the percentage of time spent in the open arms. At three weeks post-injection, two-way ANOVA also revealed a significant effect of intra-articular injection \( F(1,48) = 21.676; P < 0.001 \) and genotype \( F(1,48) = 10.943; P < 0.01 \) in the percentage of entries in the open arms, and a significant effect of intra-articular injection \( F(1,48) = 11.288; P < 0.01 \), genotype \( F(1,48) = 12.111; P < 0.01 \), and a significant interaction between these factors \( F(1,48) = 7.047; P < 0.05 \) in the percentage of time spent in the open arms. Subsequent post-hoc analysis indicated that MIA intra-articular injection produced an increase in the anxiety-like behavior in WT, as revealed by a significant decrease of the percentage of entries and time spent in the EPM open arms compared to saline at both one and three weeks post-injection (figure 1A, C). MIA injection also decreased the open arm entries in CB1KO at both time points (figure 1A, C). However, CB1KO that already presented an anxiogenic phenotype at basal level (saline group, figure 1A, C) [16] were even more anxious compared to WT one week after MIA injection (figure 1A).

In contrast, MIA injection did not modify the anxiety-like behavior in CB2KO at any time point. At one week post-injection, two-way ANOVA revealed in these mice a significant effect of intra-articular injection \( F(1,44) = 11.428; P < 0.01 \) and a significant interaction between intra-articular injection and genotype \( F(1,44) = 4.971; P < 0.05 \) in the percentage of entries in the open arms, and a significant effect of intra-articular injection \( F(1,44) = 8.297; P < 0.01 \) and a significant interaction between intra-articular injection and genotype \( F(1,44) = 7.438; P < 0.01 \) in the percentage of time spent in the open arms. At three weeks post-injection, two-way ANOVA also revealed a significant effect of
intra-articular injection in the percentage of entries ($F_{(1,38)} = 16.300; P < 0.001$) and percentage of time spent in the open arms ($F_{(1,38)} = 7.277; P < 0.05$). Subsequent post-hoc analysis indicated that MIA intra-articular injection in WT produced a significant decrease of the percentage of entries and time spent in the EPM open arms compared to saline at both one and three weeks post-injection (figure 1A, C). In contrast, no effects of MIA were revealed in CB2KO (figure 1A, C).

MIA injection similarly impaired memory functions in WT, CB1KO and CB2KO. Two-way ANOVA only revealed a significant effect of intra-articular injection in the discrimination index evaluated in the ORM in these mice at both one week (WT/CB1KO, $F_{(1,46)} = 42.722$, $P < 0.001$; WT/CB2KO, $F_{(1,41)} = 45.001$, $P < 0.001$) and three weeks post-injection (WT/CB1KO, $F_{(1,42)} = 28.737$, $P < 0.001$; WT/CB2KO, $F_{(1,38)} = 56.650$, $P < 0.001$). Thus, MIA injection produced a decrease in the ORM discrimination index in a similar way in WT, CB1KO and CB2KO at both time points (figure 2A, C).

MIA injection did not modify locomotor activity in any of the experimental groups, as no significant effects were revealed by two-way ANOVA in the total entries of the EPM (figure 1B, D) or the total time of exploration in the ORM (figure 2B, D), discarding any possible bias due to motor disruption in the different responses evaluated. These results reveal that CB1R and CB2R differentially modulate the alterations in anxiety-like behavior, but do not influence the memory impairment induced by MIA.

**CB1R and CB2R regulation of CRH and GR gene expression in osteoarthritic mice**

Several studies indicate that ECS signaling in PVN and PFC is critical for the behavioral responses and the glucocorticoid-mediated adaptation of the HPA axis activity after stress exposure [21,24]. Since the alterations promoted by osteoarthritis in
the anxiety-like behavior were differentially modulated in CB1KO and CB2KO in this study, CRH and GR gene expression were measured in the PVN and PFC of these mice to evaluate the role of CB1R and CB2R in the regulation of these key HPA axis components during osteoarthritis. Two-way ANOVA for CRH gene expression in the PVN of WT and CB1KO only revealed a significant effect of intra-articular injection (F_{(1,20)} = 59.029; P< 0.001), suggesting that MIA intra-articular injection similarly reduced CRH gene expression in the PVN of WT and CB1KO (figure 3A). In contrast, two-way ANOVA for CRH gene expression in the PVN of WT and CB2KO revealed a significant effect of genotype (F_{(1,32)} = 90.038; P< 0.001) and a significant interaction between genotype and intra-articular injection (F_{(1,32)} = 15.047; P< 0.001). Indeed, post-hoc analysis indicated that MIA produced a further increase of the higher basal levels of CRH gene expression in CB2KO (figure 3A).

Two-way ANOVA for CRH gene expression in the PFC of WT and CB1KO did not reveal any significant effect, suggesting that MIA injection did not modify CRH expression in the PFC of these animals. In contrast, two-way ANOVA for CRH gene expression in the PFC of WT and CB2KO revealed a significant effect of genotype (F_{(1,20)} = 44.659; P< 0.001) and a significant interaction between genotype and intra-articular injection (F_{(1,20)} = 11.105; P< 0.01). Post-hoc analysis showed that MIA injection significantly reduced the higher basal expression of CRH gene in the PFC of CB2KO (figure 3B).

Two-way ANOVA for GR gene expression in the PVN of WT and CB1KO revealed a significant effect of intra-articular injection (F_{(1,12)} = 10.249; P< 0.01), genotype (F_{(1,12)} = 5.731; P< 0.05) and a significant interaction between these factors (F_{(1,12)} = 7.566; P< 0.05). Two-way ANOVA for GR gene expression in the PVN of WT and CB2KO also revealed a significant effect of genotype (F_{(1,20)} = 8.548; P< 0.01) and a significant
interaction between genotype and intra-articular injection ($F_{(1,20)}= 6.680; P< 0.05$). Subsequent post-hoc analysis indicated that no changes in GR gene expression were induced by MIA in PVN of WT groups. However, MIA reversed the higher basal GR expression in CB1KO in this brain area and produced an opposite increase of this gene in CB2KO (figure 3C).

Two-way ANOVA for GR gene expression in the PFC revealed a significant interaction between genotype and intra-articular injection ($F_{(1,12)}= 5.387; P< 0.05$) in WT and CB1KO, and a significant effect of genotype ($F_{(1,20)}= 10.850; P< 0.01$) in WT and CB2KO. Post-hoc analysis indicated that MIA did not change GR gene expression in the PFC of any of the genotypes, although significant differences between MIA CB1KO and WT, and higher basal GR expression levels in CB2KO were observed in this brain area (figure 3D). These results reveal that CB1R and CB2R have an opposite role in the control of CRH and GR gene expression in brain areas involved in the regulation of HPA axis activity during osteoarthritis.

**Effects of CB1R (ACEA) and CB2R (JWH133) agonists in nociceptive, anxiety and memory alterations in osteoarthritic mice**

The effects produced by acute CB1R (ACEA) and CB2R (JWH133) agonist administration were evaluated in the nociceptive, anxiety and memory alterations induced by MIA. Thus, mice receiving ACEA (1 or 5 mg/kg), JWH133 (1 or 5 mg/kg), or vehicle were tested at both one and three weeks post-MIA injection in the following sequence: ORM, EPM and von Frey model, at 30, 45 and 60 min post-administration, respectively. These drugs did not produce any significant alteration in locomotor activity at the tested doses, as no significant effects were revealed by two-way ANOVA
in the total entries of the EPM or the total time of exploration in the ORM in these experiments (data not shown).

Baseline values in the nociceptive behavior (von Frey stimulation model) were similar in the different groups before intra-articular injection (data not shown). However, two-way ANOVA for ACEA in the withdrawal threshold of the ipsilateral paw (von Frey model) revealed a significant effect of intra-articular injection \((F_{(1,89)} = 112.051, P < 0.001)\), dose \((F_{(2,89)} = 3.791, P < 0.05)\), and interaction between these factors \((F_{(2,89)} = 8.413, P < 0.001)\) at one week post-injection, and a significant effect of intra-articular injection \((F_{(1,89)} = 80.775, P < 0.001)\), dose \((F_{(2,89)} = 6.385, P < 0.01)\), and interaction between these factors \((F_{(2,89)} = 5.718, P < 0.01)\) at three weeks post-injection. Two-way ANOVA for JWH133 in the withdrawal threshold of the ipsilateral paw also revealed a significant effect of intra-articular injection \((F_{(1,90)} = 109.74, P < 0.001)\), dose \((F_{(2,90)} = 5.418, P < 0.01)\), and interaction between these factors \((F_{(2,90)} = 6.481, P < 0.01)\) at one week post-injection, and a significant effect of intra-articular injection \((F_{(1,90)} = 147.562, P < 0.001)\), dose \((F_{(2,90)} = 8.323, P < 0.001)\), and interaction between these factors \((F_{(2,90)} = 6.008, P < 0.01)\) at three weeks post-injection. Subsequent post-hoc analysis indicated that both ACEA (1 and 5 mg/kg) and JWH133 (1 and 5 mg/kg) administration did not modify the nociceptive responses in the ipsilateral paw of control saline-injected mice in comparison with vehicle at one and three weeks (figure 4A, D). However, both doses of ACEA and JWH133 improved the mechanical allodynia by increasing the mechanical withdrawal threshold in the ipsilateral paw of MIA mice when compared to vehicle at one and three weeks (figure 4A, D). Nevertheless, the mechanical allodynia in MIA mice was not completely abolished by these doses of ACEA and JWH133 compared to saline mice (figure 4A, D). In contrast, these compounds did not produce any
modification in the contralateral nociceptive responses, as revealed by two-way ANOVA in the contralateral paw (supplemental figure S1).

At one week post-injection, two-way ANOVA for ACEA in the EPM revealed a significant effect of intra-articular injection \( (F_{(1,100)} = 10.248, P< 0.01) \), dose \( (F_{(2,100)} = 6.093, P< 0.01) \), and interaction between these factors \( (F_{(2,100)} = 4.476, P< 0.05) \) in the percentage of entries in open arms, and a significant effect of intra-articular injection \( (F_{(1,100)} = 8.185, P< 0.01) \), dose \( (F_{(2,100)} = 10.118, P< 0.001) \), and interaction between these factors \( (F_{(2,100)} = 3.822, P< 0.05) \) in the percentage of time spent in open arms. At three weeks post-injection, two-way ANOVA for ACEA in the EPM also revealed a significant effect of intra-articular injection \( (F_{(1,66)} = 8.746, P< 0.01) \) and interaction between intra-articular injection and dose \( (F_{(2,66)} = 4.178, P< 0.05) \) in the percentage of entries in open arms, and a significant effect of intra-articular injection \( (F_{(1,66)} = 5.422, P< 0.05) \) and interaction between intra-articular injection and dose \( (F_{(2,66)} = 7.551, P< 0.01) \) in the percentage of time spent in open arms. Moreover, at one week post-injection, two-way ANOVA for JWH133 in the EPM revealed a significant effect of intra-articular injection \( (F_{(1,95)} = 15.819, P< 0.001) \) and dose \( (F_{(2,95)} = 3.576, P< 0.05) \) in the percentage of entries in open arms, and a significant effect of intra-articular injection \( (F_{(1,95)} = 24.221, P< 0.001) \) and dose \( (F_{(2,95)} = 3.127, P< 0.05) \) in the percentage of time spent in open arms. At three weeks post-injection, two-way ANOVA for JWH133 in the EPM also revealed a significant effect of intra-articular injection \( (F_{(1,64)} = 6.452, P< 0.05) \), dose \( (F_{(2,64)} = 3.282, P< 0.05) \), and interaction between these factors \( (F_{(2,64)} = 3.254, P< 0.05) \) in the percentage of entries, and a significant effect of intra-articular injection \( (F_{(1,64)} = 13.714, P< 0.001) \) and dose \( (F_{(2,64)} = 4.534, P< 0.05) \) in the percentage of time spent in open arms. Post-hoc analysis indicated that ACEA (1 mg/kg) and JWH133 (1 and 5 mg/kg) administration did not modify the responses evaluated in the
EPM in control saline-injected mice in comparison with vehicle at one and three weeks post-injection (figure 4B, E; supplemental figure S2), although ACEA at 5 mg/kg produced an anxiogenic-like effect, as revealed by a decrease in the percentage of entries and time spent in the EPM open arms in these mice at both time points (figure 4B, E; supplemental figure S2), as previously described with high doses of CB1R agonists [41]. Moreover, ACEA at 1 mg/kg produced a reversion of the altered anxiety-like behavior induced by MIA at both time points (figure 4B, E; supplemental figure S2). No further modifications were observed at one week in the anxiety-like behavior of MIA mice administered with ACEA at 5 mg/kg as compared to vehicle (figure 4B; supplemental figure S2), although a significant difference was revealed in these mice at three weeks as compared to vehicle (figure 4E; supplemental figure S2). The altered anxiety-like behavior of MIA mice was improved by both doses of JWH133 at one week (Figure 4B) and was reversed at three weeks (Figure 4E).

Two-way ANOVA for ACEA in the discrimination index of ORM revealed a significant effect of intra-articular injection (F(1,109) = 40.869, P < 0.001), dose (F(2,109) = 4.104, P < 0.05), and interaction between these factors (F(2,109) = 4.445, P < 0.05) at one week post-injection, and a significant effect of intra-articular injection (F(1,66) = 41.567, P < 0.001), dose (F(2,66) = 4.059, P < 0.05), and interaction between these factors (F(2,66) = 3.899, P < 0.05) at three weeks post-injection. In contrast, two-way ANOVA for JWH133 in the discrimination index of ORM only revealed a significant effect of intra-articular injection at one week (F(1,94) = 123.070, P < 0.001) and three weeks post-injection (F(1,65) = 76.539, P < 0.001). Post-hoc analysis showed that both ACEA doses improved the memory impairment by increasing the discrimination index in MIA mice compared to vehicle at one and three weeks, although it was not completely reversed compared to saline mice (figure 4C, F). In contrast, JWH133 did not modify the
memory impairment of MIA mice at any of the doses and time points tested (figure 4C, F). Thus, both CB1R and CB2R agonists ameliorated the alterations in the nociceptive and anxiety-like behaviors, but only the CB1R agonist improved the memory deficit induced by MIA.

**Increased endocannabinoid levels in PFC and plasma of osteoarthritic mice**

We investigated the possible modulation of the endocannabinoid tone during osteoarthritis at the peripheral level and in brain areas involved in pain, emotional and cognitive processing. The levels of 2-AG and AEA were measured in the PFC, amygdala, hippocampus and plasma of MIA and saline mice. No significant differences between MIA and control groups were found in AEA levels in the different tissues evaluated. Similarly, no significant changes in 2-AG content were induced by MIA in amygdala and hippocampus. However, 2-AG levels were significantly higher in PFC and plasma of MIA mice compared to control (figure 5C, D). Therefore 2-AG tone was increased at both central (PFC) and peripheral (plasma) levels in osteoarthritic mice.

**The involvement of the ECS in human osteoarthritis**

We investigated if similar changes to those found in endocannabinoid levels in the MIA mouse model occur in human osteoarthritis and the possible correlation of these changes with clinical symptoms. As expected, significant differences between knee osteoarthritis patients and healthy subjects were observed in the scores obtained with the Huskisson scale for knee pain and the PainDETECT questionnaire for pain characteristics (figure 6A, B). A significant higher score for the depressive state (HAD_Depression) and a non significant trend for the anxiety score (HAD_Anxiety) were found in patients compared to healthy controls (figure 6C, D). Patients also
displayed more difficulties in performing the visual memory task (Rey-Osterrieth Complex Figure test; figure 6E), and lower scores in the quality of life SF36 questionnaire (figure 6F) compared to healthy volunteers. Significant correlations were observed between knee pain scores and the scores of PainDETECT, HAD_Anxiety, HAD_Depression and SF36 questionnaires in these subjects (figure 6G). Interestingly, a significant increase in 2-AG plasmatic levels, but not AEA, was revealed in osteoarthritis patients in comparison with healthy controls (figure 7A), replicating the findings obtained in osteoarthritic mice. Moreover, an up-regulation of CB1R and CB2R gene expression in blood lymphocytes was observed in osteoarthritis patients compared to control subjects (figure 7B). Significant positive correlations were found between 2-AG levels and knee pain and HAD_Depression scores, as well as significant negative correlations with SF36 and memory performance scores (figure 7C). In addition, significant positive correlations were obtained between the gene expression levels of CB1R in lymphocytes and HAD_Depression scores, and between the expression levels of CB2R and knee pain scores (Figure 7D). Therefore, key components of the endocannabinoid system were up-regulated in human osteoarthritis with significant correlations with clinical symptoms.

**DISCUSSION**

In this study, we validated a new model to evaluate the affective and cognitive alterations associated with knee osteoarthritis in mice and identified for the first time the involvement of the ECS in the affective symptoms that are crucial for the management of this chronic pain state. Osteoarthritis pain in mice was associated with increased anxiety-like behavior in the EPM and reduced memory functions in the ORM, as previously reported in other chronic pain models [35]. The EPM was the most sensitive
paradigm to reveal the alterations in the anxiety-like behavior during osteoarthritis pain under our experimental conditions. Alternative versions of the ORM paradigm have been used in other studies [2] and could also be potentially applied to evaluate the cognitive impairment produced by osteoarthritis pain. A limitation of the present study is represented by the lack of histological data to make correlations between the joint lesions induced by MIA and the behavioral findings obtained one and three weeks after MIA intra-articular injection. However, previous studies demonstrated that the histopathological changes already appear within the first week and increase progressively over time in the MIA model [10,12,31].

Although chronic pain could be considered a stressor producing similar effects to those observed in other stress-related disorders, the precise contribution of CRH and other components of the HPA axis remains unclear. Here, we found a down-regulation of CRH gene expression in PVN of osteoarthritic mice that may represent an adaptive modification to limit HPA axis activity under osteoarthritis pain and may underlie the absence of HPA neuroendocrine alterations in osteoarthritis patients [30]. In agreement, CRH signaling in the limbic system seems to contribute to nociceptive, affective and cognitive alterations in rodent chronic pain paradigms that were not associated with HPA axis dysfunctions [27,53]. In our study, osteoarthritis pain was associated with increased levels of 2-AG in PFC, a crucial brain area involved in pain, cognitive and emotional processing, that constitutes one of the primary targets of HPA axis hormones [38]. The augmented PFC endocannabinoid tone may represent a compensatory mechanism to maintain proper neuroendocrine and behavioral functions in response to persistent pain. An increase in PFC 2-AG levels with similar functional consequences has also been demonstrated in chronic stress animal models [19,38]. Indeed, the stress-induced increase of PFC endocannabinoid signaling through CB1R mediates a feedback...
mechanism to suppress HPA activity by dis-inhibiting output neurons on sub-cortical structures that regulate CRH secretion in PVN [21]. The same mechanisms may be responsible for the CRH down-regulation found in PVN of osteoarthritic mice.

The alterations induced by osteoarthritis in the anxiety-like behavior 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 other types of chronic stress. Thus, CB1KO displayed an increased sensitivity to develop anxiety and depressive-like states following repetitive stress procedures [18,37], in agreement with the expression of CB1R in cortico-limbic circuits related to stress responses [17,19,21,36,52]. Despite the protective role of CB2R in pain modulation previously described in the MIA model [32], an opposite regulation by this receptor was observed in the anxiety-like behavior. CB2R has been proposed to participate in emotional responses [44]. In line with our findings, chronic CB2R blockade produced anxiolysis and antidepressant-like effects following stress [13,14]. The opposite role of CB1R and CB2R in these affective manifestations could be related to their different role in regulating HPA axis components during osteoarthritis pain. Thus, MIA-induced CRH down-regulation 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 PVN of osteoarthritic mice. The lack of CB1R and the concomitant deregulation of GR gene expression in PVN may interfere with the ability of endocannabinoids to exert the glucocorticoid-dependent control of HPA axis [24], resulting in the altered affective responses observed in CB1KO. The lack of CB2R together with the basal CRH and GR gene expression modifications in PVN and PFC may facilitate adaptive responses during osteoarthritis pain to prevent the affective
alterations in CB2KO. The PFC seems to be particularly involved in the modulation of these responses in CB2KO. Thus, the higher basal expression of GR in PFC of CB2KO could promote the down-regulation of CRH gene expression by a glucocorticoid-mediated mechanism [40], which would limit the excitatory influence of cortical CRH on HPA axis and the anxiety-like behavior [26]. Therefore, the endocannabinoid signaling through CB1R and CB2R seems to be crucial for the emotional and stress-related responses produced by osteoarthritis pain.

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 these cognitive manifestations. Interestingly, CB1R (ACEA) or CB2R (JWH133) pharmacological activation improved the alterations in the nociceptive and anxiety-like behaviors, whereas only ACEA improved the memory impairment. Notably, ACEA and JWH133 were previously found to produce antinociception devoid of central side effects [54], which supports the interest of cannabinoid agonists for chronic pain treatment. The improvement of the affective and cognitive alterations observed in our osteoarthritis 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 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 [6,41] and cannabinoid effects on memory highly depend on the experimental conditions [1]. Both ACEA and JWH133 present a very high affinity for CB1R (ACEA $K_i$ value= 1.24 nM; JWH133 $K_i$ value= 677 nM ) and CB2R (ACEA $K_i$ value= 195 nM; JWH133 $K_i$ value= 3.4 nM), respectively [46]. The selectivity of these compounds suggests a specific activation of CB1R and CB2R under our experimental conditions.
Taken together, our results revealed that cortico-limbic endocannabinoid signaling is a key modulator of different osteoarthritis pain manifestations and participates in adaptive mechanisms that would help to maintain appropriate functions during osteoarthritis. The PFC endocannabinoid changes were reflected at periphery by a similar increase of 2-AG plasmatic levels in osteoarthritic mice. These peripheral endocannabinoid changes can provide a useful tool to investigate potential peripheral biomarkers of human osteoarthritis. Interestingly, a significant increase in the plasmatic levels of 2-AG was also revealed in osteoarthritic patients, replicating the findings obtained in the mouse model. Pain in osteoarthritis patients was associated with mood, cognitive and psychosocial alterations, as previously reported [3,15,28]. Significant correlations between knee pain scores and scores for anxiety and depressive state were observed in these subjects. Significant positive correlations between 2-AG plasmatic levels and scores of knee pain and depression, as well as significant negative correlations with memory performances and health status were observed in these subjects. In agreement, elevated 2-AG, but not AEA, serum content was found in individuals suffering from major depression and healthy individuals exposed to stress [22]. In contrast, both endocannabinoids were elevated in the synovial fluid of osteoarthritis patients [50], indicating a potentially distinct role of AEA (local structural alterations) and 2-AG (emotional and cognitive symptoms) during osteoarthritis. Although the source of circulating endocannabinoids remains unknown, immune cells could critically contribute to their release in the blood [49]. An up-regulation of CB1R and CB2R gene expression was also observed in lymphocytes of osteoarthritis patients, suggesting a generalized adaptive response of ECS in the immune system during osteoarthritis. The different CB1R and CB2R correlations with emotional and pain scores, respectively, provide further evidence of a differential role of these two receptors in the control of
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 depressive and schizophrenic patients [11,23].

The preclinical and clinical results reported in this study reveal the important role of the ECS in the emotional manifestations of osteoarthritis pain and provide clinical evidence for the modification of key ECS components during this chronic pain state. The data described here also highlight the potential translational relevance of these results and suggest that the ECS may represent a novel biomarker for osteoarthritis and an interesting pharmacological target for the management of chronic pain.
Acknowledgements

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The authors report no conflicts of interests.
References


Figure legends

Figure 1. Anxiety-like behavior associated with knee osteoarthritis in WT, CB1KO and CB2KO. The percentage (%) of entries and time spent in the open arms, and the total entries in open and closed arms of the EPM were evaluated one (A, B) and three weeks (C, D) after the intra-articular injection of MIA or saline. Data are expressed as mean ± SEM (n= 12-18 per group). ★ P < 0.05, ★★ P < 0.01, ★★★ P < 0.001 vs. saline injection (Fisher’s LSD test). ☆ P < 0.05, ☆☆ P < 0.01, ☆☆☆ P < 0.001 vs. WT (Fisher’s LSD test).

Figure 2. Memory impairment associated with knee osteoarthritis in WT, CB1KO and CB2KO. The discrimination index and the total time of exploration in the ORM were evaluated one (A, B) and three weeks (C, D) after the intra-articular injection of MIA or saline. Data are expressed as mean ± SEM (n= 12-18 per group). ★★ P < 0.01, ★★★ P < 0.001 vs. saline injection (Fisher’s LSD test).

Figure 3. Relative CRH (A, B) and GR (C, D) gene expression analysis in PVN and PFC of WT, CB1KO and CB2KO after receiving MIA or saline injection. Data are expressed as mean ± SEM (n= 5-9 per group). ★ P < 0.05, ★★ P < 0.01, ★★★ P < 0.001 vs. saline injection (Fisher’s LSD test). ☆ P < 0.05, ☆☆ P < 0.01, ☆☆☆ P < 0.001 vs. WT (Fisher’s LSD test).

Figure 4. Effects of ACEA and JWH133 in nociceptive, affective and cognitive behaviors in osteoarthritic mice. Mechanical nociceptive responses in the ipsilateral paw (A, D), anxiety-like behavior (B, E) and memory (C, F) were evaluated 60, 45 and...
30 min, respectively, after the ip administration of ACEA or JWH133, at one and three
weeks following MIA or saline injection. Data are expressed as mean ± SEM (n= 15-20
per group). ★ P < 0.05, ★★ P < 0.01, ★★★ P < 0.001 vs. saline injection (Fisher’s
LSD test). ☆ P < 0.05, ☆☆ P < 0.01, ☆☆☆ P < 0.001 vs. vehicle administration
(Fisher’s LSD test). ### P < 0.001 vs. 1mg/kg dose (Fisher’s LSD test).

Figure 5. 2-AG and AEA quantification in amygdala, hippocampus, PFC and
plasma of mice receiving MIA or saline injection. Data are expressed as mean ± SEM
(n= 8-10 per group). ★ P < 0.05, ★★ P < 0.01 vs. saline injection (Student’s t test).

Figure 6. Clinical assessment of pain (A, Huskisson scale and B, PainDETECT
questionnaire), emotional state (C, D, HAD scale), visual memory (E), and health-
related quality of life (F, SF36 questionnaire) in osteoarthritis patients and healthy
volunteers. Data are expressed as mean ± SEM (n= 14-16 per group). ★ P < 0.05, ★★
P < 0.01, ★★★ P < 0.001 vs. healthy volunteers (Student’s t test). Pearson’s
correlations between knee pain scores (Huskisson scale) and PainDETECT, HAD, and
SF36 scores are presented (G).

Figure 7. Plasmatic endocannabinoid quantification (A) and CB1R and CB2R gene
expression in lymphocytes (B) of osteoarthritis patients and healthy volunteers.
Data are expressed as mean ± SEM (n= 14-16 per group). ★ P < 0.05, ★★ P < 0.01 vs.
healthy volunteers (Student’s t test). Pearson’s correlations between 2-AG levels and
knee pain (Huskisson scale), HAD_Depression, SF36, and visual memory scores (C), as
well as correlations between CB1R or CB2R gene expression and HAD_Depression and
pain scores (Huskisson scale), respectively, are presented (D).
Summary: CB1R and CB2R differentially modulate the affective manifestations, but not the cognitive impairment associated with osteoarthritis pain.
One week post-MIA injection

Three weeks post-MIA injection

Figure 1
Figure 2

One week post-MIA injection

Discrimination index

![Graph A](image)

![Graph B](image)

Three weeks post-MIA injection

Discrimination index

![Graph C](image)

![Graph D](image)
Figure 3

(A) and (C) show the relative fold change in CRH gene expression in PVN, with WT and CB1KO mice compared to WT and CB2KO mice for Saline and MIA conditions, respectively. (B) and (D) display the relative fold change in GR gene expression in PFC, with similar comparisons for Saline and MIA scenarios.
One week post- MIA injection

A

Ipsilateral paw

Withdrawal threshold (g)

Vehicle
ACEA 1 mg/kg
ACEA 5 mg/kg
JWH133 1 mg/kg
JWH133 5 mg/kg

B

% time in open arms

Vehicle
ACEA 1 mg/kg
ACEA 5 mg/kg
JWH133 1 mg/kg
JWH133 5 mg/kg

C

Discrimination index

Vehicle
ACEA 1 mg/kg
ACEA 5 mg/kg
JWH133 1 mg/kg
JWH133 5 mg/kg

Three weeks post- MIA injection

D

Ipsilateral paw

Withdrawal threshold (g)

Vehicle
ACEA 1 mg/kg
ACEA 5 mg/kg
JWH133 1 mg/kg
JWH133 5 mg/kg

E

% time in open arms

Vehicle
ACEA 1 mg/kg
ACEA 5 mg/kg
JWH133 1 mg/kg
JWH133 5 mg/kg

F

Discrimination index

Vehicle
ACEA 1 mg/kg
ACEA 5 mg/kg
JWH133 1 mg/kg
JWH133 5 mg/kg

Figure 4
Figure 5

A. Amygdala

B. Hippocampus

C. Prefrontal Cortex

D. Plasma

- 2-AG
- AEA

Saline

MIA
PainDETECT questionnaire

Huskisson scale (knee)

HAD_Anxiety

HAD_Depression

Visual memory

SF36 questionnaire

Healthy volunteers

Osteoarthritis patients

Pearson r = 0.81
P = 0.000

Pearson r = 0.44
P = 0.014

Pearson r = 0.51
P = 0.003

Pearson r = -0.81
P = 0.000

Figure 6
Figure 7

A

2-AG levels (ng/mL)

Healthy volunteers

Osteoarthritis patients

ng/mL

**

AEA

2-AG

B

2^\(\Delta\Delta Ct\) relative fold gene expression

CB1R

CB2R

* 

C

Pearson r = 0.56

P = 0.001

Huskisson scale (knee) score

2-AG levels (ng/mL)

Pearson r = 0.47

P = 0.008

HAD_Depression score

2-AG levels (ng/mL)

Pearson r = 0.37

P = 0.04

2^\(\Delta\Delta Ct\) relative fold CB1R gene expression

D

Pearson r = 0.37

P = 0.039

Huskisson scale (knee) score

2-AG levels (ng/mL)

Pearson r = -0.37

P = 0.04

2^\(\Delta\Delta Ct\) relative fold CB2R gene expression

Pearson r = -0.47

P = 0.008

SF36 score

2-AG levels (ng/mL)

Pearson r = -0.37

P = 0.039

Visual memory score

2-AG levels (ng/mL)
Table 1. Demographic and clinical variables

<table>
<thead>
<tr>
<th></th>
<th>Osteoarthritis patients</th>
<th>Healthy volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>13 F, 3 M</td>
<td>8 F, 6 M</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>64.19 ± 2.38</td>
<td>59.79 ± 2.64</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>27.61 ± 0.89</td>
<td>25.10 ± 0.96</td>
</tr>
<tr>
<td><strong>K/L grade</strong></td>
<td>1.94 ± 0.14</td>
<td>NA</td>
</tr>
</tbody>
</table>

Demographic and clinical characteristics of knee osteoarthritis patients (n=16) and healthy volunteers (n=14) involved in the study. No significant differences in age and BMI were revealed between the two groups. F, Female; M, Male; BMI, Body Mass Index; K/L grade, Kellgren-Lawrence radiological grade; NA, not applicable.
Supplemental information

Methods

Schematic behavioral protocol

Nociceptive, affective and cognitive osteoarthritis symptoms were evaluated in mice one and three weeks after MIA or saline intra-articular injection.

First experimental sequence

Second experimental sequence

Human subject eligibility criteria

Two months inclusion/exclusion period was opened and those potential candidates fulfilling inclusion criteria were proposed to participate. Patient’s relatives or their accompanying persons, as well as volunteers from others Hospital departments, matched for age and body mass index (BMI) were included as healthy controls. After checking for inclusion/exclusion criteria (see below), demographics, medical history,
including drug abuse history and concomitant medications, vital signs, BMI and physical examination were assessed. X-ray radiographies were used to determine Kellgren-Lawrence radiological grade in osteoarthritis patients [3].

**Inclusion criteria**

Osteoarthritic patients must meet the following criteria:

- Patients of both sexes aged > 40 years old diagnosed with primary knee osteoarthritis (internal tibio-femoral compartment) fulfilling radiographic and clinical criteria of the American College of Rheumatology (ACR) [1].
- Stable pain in the last 72 hours.
- Able to understand the implications of the study and demonstrate it by voluntarily signing the informed consent.

Healthy volunteers must meet the following criteria:

- Volunteers matched for age, sex and BMI with no clinical knee osteoarthritis according to ACR criteria [1] and with no history of articular or knee pain.
- Able to understand the implications of the study and demonstrate it by voluntarily signing the informed consent.

**Exclusion criteria**

- Pregnant or breast-feeding women.
- History of drug or alcohol abuse.
- Documented cognitive impairment.
- Fibromyalgia and/or chronic fatigue syndrome.
- Concurrent articular rheumatism (history and/or current presence of signs) that could lead to a misinterpretation of pain evaluation or interfere with that assessment, such as chondrocalcinosis, Paget's disease of the ipsilateral limb in relation to the affected hand, rheumatoid arthritis, aseptic osteonecrosis, gout,
septic arthritis, ochronosis, acromegaly, hemochromatosis, Wilson’s disease, osteochondromatosis, seronegative spondyloarthropathy, mixed connective tissue disease, collagen vascular disease, psoriasis or inflammatory bowel disease.

- Other inflammatory diseases or systemic conditions affecting the joints.
- The use of corticosteroids (oral or injectable), methotrexate or hydroxychloroquine 12 weeks prior to inclusion.
- Intra-articular hyaluronic acid during the 24 weeks prior to the enrolment visit.
- NSAIDs 48h prior to the inclusion visit.
- History of other significant medical conditions that in the investigator's opinion would exclude the participation.

Endocannabinoid quantification

Chemicals. Acetonitrile, formic acid, acetic acid, ammonium acetate, trifluoroacetic acid (TFA), tert-butyl methyl ether (TBME) and ethylenediaminetetraacetic acid (EDTA) were obtained from Merck (Darmstadt, Germany). N-arachidonylethanolamide d4 (AEA-d4) and 2-arachidonoylglycerol d5 (2-AG-d5) were from Cayman Chemical (Ann Arbor, MI). Stock and working standard solutions were prepared in acetonitrile.

Mouse plasma and brain tissues. Quantification of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) levels was performed in mice that were killed at the end of the third week after receiving the intra-articular injection of MIA or saline. Blood was collected in 1.5 mL tubes containing 1.8 mg/mL EDTA, centrifuged at 2800 g at 4°C for 15 min and plasma was separated immediately and stored at -80°C until analysis. Tissues of the prefrontal cortex, amygdala and hippocampus were freshly dissected.
from the whole brain. Extracted brain areas were weighted and stored at -80ºC until analysis.

*Human plasma.* Blood from osteoarthritis and healthy subjects was collected in 10 mL K2E 18.0 mg (EDTA) Vacutainer tubes (BD, USA) and centrifuged after extraction at 2800 g at 4ºC for 15 min. Plasma was separated immediately, and 1 mL aliquots were stored at -80ºC until analysis.

**Endocannabinoid extraction from brain tissues.** Brain tissues were kept at -80ºC or in dry ice until homogenization. Tissues were homogenized on ice with a glass homogenizer in 0.02% (v/v) TFA in water (pH 3.0). The proportion used was 6 µL of 0.02% TFA per mg of tissue. Thirty movements “loose”, followed by thirty movements “tight” were used for homogenization. The mean weights of tissues were as follows: 36.1 ± 1.6 mg for hippocampus; 12.0 ± 2.0 mg for amygdala, and 6.4 ± 1.5 mg for medial pre-frontal cortex. Hippocampus tissues were homogenized individually, while two amygdala and four medial prefrontal cortex tissues from mice of the same experimental group were pooled for each sample. This was done in order to have the optimal amount of brain tissue for endocannabinoid quantification (15-25 mg). The homogenization process took less than five minutes per sample and homogenates were kept on ice until organic extraction to minimize the ex-vivo generation of endocannabinoids [2]. Aliquots of 10 µL of these brain tissue homogenates were used for 2-AG analysis, while the rest of the homogenate (50-150 µL) was used for AEA analysis. 2-AG and AEA aliquots were separately transferred to 12 mL glass tubes and diluted up to 1 mL in 0.02% TFA, spiked with internal standards (0.375 ng AEA-d5 and 14 ng 2-AG-d5), extracted 20 min with TBME (6 mL), and centrifuged (2800 g, 5 min, room temperature). The organic phase was transferred to clean tubes, evaporated (40ºC, 20 min) under a stream of nitrogen, and extracts were reconstituted in 100 µL of a
mixture water:acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to HPLC vials. Twenty μL were injected into the liquid chromatography–mass spectrometry (LC-MS-MS) system.

*Endocannabinoid extraction from plasma.* Plasma samples were thawed in less than 30 min at room temperature and were swiftly processed on ice until the organic extraction to minimize the ex-vivo generation of endocannabinoids [5]. Aliquots of 0.5 mL of human plasma or aliquots of 100 μL of mouse plasma were transferred to 12 mL glass tubes spiked with internal standards (0.25 ng AEA-d4 and 5 ng 2-AG-d5) diluted up to 1 mL with 0.1M ammonium acetate buffer (pH 4.0) extracted 20 min with 6 mL of TBME and centrifuged (2800 g, 5 min) at room temperature. The organic phase was transferred to clean tubes, evaporated (40ºC, 20 min) under a stream of nitrogen, and extracts were reconstituted in 100 μL of a mixture water:acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to HPLC vials. Twenty μL were injected into the LC/MS-MS system.

*LC-MS/MS analysis.* An Agilent 6410 triple quadrupole (Agilent Technologies, Wilmington, DE) equipped with a 1200 series binary pump, a column oven and a cooled autosampler (4ºC) was used. Chromatographic separation was carried out with a Waters C18-CSH column (3.1 x 100 mm, 1.8 μm particle size) maintained at 40ºC with a mobile phase flow rate of 0.4 mL/min. The composition of the mobile phase was: A: 0.1% (v/v) formic acid in water; B: 0.1% (v/v) formic acid in acetonitrile. The initial conditions were 40% B. The gradient was first increased linearly to 90% B over 4 minutes, then increased linearly to 100% B over 5 minutes and maintained at 100% B for 3 minutes, to return to initial conditions for a further 4 minutes with a total run time of 16 minutes. The ion source was operated in the positive electrospray mode. Desolvation gas temperature of 350ºC and a gas flow rate of 10 L/min were used. The
pressure of the nebulizer was set at 40 psi and the capillary voltage at 4,000 V. The collision energies were set at 12 V for all compounds. The selective reaction monitoring mode was used for the analysis with the following precursor to product ion transitions: m/z 348→62 for AEA, m/z 352→66 for AEA-d4, m/z 379.2→287 for 2-AG and m/z 384→287 for 2-AG-d5. 2-AG levels were expressed as the sum of isomer 1 and isomer 2, due to the instability of isomer 2 to isomerization. Quantification was done by isotope dilution.

**Gene expression analysis by real-time PCR**

At the end of the behavioral experiments, corticotropin releasing hormone (CRH) and glucocorticoid receptor (GR) gene expression were evaluated in PVN and PFC of CB1KO, CB2KO and WT. Mice were killed by cervical dislocation and brains were rapidly removed, fresh-frozen and stored immediately at -80°C until use. Total RNA was isolated from frozen (-80 °C) paraventricular nucleus (PVN) and prefrontal cortex (PFC) micropunches with Tri Reagent (Ambion) and subsequently reverse transcribed to cDNA. CRH and GR gene expression was measured with the following TaqMan® Gene Expression assays: ‘‘Mm01293920_s1’’ for CRH and ‘‘Mm00433832_m1’’ for GR (Life Technologies, Spain). Real time PCR experiments were performed on the StepOne Plus system (Life Technologies) and the reference gene used was Rn18SrRNA, detected with TaqMan® ribosomal RNA control reagent ‘‘Mm03928990_g1’’.

CB1R and CB2R gene expression were evaluated in lymphocytes of osteoarthritis patients and healthy controls. For peripheral lymphocytes isolation, blood samples from osteoarthritis patients and healthy controls were extracted in Vacutainer® CPT™ (Sodium Citrate: 1.0 mL, FICOLL: 2.0 mL) tubes (BD, USA). After 20 min incubation with Rosette Sep™ (*Human total lymphocyte enrichment cocktail*) (STEMCELL Technologies, France), samples were centrifuged (1747 g, 25 min, room temperature)
and the lymphocyte fraction at the layer between plasma and the density gradient barrier was collected. Lymphocytes were resuspended and washed twice with PBS 1X (300 g, 15 min, room temperature). The cell pellet was subsequently resuspended in RNAProtect Cell Reagent (QIAGEN, Germany) and stored at -20° C until use. Total RNA from lymphocytes of osteoarthritis patients and healthy controls was obtained using RNeasy Mini kit (QIAGEN), according to the manufacturer’s instructions, and subsequently reverse transcribed to cDNA. CB1R and CB2R gene expression was determined by real-time PCR carried out with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Spain) using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. The endogenous reference gene used was HPRT (hypoxanthine guanine phosphoribosyl transferase). The following human specific primers were used:

5’-CTTCACGGTCTGAGAAGCT-3’ (CB1R forward);
5’-GTGGAAGTCAATGACTGAGAAGCTG-3’ (CB1R reverse);
5’-TGACCTTCAGCCTCTGTGG-3’ (CB2R forward);
5’-GCAGGTAGGAGACTAGTGCTGAGAAGCTG-3’ (CB2R reverse);
5’-GGCCAGACTTTGGATTGATTG-3’ (HPRT forward);
5’-TGCCTCTCATCTAGTTGATTG-3’ (HPRT reverse).

Data for each target gene were normalized to the endogenous reference gene, and the fold change in target gene mRNA abundance was determined by the $2^{ΔΔCt}$ method [4].
Results

S1: Effects of ACEA and JWH133 in contralateral paw nociceptive responses of osteoarthritic mice.

Figure S1: Mechanical nociceptive responses in the contralateral paw were evaluated 60 minutes after the ip administration of ACEA and JWH133, one or three weeks after intra-articular injection of MIA or saline in mice (n= 15-20 per group). The mechanical threshold (g) required to elicit paw withdrawal is expressed as mean ± SEM.

S2: Effects of ACEA and JWH133 in the percentage of EPM open arm entries in osteoarthritic mice.
Figure S2: The percentage of entries in the open arms of EPM was evaluated 45 minutes after the ip administration of ACEA or JWH133, one or three weeks after intra-articular injection of MIA or saline in mice (n= 15-20 per group). Data are expressed as mean ± SEM. ★★★ P < 0.001 vs. saline injection (Fisher’s LSD test). ☆ P < 0.05, ☆☆ P < 0.01, ☆☆☆ P < 0.001 vs. vehicle treatment (Fisher’s LSD test). # P < 0.05 vs. 1mg/kg dose (Fisher’s LSD test).

References


