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**Mechanism and pharmacotherapy of osteoarthritis - recent
advances in the endocannabinoid system targeting**

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List of publications constituting the doctoral dissertation

1. **Marta Bryk**, Jakub Chwastek, Jakub Mlost, Magdalena Kostrzewa, Katarzyna Starowicz. *Sodium Monoiodoacetate Dose-Dependent Changes in Matrix Metalloproteinases and Inflammatory Components as Prognostic Factors for the Progression of Osteoarthritis*. *Frontiers in Pharmacology* 2021, 12:643605, doi: 10.3389/fphar.2021.643605, IF: 5.988, MEiN points: 100
2. **Marta Bryk**, Jakub Chwastek, Magdalena Kostrzewa, Jakub Mlost, Aleksandra Pędracka, Katarzyna Starowicz. *Alterations in Anandamide Synthesis and Degradation during Osteoarthritis Progression in an Animal Model*. *International Journal of Molecular Sciences* 2020, Oct 6;21(19):E7381, doi: 10.3390/ijms21197381, IF: 6.208, MEiN points: 140
3. **Marta Kędziora**, Serena Boccella, Jakub Mlost, Ida Marabese, Rosmara Infantino, Sabatino Maione, Katarzyna Starowicz. *Inhibition of anandamide breakdown reduces pain and restores LTP and monoamine levels in the rat hippocampus via the CB1 receptor following osteoarthritis*. *Neuropharmacology*, Volume 222, 1 January 2023, 109304, doi: 10.1016/j.neuropharm.2022.109304, IF: 5.273, MEiN points: 140

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Abbreviations

2-AG – 2-arachidonylglycerol

AA – arachidonic acid

ABHD – α/β -hydrolase domain

AEA – anandamide

ACL – anterior cruciate ligament

ACLT – anterior cruciate ligament transection

ADAM – adamalysins

ADAMTS – adamalysins with thrombospondin motifs

CB – cannabinoid receptor

CBD – cannabidiol

CBN – cannabinol

CNS – central nervous system

COX – cyclooxygenase

CXCL – chemokine

DAG – diacylglycerol

DAGL – diacylglycerol lipase

DRG – dorsal root ganglion

ECM – extracellular matrix

ECS – endocannabinoid system

EET-EA – epoxyeicosatrienoyl-ethanolamide

EtA – ethanolamine

FAAH – fatty acid amide hydrolase

G – glycerol

GDE1 – glycerophosphodiester phosphodiesterase 1

GDPD – lysophospholipase D isoform

GPCR – G-coupled receptor

HA – hyaluronic acid

HETE – hydroxyeicosatetraenoic acid

HETE-EA – hydroxyeicosatetraenoyl-ethanolamides

HFLS – human fibroblast-like synoviocytes

HFLS-OA – human osteoarthritic fibroblast-like synoviocytes

i.a. – intra-articular injection
IL – interleukin
KWB – Kinetic Weight Bearing
L – leukotriene
LOX – lipoxygenase
LPS – lipopolysaccharide
LTP – long term potentiation
MAGL – monoacylglycerol lipase
MAPK – mitogen activated protein kinase
MIA – sodium monoiodoacetate
MMP – matrix metalloproteinase
MMT – medial meniscal transection
MMx – medial meniscectomy
mPFC – medial prefrontal cortex
NAAA – N-acylethanolamine acid amide hydrolase
NAPE – N-acyl-phosphatidylethanolamine
NAPE-PLD – N-acylphosphatidylethanolamine phospholipase D
OA – osteoarthritis
OARSI – Osteoarthritis Research Society International
PAM – Pressure Application Measurement
PG – prostaglandins
PG-EA – prostaglandin ethanolamides
PLC – phospholipase C
PM – prostamides
PTPN – phosphatase
RA – rheumatoid arthritis
SHIP1 – inositol 5' phosphatase
SNI – spared nerve injury
sPLA2 – soluble phospholipase A2
THC – tetrahydrocannabinol
TNF α – tumor necrosis factor α
TRPV1 – transient receptor potential cation channel subfamily V member 1

Streszczenie

Choroba zwyrodnieniowa stawów (osteoartroza, OA) jest coraz częstszym schorzeniem dotyczącym osoby powyżej 65 roku życia, które powoduje utrudnienia w poruszaniu, sztywność stawów i przewlekły ból. Aktualnie dostępne możliwości terapeutyczne są ograniczone, powszechnie stosowym lekiem pierwszego wyboru jest dostawowe podanie kwasu hialuronowego poprawiające ruchomość stawu dotkniętego chorobą, natomiast w celu bieżącego uśmierzania bólu stosowane są niesteroidowe leki przeciwzapalne. W zaawansowanych stadiach OA niezbędna jest alloplastyka stawu, jednak nawet po przejściu operacji spora część pacjentów wciąż odczuwa przewlekły ból. Może on być powodem pojawienia się chorób współistniejących, takich jak zaburzenia nastroju czy depresja. Bezpośrednim powodem progresji OA jest degeneracja powierzchni chrząstki stawowej oraz miejscowy stan zapalny, co związane jest z podniesieniem poziomu czynników zapalnych i enzymów degradujących macierz zewnątrzkomórkową w tkankach stawu. Najnowsze badania wskazują także na udział układu endokanabinoidowego w przebiegu OA oraz związanych z nią zaburzeń nastroju, jednak dokładny mechanizm nie jest znany.

Celem niniejszej pracy było zbadanie w zwierzęcym modelu OA molekularnych mechanizmów leżących u podstawy progresji choroby, ze szczególnym uwzględnieniem zmian w poziomie czynników zapalnych w obrębie stawu kolanowego (chrząstka, płyn maziowy, błona maziowa) w czasie 28 dni po podaniu różnych dawek monojodooctanu sodowego (MIA), a także zmian w poziomie enzymów biorących udział w ścieżkach syntezy i rozkładu pierwszego opisanego endokanabinoidu, anandamidu, w rdzeniu kręgowym, chrząstce i błonie maziowej. Dodatkowo, zbadano również zmiany w zaburzeniach uwagi i pamięci 28 dni po wywołaniu OA i sprawdzono wpływ inhibitora enzymu FAAH (odpowiedzialnego za rozkład anandamidu) na redukcję objawów OA i długotrwałe wzmocnienie synaptyczne (LTP) w hipokampie.

Uzyskane wyniki wskazują na zmiany w poziomie czynników zapalnych (CCL2, CXCL1, IL-1 β) oraz metaloproteinaz macierzy zewnątrzkomórkowej (MMP-2, -3, -9, -13) w tkankach stawu szczurów z OA, które były zależne od dawki MIA i czasu jaki upłynął od indukcji modelu. Uzyskane dane pozwalają na wybranie odpowiedniej dawki do wystandaryzowania dalszych badań, ponieważ stosowane u szczurów dawki MIA różnią się w literaturze, co może powodować problemy z porównaniem wyników badań. Wykazano również zmiany w poziomie enzymów uczestniczących w szlakach syntezy i rozkładu anandamidu, które wskazują na zaangażowanie układu endokanabinoidowego w proces rozwoju OA oraz jego potencjał w poszukiwaniu skuteczniejszych strategii terapeutycznych. Potwierdzono także, że zwierzęta

z OA mają znacząco zaburzoną pamięć (obniżenie LTP w hipokampie) oraz wykazują cechy obniżenia nastroju (zaburzenia poziomu monoamin: dopaminy i serotoniny). Zahamowanie rozkładu anandamidu poprzez podanie inhibitora enzymu FAAH (URB597) spowodowało odwrócenie tych zmian oraz redukcję objawów obserwowanych na poziomie behawioralnym.

Podsumowując, wyniki uzyskane w czasie realizacji projektu sugerują, że układ endokannabinoidowy pełni ważną rolę w procesie rozwoju OA, jak również może stanowić narzędzie terapeutyczne do hamowania objawów bólowych i poznawczych, które towarzyszą progresji choroby.

Abstract

Osteoarthritis (OA) is one of the most common joint diseases and the major cause of disability in people aged >65 years. OA results in impaired mobility, joint stiffness and chronic pain and can be accompanied by comorbid conditions, increased mortality, and decreased quality of life. Current therapeutic possibilities are limited, a common first-line treatment is an intra-articular injection of hyaluronic acid to improve joint mobility, while non-steroidal anti-inflammatory drugs are used for ongoing pain relief. In the advanced stages, joint arthroplasty is necessary, but even after undergoing the surgery, a large number of patients still experience chronic pain. This can be the cause of the onset of comorbidities such as mood impairment or depression. The direct cause of OA progression is degeneration of the articular cartilage surface and local inflammatory state, which is associated with elevated levels of inflammatory factors and extracellular matrix-degrading enzymes in the joint tissues. Recent studies also confirm the involvement of the endocannabinoid system (ECS) in OA symptoms and associated mood disorders; however, the exact mechanism remains unclear.

The aim of the present study was to investigate, in an animal model of OA, the molecular mechanisms underlying the progression of the disease, with a particular focus on changes in the levels of inflammatory factors within the knee joint (cartilage, synovial fluid, synovial membrane), over 28-day period following administration of various doses of sodium monoiodoacetate (MIA). Additionally, changes in the levels of synthetic and degradative enzymes regulating the first discovered endocannabinoid, anandamide (AEA) in the spinal cord, cartilage and synovial membrane were studied. In addition, impaired memory and attention were also examined 28 days after OA induction, and the effect of an inhibitor of fatty

acid amide hydrolase (FAAH), the primary enzyme responsible for the AEA degradation, was assessed on the reduction of OA-induced symptoms and hippocampal long-term potentiation (LTP).

The results show dose- and time-dependent changes in the levels of pro-inflammatory factors (CCL2, CXCL1, IL-1 β) and matrix metalloproteinases (MMP-2, -3, -9, -13) in the joint tissues of rats with MIA-induced OA. Obtained data allow for the selection of an appropriate dose to standardize further research, since the MIA doses used in rat studies vary, which present a challenge in comparing the results from different experiments. Changes in the levels of enzymes involved in the AEA synthesis and degradation pathways were also found, indicating the involvement of the ECS in the development of OA and potential therapeutic targets in the search for more effective treatment strategies. These findings also support that animals with MIA-induced OA have significantly impaired memory (decreased LTP in the hippocampus) as well as impaired levels of monoamines (i.e., dopamine and serotonin), which are implicated in mood. Inhibition of AEA breakdown by administration of a FAAH enzyme inhibitor (URB597) reversed these changes and reduced the signs observed at the behavioral level.

To conclude, the results obtained during the current project implementation suggest that the ECS plays an important role in the development of MIA-induced OA, as well as identify potential novel therapeutic approaches to treat pain and cognitive symptoms that accompany disease progression.

Introduction

1. Introduction

1.1. Osteoarthritis etiology and symptoms

Osteoarthritis (OA) is a degenerative joint disease, one of the most common disorders among middle-aged and older adults (Deshpande *et al.*, 2016). Due to its prevalence in an aging society affected by civilization diseases such as type II diabetes or obesity, OA has become a global healthcare problem. It is estimated that OA ranks at the top of healthcare costs in Europe (Bedenbaugh *et al.*, 2021). Approximately 30% of people aged 18-64 and up to 60% of people over 65 years old can be affected by various forms of arthritis indicating its tremendous prevalence (Jafarzadeh & Felson, 2018). An official definition of OA provided by the Osteoarthritis Research Society International (OARSI) describes OA as *“a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness”* (“Standardization of Osteoarthritis Definitions,” 2023). The disease onset might be a result of a joint injury (such as a fall or accident) – in this case OA beginning can be precisely determined and treatment strategy may be implemented from the very early stages. However, in many cases the direct onset of OA remains unclear and is a consequence of prolonged joint overloading through excessive sport, being overweight or elderly age. In this type of OA (called primary or idiopathic), the disease has no strictly defined beginning and therefore is more difficult to treat, since patients tend to ignore early symptoms and seek doctor’s advice when the pain becomes unbearable. The main symptoms of both OA types are movement difficulties, joint stiffness (especially in the morning), cracking sounds while moving and chronic pain, that impedes daily functioning. In more severe cases, OA can be a cause of a permanent disability, forcing the patient to undergo a total joint arthroplasty. The direct cause of the abovementioned symptoms is cartilage and subchondral bone degeneration, which leads to the local chronic inflammatory state (synovitis). Inflamed synovial membrane overproduces several factors, such as chemokines, cytokines, aggrecanases and matrix metalloproteinases (MMPs), which cause extracellular matrix (ECM) components (collagen, proteoglycans, aggrecan) degradation and lead to subsequent cartilage degradation, creating a vicious circle of the OA progression (Scanzello & Goldring, 2012). A schematic representation of joint changes during OA progression is showed on Fig. 1.

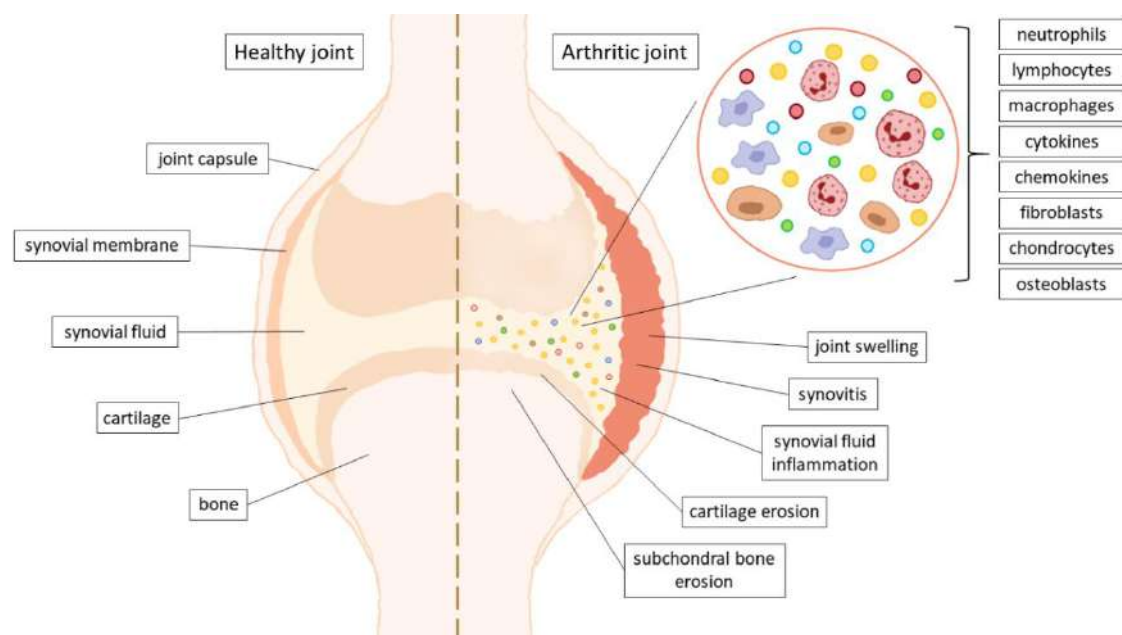


Figure 1: Schematic summary of OA pathogenesis; adapted from Author's publication: (Bryk & Starowicz, 2021). OA progression is associated with cartilage and subchondral bone degradation, swelling, synovial membrane outgrowth, synovitis, an increase of inflammatory factors (such as cytokines, chemokines, macrophages, neutrophils) and degraded cartilage fragments in the synovial fluid.

Elevation of matrix degradation enzymes (MMPs) represents a hallmark of OA. MMPs play an important role in various cellular processes, such as cell development, morphogenesis, angiogenesis, tissue remodeling and repair, innate immunity and inflammation (Yamamoto *et al.*, 2021). MMPs, along with adamalysins (ADAMs), and ADAM with thrombospondin motifs (ADAMTSs) are members of zinc-dependent endopeptidases and belong to several groups. Most important are the: gelatinases (e.g. MMP-2, MMP-9), which are responsible for the digestion of denatured collagens and gelatins, as well as inflammatory responses (Kim *et al.*, 2012; Hannocks *et al.*, 2019); stromelysins (e.g. MMP-3, MMP-10, MMP-11), which digest ECM components and activate a number of proMMPs (Li *et al.*, 2012); and collagenases (e.g. MMP-1, MMP-8, MMP-13) which cleave different types of collagens (Neuhold *et al.*, 2001; Ma *et al.*, 2017). MMPs activity is strictly regulated by endogenous inhibitors (tissue inhibitors of metalloproteinases, TIMPs) (Laronha & Caldeira, 2020). In the healthy joint MMPs and TIMPs balance is maintained, however during pathological states it can be disrupted and promote disease progression. Various MMPs levels are upregulated in the synovial fluid of advanced OA patients (Tchetverikov *et al.*, 2005; Heard *et al.*, 2012). In particular, MMP-3 protein abundance in the synovial tissue of OA patients was positively correlated with the OA severity (Chen *et al.*, 2014). A decrease in serum levels of MMP-1 and MMP-3 correlated with

a reduction in cartilage volume loss and the effect of OA treatment (Pelletier *et al.*, 2010). Fibroblast activation protein (Fap), responsible for the degradation of denatured type II collagen and MMP-13-cleaved native collagen was significantly elevated in OA synovium. Moreover, in a mouse model of OA, genetic deletion or pharmacological inhibition of Fap significantly ameliorated posttraumatic OA signs (Fan *et al.*, 2023).

Importantly, OA joint tissues differ from those from healthy patients in terms of susceptibility to pro-inflammatory stimulation. Human synovial fibroblast cells collected from patients suffering from OA (HFLS-OA) are more vulnerable to pro-inflammatory stimulation with tumor necrosis factor α (TNF α) or lipopolysaccharide (LPS) than cells from healthy controls (HFLS). Selected chemokines (CXCL6, CXCL10, CXCL16), were overproduced after pro-inflammatory stimulation, but in OA cells this increase was significantly higher, in comparison to HFLS collected from the healthy controls (Fig. 2, for details, see: (Chwastek *et al.*, 2022)). This proves, that during OA development joint tissue cells switch into a pro-inflammatory state, which facilitates further cartilage degradation and disease progression. Therefore, in the early stages of the disease, non-steroidal anti-inflammatory drugs (NSAIDs) are effective in relieving OA pain, but when cartilage degradation is at its advanced degree this type of drugs proves insufficient and stronger analgesics, such as opioids, are necessary.

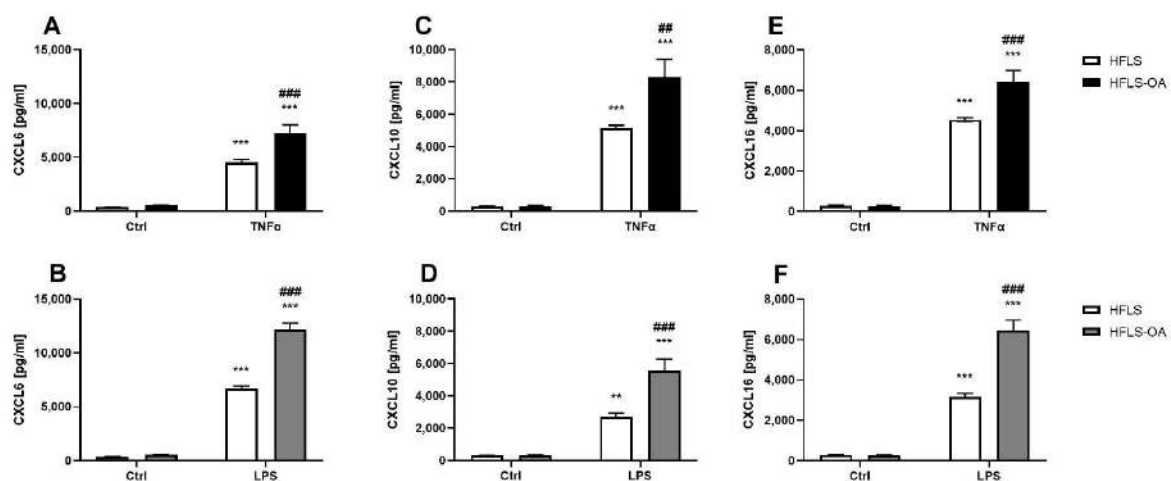


Figure 2: Chemokine (CXCL6, CXCL10, CXCL16) protein secretion by HFLS or HFLS-OA cells after 24 h TNF α (A, C, E) or LPS (B, D, F) stimulation. Adapted from: (Chwastek *et al.*, 2022), where full description including p values, sample size etc. can be found.

Current therapeutic strategies for OA treatment are very limited. The first-line therapy to maintain joint functionality is intra-articular (i.a.) hyaluronic acid (HA) injection. This biopolymer increases lubrication and therefore facilitates joints' movement. However,

the effect is transient due to HA degradation by enzymes such as hyaluronidase; therefore, repeated injections are required. The general safety of HA injection is confirmed by various studies (Altman *et al.*, 2018), however, its efficacy is not clear (Pereira *et al.*, 2022). Besides improving joint functionality, another important component of OA treatment is chronic pain alleviation. In early OA stages, NSAIDs are widely used, while opioids are required for more severe pain. Both groups of medications possess serious side effects when used chronically, such as bleeding from the gastrointestinal tract or addiction, respectively. Because there are currently no effective therapies that stop the progression of OA, patients take pain medications for a prolonged period, subjecting them to adverse effects and addiction (Bindu *et al.*, 2020). Arthritis is one of the most common disorders among chronic opioid users (Hudson *et al.*, 2008). In the US, chronic opioid use has contributed to an “opioid epidemic”. It is estimated that the total number of drug overdose deaths has quintupled from 1999 till today (Hedegaard *et al.*, 2021). As there is currently no approved therapy that prevents or reverses OA progression, identifying novel therapeutic strategies is urgently required.

1.2. Animal model of osteoarthritis

To investigate OA development, as well as novel pharmacological treatment strategies in preclinical studies, animal models are indispensable. There are several OA models described in the literature, which can be divided into two categories: surgical and chemical. Surgically-induced models mimic the clinical situation of OA development after joint injury and in this category may include: the anterior cruciate ligament transection (ACLT) model, medial meniscectomy (MMx) model, articular groove model, medial meniscal transection (MMT) model, partial medial meniscectomy model or ACL injury model. In turn, chemical compounds that are used to trigger OA in rodents include sodium monoiodoacetate (MIA), steroids, cytokines, collagenase, papain or immunotoxin (Cope *et al.*, 2019) (see Fig. 3 for details).

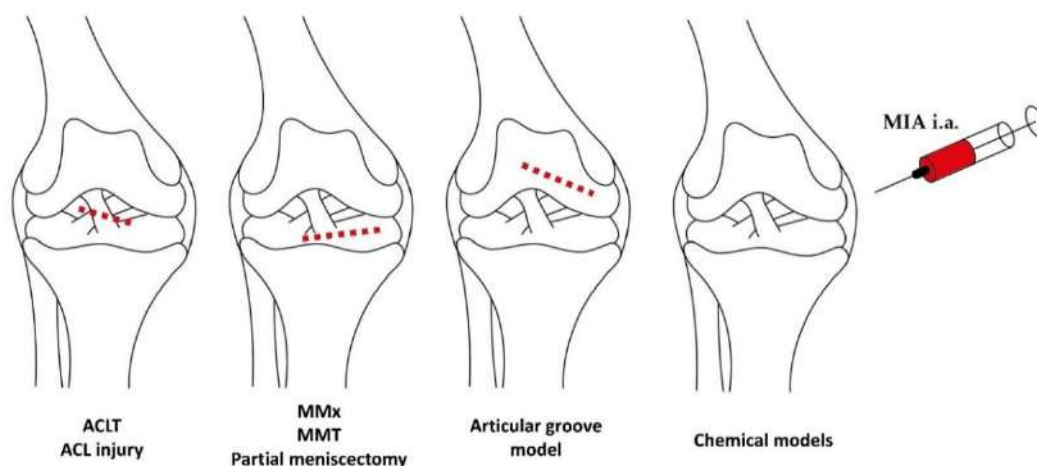


Figure 3: Schematic representation of the sites of surgical/chemical intervention for OA induction.

Chemically-induced OA reflects better idiopathic type of the disease, because there is no initial injury within the joint and the symptoms, as well as cartilage degeneration occur gradually and remain stable, because cartilage has little or no regenerative ability. In MIA-induced OA, which is employed in this dissertation, the disease is triggered by i.a. injection of 1 or 2 mg of MIA. MIA inhibits glycolysis in chondrocytes and therefore leads to cartilage degeneration. The MIA doses used to induce OA in rodents vary in the literature between 0.5 and 4.8 mg (Allen *et al.*, 2017; Haywood *et al.*, 2018; Lee *et al.*, 2018; Piao *et al.*, 2020), which presents a challenge in comparing drug efficacy between experiments. After injection, a transient decrease in nociceptive threshold occurs in the first few days, which can be attributed to the i.a. injection itself and the temporary inflammatory state caused by MIA. Then, significant cartilage and subchondral bone degeneration, followed by chronic pain development is observed from day 28 post-injection and remains stable for at least up to week 10 (Fig. 4, Author's unpublished data).

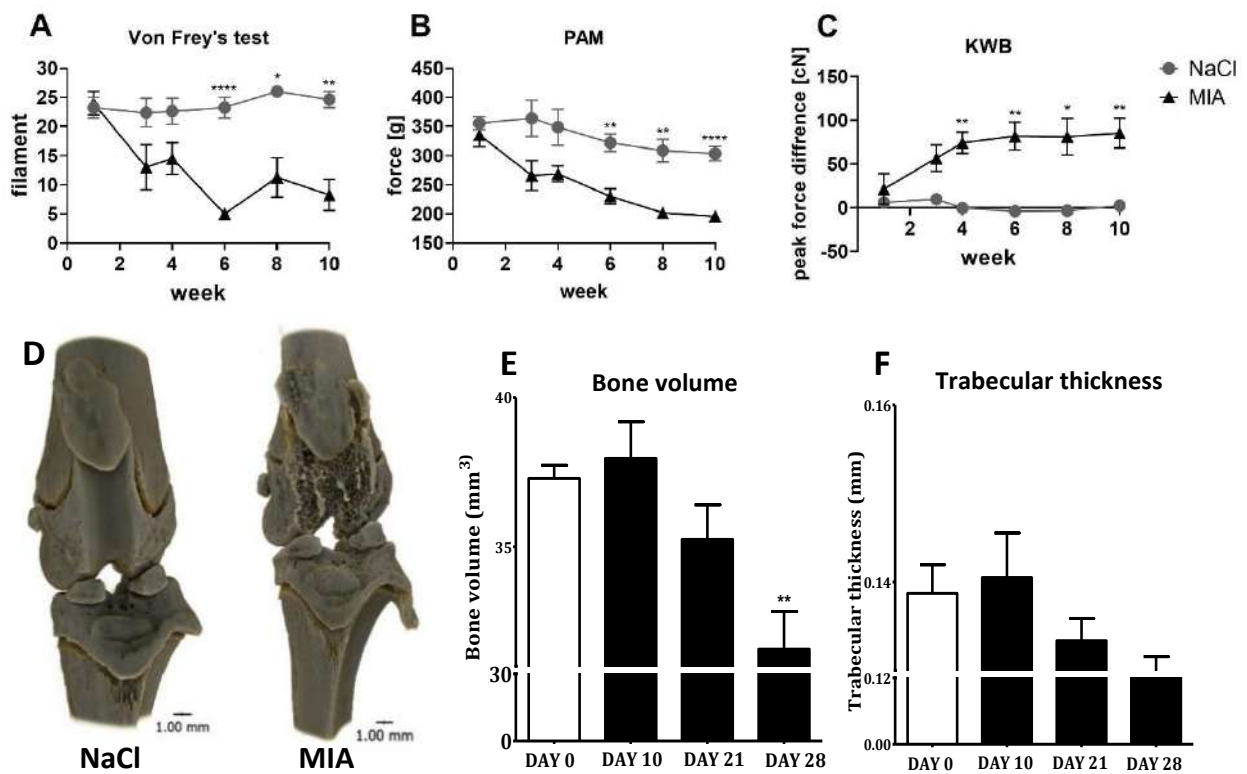


Figure 4: Development of chronic pain associating OA in a rat MIA-induced model (Author's unpublished data). A: Von Frey's test results (allodynia); B: PAM test results (joint hypersensitivity); C: KWB test results (gait analysis). Two-way ANOVA (or mixed model) and Šidák's post hoc test. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$; **** $p < 0.0001$ difference between NaCl and MIA rats. D: Microtomographic pictures visualizing cartilage degradation 6 weeks post-MIA injection in sham (NaCl) and MIA-treated group. E-F: bone volume (E) and bone trabecular thickness (F) decrease in three time points after MIA injection.

Persistent pain in OA rodent models can be defined by various methods (Deuis *et al.*, 2017). Von Frey's test measures allodynia – a condition when a neutral (non-noxious) stimulus (e.g., touch) becomes painful. Knee joint hypersensitivity to an external force applied by the experimenter can be measured by Pressure Application Measurement (PAM) test. Also, for knee OA studies it is important to analyze gait and determine differences in paw loading during walking. In general, if OA affected joint causes pain, animal gait balance should be disrupted in favor of the healthy joint. The Kinetic Weight Bearing (KWB) test allows for the assessment of the difference between the rear left and right paw force applied to the ground during a free walking sequence. Microtomographic pictures of the whole knee joint allow for visualization of cartilage and subchondral bone changes occurring within the joint and quantification of bone density, bone volume, trabecular thickness, spacing, etc.

1.3. Osteoarthritis pain-related comorbidities

Irrespective of their underlying mechanisms, many chronic pain syndromes share several comorbidities. Cartilage degeneration and local inflammatory state cause movement difficulties and chronic pain, which besides unpleasant sensations can contribute to the mood impairment or emotional problems. In general, chronic pain and depression have high rates of co-morbidity, depression may co-occur in up to 49% of chronic pain patients (Meda *et al.*, 2022). Chronic pain patients are also at high risk of cognitive disorders, including anxiety and depression (Fisher *et al.*, 2018). When compared to the control group of the same age, gender and education level, chronic pain patients had a higher level of depressive symptoms and anxiety scores, accompanied with lower estimated IQ and spatial and verbal memory (Moriarty *et al.*, 2017). Chronic pain is a major stressor, which can influence neuronal circuits in the brain. The mechanism might be mediated by long-term potentiation (LTP) in the anterior cingulate cortex (ACC), which is involved in pain perception (Zhuo, 2016). Moreover, the hippocampus plays an important role in memory formation, but also belongs to the limbic system which is involved in mood regulation. Chronic pain via the TNF α -dependent mechanism can induce structural and chemical plasticity or flexibility in hippocampal neurons and contribute to depression development (Fasick *et al.*, 2015). In a mouse model, neuropathic pain disrupted synaptic plasticity and neurogenesis (Mutso *et al.*, 2012) and impaired LTP in the hippocampus (Kodama *et al.*, 2007). A schematic representation of relation between chronic pain in OA and brain disturbances is presented on Fig. 5.

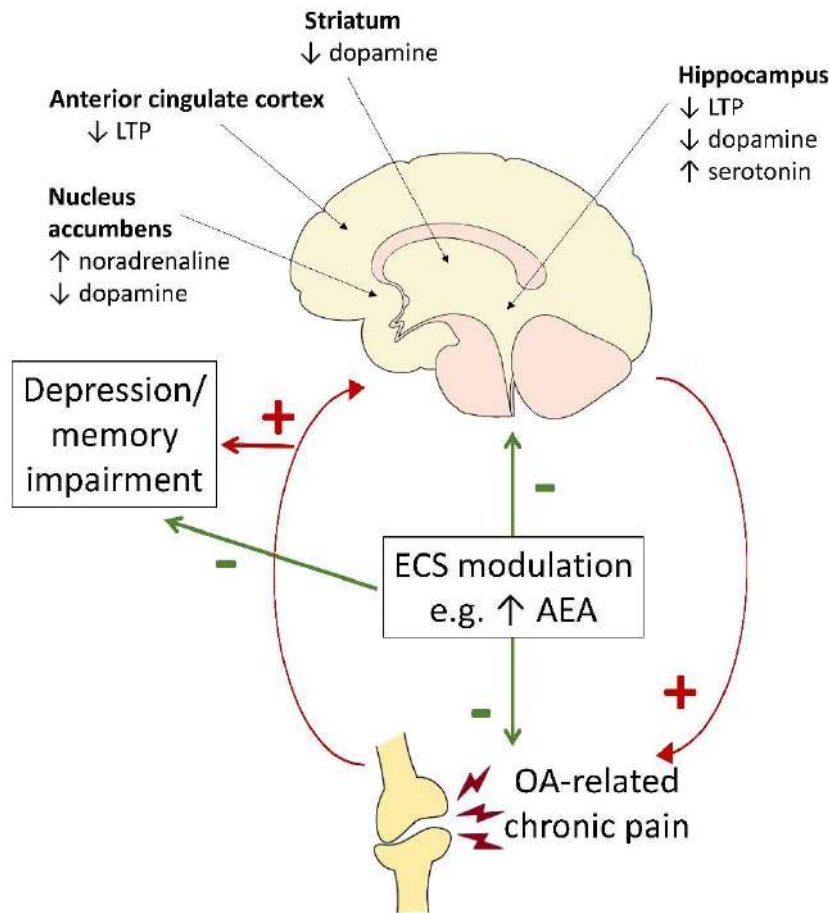


Figure 5: Schematic representation of the link between OA-related chronic pain, brain disturbances that contribute to cognitive changes (mood and memory impairment) and the potential modulation of ECS as a therapeutic strategy.

One-fifth of OA patients also suffer from depression (Stubbs *et al.*, 2016). According to Zheng *et al.*, the presence of depression in knee OA patients was associated with having younger age, a higher body mass index, greater pain, stiffness, lower education level, having more than one comorbidity and having two or more painful sites (Zheng *et al.*, 2021). OA pain also causes increased fatigue and reduced sleep quality, which can contribute to depression development (Fertelli & Tuncay, 2019). Hip-OA patients had decreased performance in neuropsychological tests (including verbal and visual short-term and long-term memory, and selective attention) compared to healthy controls (Kazim *et al.*, 2022). Six months after a total hip arthroplasty, which was connected with pain reduction, OA patients showed improvement in attention, working speed, concentration, visual construction and memory (semantic and episodic) (Strahl *et al.*, 2022). There is evidence that OA pain may disrupt neural circuits within the brain and neurotransmitters levels, in different pain models a disrupted monoamine transmission within the brain was reported. For example, mesostriatal dopaminergic neurons degradation

contributes to chronic pain development in rats (Takeda *et al.*, 2005), while in humans, diminished dopaminergic activity may be observed during chronic pain conditions (Taylor *et al.*, 2016). MIA-induced OA triggered anxiety-like behavior measured by the Elevated Plus Maze test as well as changes in noradrenaline and serotonin levels in the striatum, frontal cortex and nucleus accumbens. Moreover, advanced network analysis revealed that noradrenaline and serotonin neurotransmission in the nucleus accumbens are key circuits affected by chronic pain (Fig. 6, unpublished data, submitted to the Journal of Neurochemistry in January 2023: Mlost, J., Białoń, M., Kędziora, M., Wąsik, A., Warzecha, Ż., Starowicz, K., manuscript number: JNC-2023-0057). Moreover, recent findings confirm the role of inflammation and pro-inflammatory factors in depression development (Lee & Giuliani, 2019), whereas in the OA local inflammatory state is permanent. The research on OA and mood disorders are increasingly intensively studied, however still the direct mechanism of these changes as well as methods of its prevention are not well described.

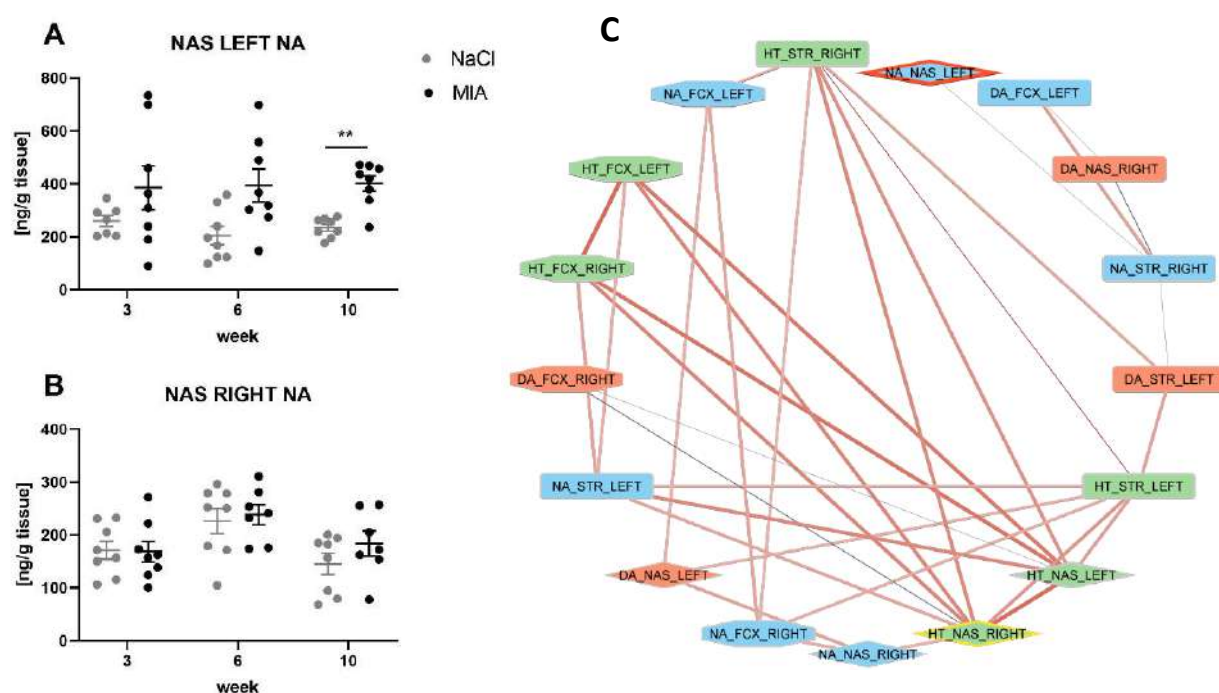


Figure 6: Monoamine levels and correlation graph in OA animal model (unpublished data, Mlost et al. manuscript number: JNC-2023-0057). Noradrenaline levels in left (A) and right (B) nucleus accumbens 3, 6 and 10 weeks post-MIA injection. C: Correlation networks based on Pearson's analysis in MIA animals. Red color: a positive correlation, blue: a negative correlation. Edge width represents the strength of the correlation. Yellow borders denote nodes with the highest vertex degree, red borders denote nodes selected by the Boruta algorithm.

1.4. The endocannabinoid system

The term “cannabinoids” covers a broad group of compounds, both naturally occurring and synthetic. More than 420 different compounds can be found in *Cannabis sativa*, the best known are: Δ 9-tetrahydrocannabinol (Δ 9-THC), Δ 8-tetrahydrocannabinol (Δ 8-THC), cannabidiol (CBD) and cannabinol (CBN). The oldest evidence of cannabis seeds presence is dated to 12000 years ago, in the Altai Mountains in Central Asia (Crocq, 2020). The role of cannabinoids in the treatment of pain was known as far back as ancient China, 4000 years ago (Brand & Zhao, 2017). Despite the fact, that cannabis was used for various pain-related disorders, the chemical structure and mechanism of its function remained unknown. The history of cannabinoid pharmacology discovery dates back to the 20th century, when Raphael Mechoulam described the CBD structure in 1963 and two years later solved the chemical structure of THC (Mechoulam & Gaoni, 1965). In 1988, Devane et al. characterized in the human and rat brain a receptor involved in cannabis effects and called it cannabinoid receptor type 1 (CB1) (Devane *et al.*, 1988), two years later it was cloned by L. Matsuda et al. (Matsuda *et al.*, 1990). This discovery has brought a highly important question about the existence of an endogenous ligand for the CB1 receptor, since until then only plant-derived compounds acting on CB1 have been known. This prompted intensified laboratory research on the endocannabinoid system (ECS) and led to the discovery of the first endocannabinoid: anandamide (AEA) (Devane *et al.*, 1992) and cannabinoid receptor type 2 (CB2) (Munro *et al.*, 1993). The discovery of endogenous cannabinoid ligands initiated a new chapter in pharmacological research. Over the past 30 years, the knowledge about the ECS has expanded significantly. A precise pathway of synthesis and degradation of two main endocannabinoids: AEA and 2-arachidonyloglycerol (2-AG), as well as molecular targets on which they act have been described.

Cannabinoid receptors are 7-transmembrane $G_{i/o}$ -coupled receptors (GPCR), which (in contrast to most GPCRs) have more than one endogenous ligand (Di Marzo & De Petrocellis, 2012). Their activation leads to the inhibition of adenylate cyclase and stimulation of mitogen-activated protein kinases (MAPKs). CB1 receptors also cause inhibition of voltage-gated calcium channels, activation of K^+ channels and neurotransmitter release on the presynaptic site (Kendall & Yudowski, 2017). CB1 receptors were first discovered in the brain tissue, since they are mostly localized in the central nervous system (CNS). It was initially thought that CB1 receptors are absent at the periphery, however, more detailed studies confirmed their presence in other than nervous tissues. Due to the central localization, CB1 receptors are primarily

responsible for THC adverse effects, such as euphoria, heightened sensory perception, altered perception of time, catalepsy, or hypothermia (Danovitch & Gorelick, 2012). In turn, CB2 receptors and mostly peripherally located, however, a smaller amount was also found in the CNS (Shahbazi *et al.*, 2020). This type of receptor was originally found in the immune cells and that is why CB2 agonists may play an important role in immunological response modulation (Munro *et al.*, 1993). Because CB2 receptors are localized in a very small quantity in the CNS, CB2 modulators offer greater hope for clinical usage, since they are devoid of many of the side effects that CB1 agonists possess.

Endocannabinoids are lipid compounds which bind and activate CB1 and CB2 receptors. They are not stored in the cell, but synthesized and released on demand and mediate short- and long-term synaptic plasticity via retrograde signaling at both excitatory and inhibitory synapses. However, there is evidence that endocannabinoids may also signal in a non-retrograde manner (Castillo *et al.*, 2012). The synthesis and degradation processes are enzymatically regulated. AEA is produced from N-acyl-phosphatidylethanolamine (NAPE) with N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) involvement and acts on both CB1 and CB2 receptors, as well as other receptors such as transient receptor potential cation channel subfamily V member 1 (TRPV1) and orphan receptors (e.g., GPR55). There are also several alternative pathways of AEA synthesis, for example by phospholipase C (PLC), inositol 5' phosphatase (SHIP1) and non-receptor protein tyrosine phosphatase 22 (PTPN22). Additionally, AEA can be synthesized from NAPE with α/β -hydrolase domain 4 (ABHD4) and lysophospholipase D isoforms 1 and 3 (GDPD1,3), or soluble phospholipase A2 (sPLA2), glycerophosphodiester phosphodiesterase 1 (GDE1) and GDPD1,3. AEA's main degradation pathway is mediated by fatty acid amide hydrolase (FAAH) and N-acylethanolamine acid amide hydrolase (NAAA) enzymes to arachidonic acid (AA), glycerol (G) and ethanolamine (EtA). On the alternative pathway AEA is degraded by cyclooxygenase 2 (COX-2) to prostaglandins (PG), prostamides (PM) and prostaglandin ethanolamides (PG-EA); by P450 cytochrome monooxygenases (P450s) to epoxyeicosatrienoyl-ethanolamide (EET-EA) and by 5-, 12-, 15-lipoxygenases (LOXs) to hydroxyeicosatetraenoic acids (HETEs), hydroxyeicosatetraenoyl-ethanolamides (HETE-EAs). These products of AEA degradation might be responsible for the inflammatory reaction (Blankman & Cravatt, 2013).

2-AG is synthesized from diacylglycerols (DAGs) with diacylglycerol lipase (DAGL) α and β . 2-AG acts via CB1 and CB2 receptors and is degraded to AA, G and EtA mainly by monoacylglycerol lipase (MAGL), but also on additional pathways by the hydrolase domain

containing 6/12 (ABHD6/12). Similarly to AEA, 2-AG can also be degraded by COX-2 enzyme to PG and PM. AA can also be degraded by LOXs to HETEs, HETE-EAs and leukotrienes (L) (Maccarrone, 2017; Donvito *et al.*, 2018; Biringer, 2021). A schematic representation of AEA and 2-AG synthesis and degradation pathways is presented in Fig. 7.

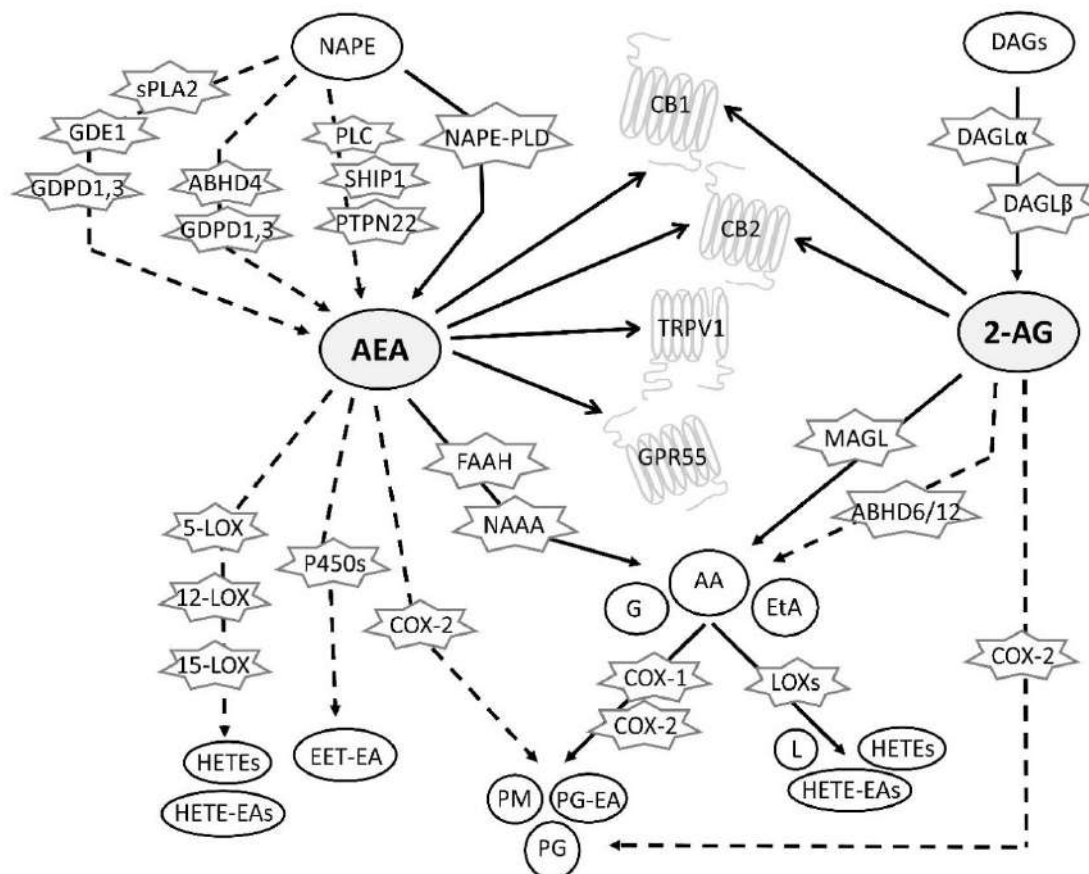


Figure 7: Schematic representation of two main endocannabinoids, AEA and 2-AG, synthesis and degradation pathways. Main synthesis and degradation pathways are marked as solid lines, alternative pathways are marked with dashed lines. Detailed description in the above text.

NAPE-PLD is also responsible for the synthesis of other (besides AEA) N-acylethanolamines: palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) – endocannabinoid-like lipid mediators responsible for example for the inflammatory processes control (Petrosino & Di Marzo, 2017; Payahoo *et al.*, 2018).

1.5. Endocannabinoid system changes during chronic pain and osteoarthritis

It is well established that the ECS is involved in the modulation of various physiological processes such as memory, temperature, mood, food intake, pain and cognition. During a chronic pain state, the ECS balance is disrupted (Sagar *et al.*, 2012). A diminished level of endocannabinoids was found in the saliva of chronic orofacial pain patients compared to controls (Haviv *et al.*, 2022). Chronic migraine patients have disrupted levels of enzymes

responsible for endocannabinoids synthesis and degradation in the peripheral blood (Greco *et al.*, 2021). An expression of enzymes responsible for AEA degradation during pain development in the chronic constriction injury (CCI) model was proven to be elevated in the dorsal root ganglia (DRGs) and spinal cord (Malek *et al.*, 2014). In a spared nerve injury (SNI) rat model of chronic pain, an initial increase in 2-AG release in the medial prefrontal cortex (mPFC) resulted in a loss of CB1 receptors function on GABA-ergic interneurons in the mPFC, which triggered depressive-like behavior in animals (Mecca *et al.*, 2021). It was proven, that endocannabinoids are present in the synovial fluid of OA dogs, moreover, 2-AG and OEA levels were elevated in the OA knee in comparison to the contralateral knee (Valastro *et al.*, 2017). In the synovial tissue of human OA and rheumatoid arthritis (RA) patients, both CB1 and CB2 receptors are present, while in their synovial fluid AEA and 2-AG were identified. Neither AEA nor 2-AG was detected in synovial fluid from healthy controls (Richardson *et al.*, 2008). Synovial fibroblasts from RA patients produce AEA, and this endocannabinoid is likely responsible for a cortisol-mediated increase of cellular adhesion (Lowin *et al.*, 2012). In obese OA patients, a negative correlation between cerebrospinal fluid 2-AG and leptin levels was found (Nicholson *et al.*, 2015).

The presented literature data indicate the role of ECS in OA progression and chronic pain development, nevertheless, studies investigating the role of ECS components in OA development are still limited. Therefore, the present thesis preparation investigated this area, with a particular focus on the description of AEA changes during OA development in a rat model.

2. Aim of the project and research hypothesis

OA-related chronic pain treatment is a complex problem. The details of the mechanism of pain development are not well-described, especially the role of ECS in OA development is highly understudied. On January 2023, a PubMed search for the phrases “(osteoarthritis) and (endocannabinoid)” showed 57 results, of which only 37 were research articles. Even less research evaluates ECS’s role in mood impairment during OA. The available studies suggest that ECS is involved in OA pathology, however, the details of this link remain unclear. The changes within the ECS can be evoked by OA itself, e.g., by inflammatory reaction occurring within the affected joint (see Fig. 8A). Cannabinoid receptors, especially CB2 type, are present in a great extent on the immune cells, endocannabinoids are also metabolized

by COXs and LOXs to prostaglandins, prostanoids and leukotrienes which can intensify immune reaction. On the other hand, ECS changes can develop as a result of adaptive shifts caused by the OA-related chronic pain, which can cause nerve sensitization and therefore induce changes within the ECS (see Fig. 8B). Disrupted ECS balance can also work in a vicious cycle and therefore contribute to pain progression and impede treatment possibilities (see Fig. 8B). What is more, very little is yet known about the role of ECS disruption in the accompanying OA patients' mood impairment, which may be connected with chronic pain and ECS imbalance in the CNS (see Fig. 8C).

Therefore, the main aim of this dissertation was to describe the changes occurring during OA progression in terms of local (within the joint) inflammatory changes and the ECS central alterations in a rat model, as well as to evaluate the role of ECS modulators in OA-related chronic pain and mood impairment treatment. The hypothesis of this research implies that chronic pain development, mood impairment and ECS balance disruption are connected and may influence each other. Moreover, ECS modulation can inhibit chronic pain development (or diminish existing pain), as well as prevent mood impairment.

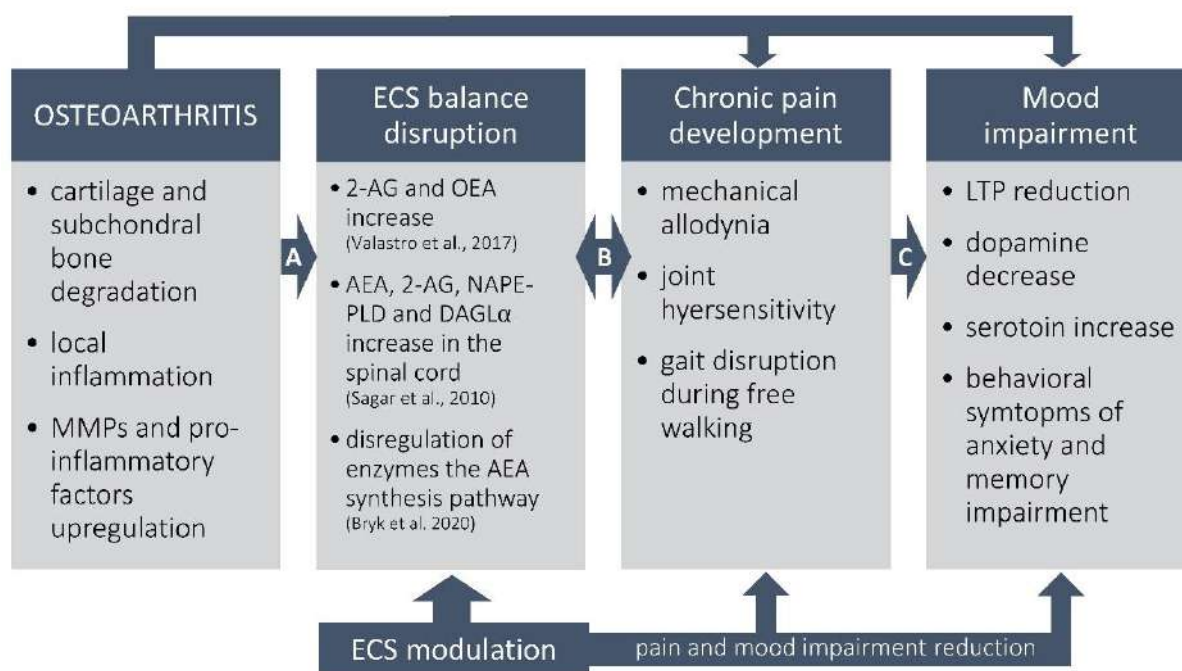


Fig. 8: Schematic representation of the research problem and the aim of the project.

In sum, the main objectives of this project were to:

- 1) describe degenerative and inflammatory alterations that occur during OA progression in the joint tissues for a better understanding of the pathology and mechanism of disease development in the MIA-induced rat OA model;

- 2) elucidate the role of ECS in the OA pathogenesis, by investigating the role of multiple AEA production and degradation pathways during disease progression;
- 3) examine the effect of AEA degradation inhibition on pain and on concomitant changes in LTP and monoamine levels in the hippocampus of OA animals.

Scientific publications



Sodium Monoiodoacetate Dose-Dependent Changes in Matrix Metalloproteinases and Inflammatory Components as Prognostic Factors for the Progression of Osteoarthritis

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Osteoarthritis (OA) is a degenerative joint disease that primarily affects people over 65 years old. During OA progression irreversible cartilage, synovial membrane and subchondral bone degradation is observed, which results in the development of difficult-to-treat chronic pain. One of the most important factors in OA progression is joint inflammation. Both proinflammatory and anti-inflammatory factors, as well as extracellular matrix degradation enzymes (matrix metalloproteinases (MMPs)), play an important role in disease development. One of the most widely used animal OA models involves an intra-articular injection of sodium monoiodoacetate (MIA) directly into the joint capsule, which results in glycolysis inhibition in chondrocytes and cartilage degeneration. This model mimics the degenerative changes observed in OA patients. However, the dose of MIA varies in the literature, ranging from 0.5 to 4.8 mg. The aim of our study was to characterize grading changes after injection of 1, 2 or 3 mg of MIA at the behavioral and molecular levels over a 28-day period. In the behavioral studies, MIA injection at all doses resulted in a gradual increase in tactile allodynia and resulted in abnormal weight bearing during free walking sequences. At several days post-OA induction, cartilage, synovial membrane and synovial fluid samples were collected, and qPCR and Western blot analyses were performed. We observed significant dose- and time-dependent changes in both gene expression and protein secretion levels. Inflammatory factors (CCL2, CXCL1, IL-1 β , COMP) increased at the beginning of the experiment, indicating a transient inflammatory state connected to the MIA injection and, in more severe OA, also in the advanced stages of the disease. Overall, the results in the 1 mg MIA group were not consistently clear, indicating that the lowest tested dose may not be sufficient to induce long-lasting OA-like changes at the molecular level. In the 2 mg MIA group, significant alterations in the measured factors were observed. In the 3 mg MIA group, MMP-2, MMP-3, MMP-9, and MMP-13 levels showed very strong upregulation, which may cause overly strong reactions in animals. Therefore, a dose of 2 mg appears optimal, as it induces significant but not excessive OA-like changes in a rat model.

Keywords: osteoarthritis, chronic pain, matrix metalloproteinases, cartilage, synovial membrane, synovial fluid, inflammation, pain

INTRODUCTION

Knee osteoarthritis (OA) is one of the leading causes of global chronic disability. A disease that affects joint tissues, including cartilage, subchondral bone, ligaments, and muscles, OA manifests with chronic pain, joint stiffness/tenderness, loss of flexibility, and cracking sounds while moving. In the United States in 2016, 14 million people suffered from symptomatic knee OA. More than half of all patients were <65 years old (Deshpande et al., 2016). Risk factors favoring disease development include age, obesity, joint injuries, abnormal joint loading (e.g., excessive sports), gender (women are more likely to develop foot, hip and knee OA than men) and genetic factors (Vina and Kent Kwoh, 2018). Recently, the Osteoarthritis Research Society International (OARSI) revised the OA definition, adding an inflammatory component as a crucial factor contributing to disease development. An important process reported in OA progression is synovitis, during which a number of inflammatory changes occur in the synovial membrane. This contributes to local inflammation, which leads to further cartilage degeneration and inflammatory factor influx (Mathiessen and Conaghan, 2017).

Several of the most important factors are cytokines, both proinflammatory, e.g., interleukin 1 β (IL-1 β), tumor necrosis factor α (TNF α), IL-6, IL-15, IL-17, and IL-18, and anti-inflammatory, such as IL-4, IL-10, and IL-13 (Wojdasiewicz et al., 2014). Importantly, a low-grade inflammatory state is present in OA from the very early stages, long before external symptoms can be detected by patients (Scanzello, 2017), and contributes to pain sensitization. Knee synovitis can also be used as an OA diagnostic tool (Berlinberg et al., 2019). Moreover, inflammation involves activation of enzymes involved in articular extracellular matrix (ECM) degradation – the matrix metalloproteinases (MMPs), e.g., MMP-2, MMP-3, MMP-9, MMP-13. These enzymes are members of a large family of zinc-dependent proteolytic enzymes. They are involved in shaping collagen, proteoglycan aggrecan, and non-collagen matrix components in joint degradation (Mehana et al., 2019). MMPs belong to one of three groups. MMP-2 and MMP-9 are gelatinases that digest denatured collagens and gelatins. MMP-2 (but not MMP-9) digests type I, II, and III collagens. MMP-2 in humans is important for osteogenesis, as mutations in human MMP-2 result in an autosomal recessive form of multicentric osteolysis (Martignetti et al., 2001). Both MMP-2 and MMP-9 are involved in inflammatory processes (Hannocks et al., 2019). MMP-3, also called Stromelysin 1, is responsible for digesting ECM components and activates a number of proMMPs (e.g., proMMP-1). MMP-13 is a collagenase that cleaves interstitial collagens type I, II, and III at a specific site three-quarters of the way from the N-terminus and digests both ECM and non-ECM components (Visse and Nagase, 2003).

Unfortunately, current OA therapeutics are limited to reducing pain and relieving bothersome symptoms only. A greater understanding of the mechanisms underlying the disease is necessary for creating more effective therapies. An important strategy for OA investigation is the creation of more representative animal models. Among several animal OA models,

the sodium monoiodoacetate (MIA) intra-articular (i.a.) injection model is widely used for pain research and efficacy evaluation of therapeutic interventions (Lampropoulou-Adamidou et al., 2014). This chemical model mimics changes observed in patients very well. Malek et al. described changes in knee sensitivity and knee morphometric analysis in response to 3 mg of MIA i.a. injection up to 28 days post-injection (Malek et al., 2015). Knee changes 28 days post-MIA injection were irreversible and closely reflected changes in severe, advanced OA stages in human patients. In turn, Pajak et al. demonstrated biphasic pain progression in both behavioral and biochemical tests. An initial pain threshold lowering was associated with an inflammatory reaction caused by i.a. injection. Nevertheless, after 14 days, a stable, lasting reduction in pain threshold was observed (Pajak et al., 2017). The dose of MIA used to induce OA in rats varies in the literature from 0.5 to 4.8 mg; however, 1 or 3 mg is the most commonly used dose (Havelin et al., 2016; Allen et al., 2017; Haywood et al., 2018; Lee et al., 2018; Lockwood et al., 2019; Que et al., 2019; Piao et al., 2020). This could result in either peripheral mechanisms of joint damage in OA development and/or centrally mediated mechanisms, both of which may be important for future disease-modifying drug design. Therefore, the aim of our study was to assess the complete characteristics of grading MIA-induced OA (1, 2 or 3 mg of MIA i.a.) in a rat model at both the behavioral and molecular levels over a 28-day period. The role of inflammatory components and ECM MMPs in a rat joint model with progressive OA severity was investigated. Differences elicited by selected MIA doses were noted; our research allowed us to identify the smallest dose needed to effectively induce OA in a rat model. Overall, the obtained data allow for detailed description of changes occurring in joint tissues (synovial membrane, cartilage and synovial fluid) during disease progression, which provides a superior understanding of OA pathology and mechanisms.

MATERIALS AND METHODS

Animals

In all experiments, male Wistar rats initially weighing 225–250 g were used (Charles River, Hamburg, Germany). Animals were housed 5 per cage in a 12/12 h light/dark cycle with food and water *ad libitum*. Behavioral experiments were performed between 9:00 and 12:00 am. Experiments were approved by the Local Bioethics Committee of the Maj Institute of Pharmacology (Cracow, Poland), approval numbers: 938/2012, 125/2018. Separate sets of animals were used for behavioral and biochemical studies. Depending on the experimental group, on days 2, 7, 21 or 28 of the experiment, animals were sacrificed, and joint tissue samples (cartilage, synovial membrane, synovial fluid) were collected. Healthy animals (examined via biochemical analysis) were sacrificed on various days (1 or 2 animals in each experimental day) to minimize the differences associated with the duration of the experiment. No differences in biochemical analysis were observed in the healthy group. In behavioral tests, animals were tested prior to MIA injection (day 0); to apply the 3R principle of animal testing, we

determined that no healthy group was necessary. Moreover, according to the 3R principle and based on our previous behavioral research were 1 mg (Mlost et al., 2018b; Mugnaini et al., 2020; Mlost et al., 2021) or 3 mg of MIA (Malek et al., 2015; Pajak et al., 2017; Mlost et al., 2018a; Bryk et al., 2020) were successfully used, we decided to examine only 1 and 3 mg MIA doses in behavioral tests to minimize the number of animals exposed to painful procedures.

Induction of OA

Animals were briefly anesthetized with 5% isoflurane (Aerrane, Baxter, United States) in 100% oxygen (3.5 L/min). The rear right knee skin surface was shaved and swabbed with 75% ethanol. Joint damage was induced by a single i.a. injection of 1, 2 or 3 mg of MIA (Sigma-Aldrich, Saint Louis, United States) dissolved in 50 μ l of 0.9% physiological saline via a 30 G \times 1/2" needle. All surgical procedures were performed under sterile conditions. After OA induction, animals were moved back into their home cages and observed until full recovery from anesthesia. Healthy animals did not undergo any injection. After i.a. injection, rats were maintained under the same conditions as the preoperative period. Changes in kinetic weight bearing (evaluated in the kinetic weight bearing test; KWB) and mechanical withdrawal thresholds (evaluated in von Frey's test) were recorded prior to i.a. injection (day 0) and 2, 7, 14, 21 and 28 days post-injection. Experimental groups consisted of $n = 5-8$ animals (for KWB test), $n = 8$ animals (for Von Frey test) and $n = 5-6$ animals for biochemical studies. In rare cases, individual samples had to be excluded from the analysis due to abnormalities during isolation or sample contamination, and groups were indicated by an appropriate n number.

Von Frey Test

For the assessment of mechanical allodynia, calibrated von Frey monofilaments (Bioseb, France) were used. Rats were placed in Plexiglas cages with a wire net floor 5 min before the experiment. Von Frey filaments were applied to the mid plantar surface of the ipsilateral hind paw according to the up and down method (Chaplan et al., 1994; Deuis et al., 2017). Each filament was applied three times for an approximately 2–3 s period or until a withdrawal response was evoked. After response, the paw was retested with monofilaments in descending order until no response occurred, at which point monofilaments were again applied in ascending order until the response could once again be evoked. The monofilament that evoked the final reflex was noted as the paw withdrawal latency. The strength of the von Frey monofilament bending forces was as follows: 0.4; 0.6; 1.0; 1.4; 2; 4; 6; 8; 10; 15 and 26 g as a cut-off for response.

Kinetic Weight Bearing

To characterize pain behavior in the MIA model, KWB, a novel instrument developed by Bioseb (France), was used. Sensors placed on the ground measure the weight borne by each individual paw during the walking sequence of a freely moving animal, while a built-in camera detects the center of gravity of the animal. Data collection was terminated when 5 validated runs were obtained, or after 6 min of acquisition. If the animal did not run during this time window, the measurement was repeated at

the end of the session. Those who failed to make at least one validated run during the second session were excluded from the analysis (one animal in 3 mg of MIA group in day 7 and 28). Rats were trained to move through the test corridor (50 \times 130 cm) for a week before the actual experiment. Measurements were made directly before MIA administration and 2, 7, 21 and 28 days post-MIA treatment. All recorded data were validated by an observer blinded to the study. The final results include information about the mean peak force and surface area applied by each rear paw. The presented results discuss only the most relevant parameters in the context of OA research: peak force (centinewton, cN) – the mean of the maximum forces of each rear paw and peak surface (cm²) – the mean of the maximum surface of each rear paw.

RNA and Protein Isolation

Cartilage from the medial femoral condyle and synovial membrane fragments were collected with surgical scissors. After tissue collection, each sample was placed in RNA-later solution (Invitrogen, United States) and stored at -80°C . RNA and protein from the synovial membrane were isolated using TRIzol Reagent (Invitrogen, United States) according to the manufacturer's protocol. Extraction of high-quality RNA from cartilage was performed according to a protocol published by Le Bleu et al. (2017). Synovial fluid was collected by rinsing the joint capsule with 50 μ l of physiological saline and aspirating the fluid with a syringe with a 25 G \times 5/8" needle. Then, synovial fluid was immediately placed on ice and frozen at -80°C . Equal volumes of samples were lyzed with 2% SDS and centrifuged at 12,000 \times g. The supernatant was collected for immunoblotting.

Total RNA levels were measured with a Nanodrop spectrophotometer (ND-1000, Nanodrop; Labtech International, United Kingdom). Each sample was equalized to a concentration of 1 μ g/ μ l and reverse transcribed to cDNA using an NG dART RT kit (EURx, Poland) according to the manufacturer's protocol. qPCR reactions were carried out using iTaq Universal Probes Supermix (Bio-Rad, United States) and TaqMan Assays (Thermo Fisher, Applied Biosystems, United States) in a Thermal Cycler CFX96 (Bio-Rad, United States). Cycle threshold values were calculated automatically via CFX Manager software. RNA abundance was calculated as $\text{ddCT } 2^{-(\text{threshold cycle})}$ and *B2m* normalized. The TaqMan assay protocols used in the study are presented in the **Supplementary Material**.

Western Blot

The protein levels in the cartilage and synovial samples were estimated via BCA kit (Thermo-Fischer, United States), and equal amounts of proteins were denatured in 4 \times Laemmli sample buffer (Bio-Rad, United States) and denatured at 96°C for 6 min. Equal volumes of synovial fluid samples were lyzed with 2% SDS and denatured with Laemmli buffer, such as cartilage and synovial samples. An equal amount of protein from the various experimental groups was separated on Criterion TGX 4–20% precast gels (Bio-Rad, United States) and transferred onto a PVDF membrane (Roche, Switzerland). After blocking with blocking reagent from the BM Chemiluminescence Western Blotting Kit (Roche, Switzerland), membranes were incubated

overnight with primary antibodies (detailed information in the **Supplementary Material**) using a SignalBoost Immunoreaction Enhancer Kit (Merc, Germany). The amount of β -actin was measured on the same membrane on which the other proteins were measured by mild stripping (using a protocol published by Abcam). In control samples of synovial fluid concentration of proteins was very low, so this tissue was normalized to volume and detection of reference proteins was not possible.

Statistical Analysis

Statistical analysis was performed using Statistica 13 (SatSoft Software, Tulsa, Oklahoma, United States), and graphs were generated using Prism 8 (GraphPad Software La Jolla, California, United States). Data are presented as the mean \pm SEM; whiskers on the boxplot graphs show the minimum and maximum values. The results of the von Frey test were evaluated by two-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test. The results from the KWB test were evaluated by one-way ANOVA on day 0 or two-way ANOVA on the following experimental days, followed by Tukey's HSD post-hoc test. The qPCR and Western blot results were evaluated by one-way ANOVA followed by Dunnett's post-hoc test, treating healthy animals as the control group. In behavioral tests, each group consisted of eight animals (von Frey test) or 5 animals (KWB test). In biochemical analyses, the experimental group sizes consisted of 3-6 samples per group (qPCR) or 4-5 samples per group (Western blot). Values of $p < 0.05$ (denoted * or #), $p < 0.01$ (denoted ** or ##), and $p < 0.001$ (denoted *** or ###) were considered to be statistically significant.

RESULTS

A significant difference in pain threshold was observed in both MIA doses, using the von Frey and KWB tests. Differences in tactile allodynia, as well as in paw pressure and surfaces applied to the ground between the rear right and rear left paws on subsequent experimental days were observed. Significant signs of pain were noticed from the 2nd day post-MIA treatment and persisted until the day 28. In biochemical analyses, statistically significant dose- and time-dependent changes in MMP and inflammation-related gene expression (qPCR) and protein production (Western blot technique) were observed. The results will be explained in details in the following sections.

Development of OA-Related Allodynia

MIA injection at both investigated doses (1 and 3 mg MIA i.a.) resulted in the development of mechanical allodynia in the ipsilateral hind paw (rear right) on day 2 ($p < 0.05$ for 1 mg of MIA and $p < 0.001$ for 3 mg of MIA; **Figure 1**), a phenomenon not observed on day 0. On day 2 a significant difference between tested doses was also observed ($p < 0.05$; **Figure 1**), which was not observed in the following days. OA rats showed a significant gradual reduction in withdrawal threshold that progressed with time in both experimental groups. The lowest mechanical withdrawal latency was observed at day 28. From day 7 on, the effect was similar in both groups (1 and 3 mg of MIA; $p <$

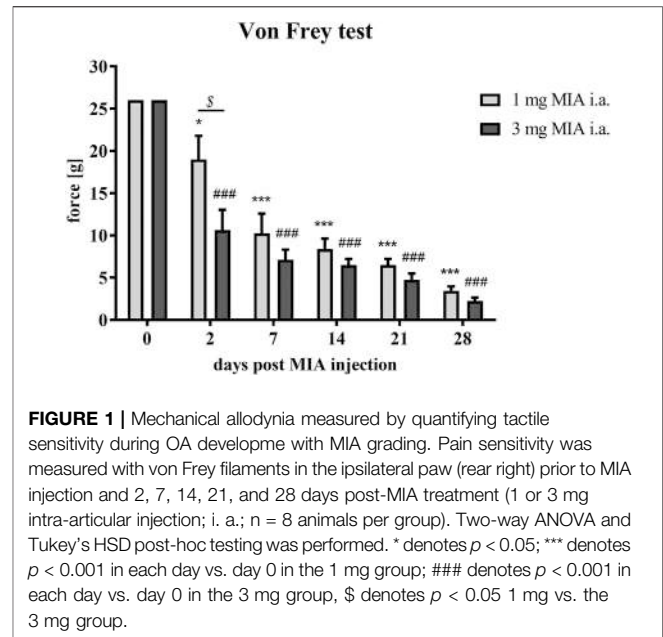


FIGURE 1 | Mechanical allodynia measured by quantifying tactile sensitivity during OA development with MIA grading. Pain sensitivity was measured with von Frey filaments in the ipsilateral paw (rear right) prior to MIA injection and 2, 7, 14, 21, and 28 days post-MIA treatment (1 or 3 mg intra-articular injection; i. a.; $n = 8$ animals per group). Two-way ANOVA and Tukey's HSD post-hoc testing was performed. * denotes $p < 0.05$; *** denotes $p < 0.001$ in each day vs. day 0 in the 1 mg group; ### denotes $p < 0.001$ in each day vs. day 0 in the 3 mg group; \$ denotes $p < 0.05$ 1 mg vs. the 3 mg group.

0.001; **Figure 1**). These results indicate that animals in whom OA symptoms were induced with both MIA doses, developed an OA-associated allodynia.

Paw Weight Bearing Differences During Free Walk

Rats were evaluated for paw force and surface distribution during a free walking sequence. A significant difference in both paw force (**Figures 2A–F**) and surface (**Figures 3A–F**) applied to the ground during free walking was observed for both MIA doses used to induce OA. Differences were observed in both experimental groups (1 or 3 mg MIA i.a.) on almost all experimental days (except days 7 and 14 in the peak surface analysis of the 1 mg MIA group); however, a stronger effect was elicited by 3 mg of MIA. Therefore it can be concluded that both MIA doses induced weight bearing differences in MIA-treated rats.

Inflammation During OA Progression

In the present study, cartilage oligomeric matrix protein (COMP) levels were measured in joint tissues isolated from osteoarthritic animals. In cartilage samples, a U-shaped pattern of protein production was observed following injection, with an initial decrease (significant only on day 7 in the 3 mg MIA group; $p < 0.05$; **Figure 4C**) and subsequent return to baseline (in the 2 and 3 mg MIA groups) or an increase (in the 1 mg MIA group; $0.01 > p > 0.001$; **Figures 4A–C**). In synovial membranes, in contrast, an initial increase (day 2) was solely observed in all experimental groups (MIA 1 mg; $0.01 > p > 0.001$; MIA 2 mg; $p < 0.05$; MIA 3 mg; $p < 0.001$; **Figures 4D–F**). In synovial fluid samples, an increase in COMP levels was detected in the latter stages of OA – exclusively on day 28 post-MIA treatment – in all groups (MIA 1 mg; $0.01 > p > 0.001$; MIA 2 mg; $p < 0.05$; MIA

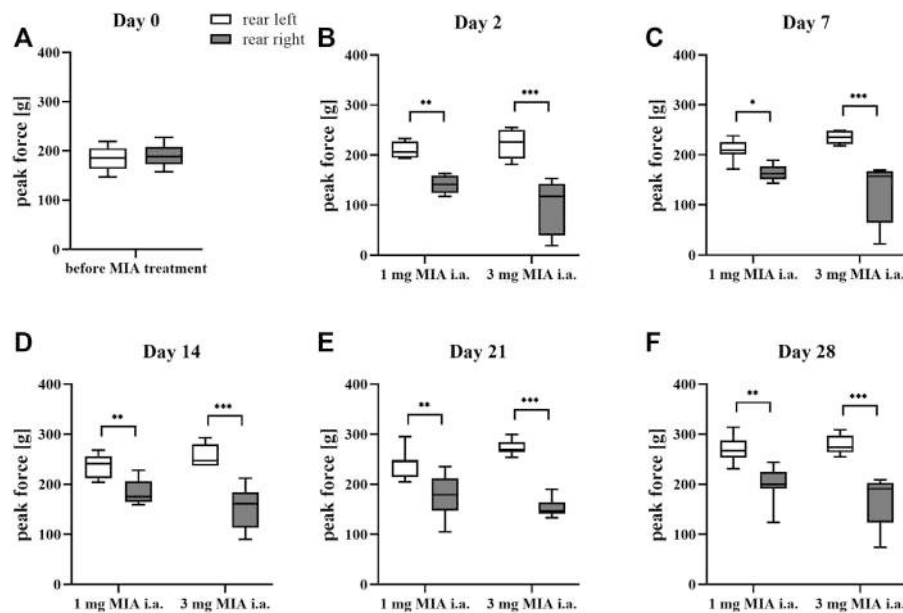


FIGURE 2 | Gait analysis in OA rats recorded with KWB by measuring the distribution of paw force applied to the ground during a free walk (for 1 and 3 mg of MIA i.a. groups) during a free walk. Measurements taken prior to the MIA injection (day 0; **A**) and 2, 7, 14, 21, and 28 days (**B–F**) post-MIA treatment ($n = 5–8$ animals per group). One-way ANOVA on day 0 or two-way ANOVA on the following experimental days and Tukey's HSD post-hoc tests were performed. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$.

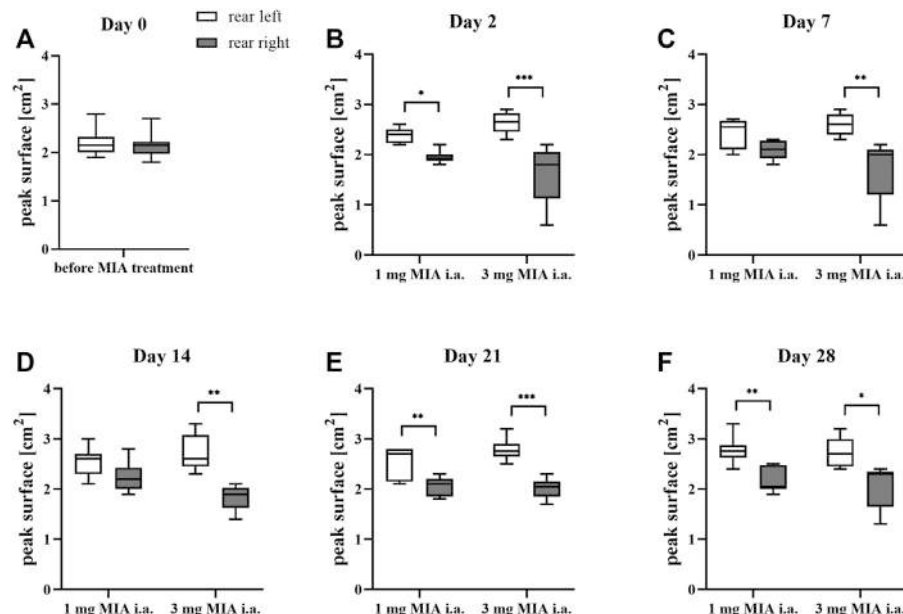


FIGURE 3 | Gait analysis in OA rats recorded with KWB by measuring the distribution of paw surfaces applied to the ground during free walks (for 1 and 3 mg of MIA i.a. groups) during a free walk. Measurements taken prior to the MIA injection (day 0; **A**) and 2, 7, 14, 21, and 28 days (**B–F**) post MIA treatment ($n = 5–8$ animals per group). One-way ANOVA on day 0 or two-way ANOVA on the following experimental days and Tukey's HSD post hoc test were performed. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$.

3 mg; $p < 0.001$; **Figures 4G–I**). The levels of various inflammation-related factors were measured only in the synovial fluid. Chemokine (C-C motif) ligand 2 (CCL2) levels

showed an initial increase (day 2 at all MIA doses; $0.01 > p > 0.001$; **Figures 5A–C**). In the 1 mg MIA group, the CCL2 level returned to baseline, whereas in the 2 mg MIA group, it remained

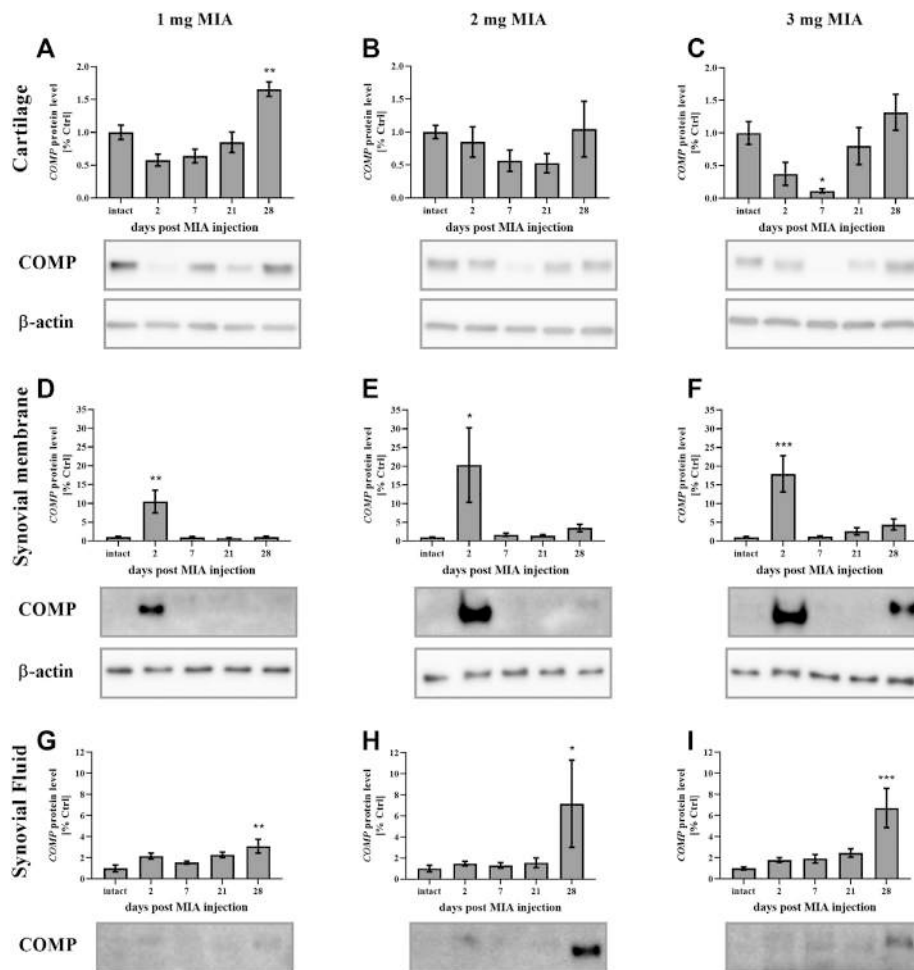


FIGURE 4 | Changes in COMP protein levels in cartilage (A–C), synovial membrane (D–F) and synovial fluid (G–I) samples from osteoarthritic rats as measured by Western blot assay. Tissues were collected 2, 7, 21 or 28 days after OA induction. Results are presented as mean group fold change \pm SEM in comparison to the control group (healthy animals), $n = 4$ –5 samples per group. Data were analyzed with one-way ANOVA followed by Dunnett's post-hoc test. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

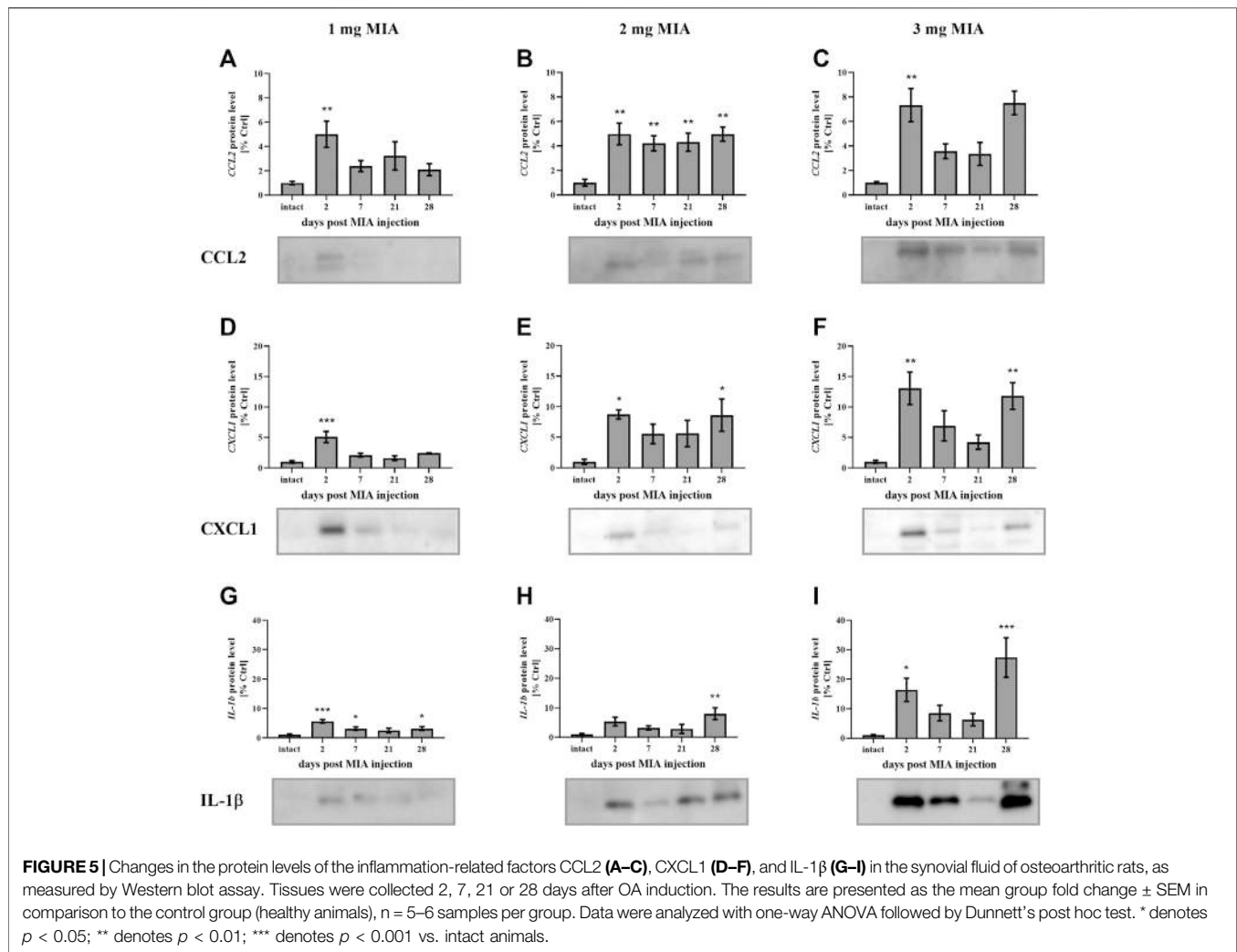
elevated throughout the experiment ($0.01 < p < 0.001$; **Figures 5A,B**). In the 3 mg MIA group, the CCL2 level was increased throughout the entire experimental period; however, these differences reached statistical significance only on day 2 ($0.01 > p > 0.001$; **Figure 5C**). A similar pattern was observed for chemokine (C-X-C motif) ligand 1 (CXCL1). The protein level was increased in synovial fluid by the 2nd day post-OA induction in all groups (MIA 1 mg; $p < 0.001$; MIA 2 mg; $p < 0.05$; MIA 3 mg; $0.01 > p < 0.001$; **Figures 5D–F**). In the 1 mg MIA group, this increase returned to baseline, whereas in the 2 and 3 mg MIA groups, it remained significantly upregulated on day 28 post-MIA treatment (MIA 2 mg; $p < 0.05$; MIA 3 mg; $0.01 > p > 0.001$; **Figures 5D–F**). The IL-1 β levels in the synovial fluid in the 1 mg MIA group were increased on days 2 ($p < 0.001$), 7 ($p < 0.05$) and 28 ($p < 0.05$) post-MIA treatment (**Figure 5G**). In the 2 mg MIA group, this expression level was increased only on day 28 post-MIA treatment ($0.01 > p > 0.001$; **Figure 5H**). At the highest MIA dose (3 mg), IL-1 β levels were increased on days 2

($p < 0.05$) and 28 ($p < 0.001$) post-OA induction (**Figure 5I**). The above results show a dose-dependent inflammation in joint tissues isolated from osteoarthritic animals. The strongest effect was observed in the group in which OA was induced with MIA at a dose of 3 mg, however, a comparable effect was triggered by 2 mg of MIA.

Matrix Metalloproteinase Expression Changes During OA Progression

Changes in MMP Gene Expression During OA Development

MMP gene expression was measured in synovial membrane and cartilage tissues of osteoarthritic rats. *Mmp-2* gene expression significantly increased in the later stages of OA. In the synovial membrane samples, an increase was observed 7 days post-MIA injection (at all MIA doses; MIA 1 mg; $0.01 > p > 0.001$; MIA 2 mg; $p < 0.001$; MIA 3 mg; $p < 0.001$; **Table 1**), 21 days post-MIA



injection (2 and 3 mg MIA groups; $0.01 < p < 0.001$ and $p < 0.001$ respectively) and 28 days post-MIA injection (1 and 3 mg MIA groups $0.01 < p < 0.001$ and $p < 0.001$ respectively). In the 2 mg MIA group, the *Mmp-2* expression level remained elevated on day 28 (similar to the 1 mg MIA group); however, these differences did not reach statistical significance. Similar results were observed in cartilage samples—an increase in *Mmp-2* abundance was observed starting at day 7 (in the 2 mg MIA group; $p < 0.05$) or day 21 (in the 1 and 3 mg MIA groups; $0.01 > p > 0.001$ and $p < 0.001$ respectively) that persisted until the end of the experiment (MIA 1 mg; $p < 0.05$; MIA 2 mg; $0.01 > p > 0.001$; MIA 3 mg; $p < 0.001$). The *Mmp-3* expression level in the 1 mg MIA group was elevated in the initial stages of OA (days 2 and 7; $0.01 > p > 0.001$; in the synovial membranes or day 2 in cartilage; $p < 0.05$). In the synovial membranes of the 2 mg MIA group, an increase in *Mmp-3* gene expression was observed starting at the beginning of the experiment; however, significance was reached only on day 21 ($p < 0.05$). In the cartilage samples, significant upregulation was observed on days 7 ($p < 0.05$) and 28 ($p < 0.001$) post-MIA treatment. In the synovial membranes samples of the 3 mg MIA group, *Mmp-3* expression was elevated throughout the

entire experimental period (day 2; 7; 21; $0.01 > p > 0.001$; day 28 $p < 0.001$); in cartilage, it was elevated only in the later stages (days 21 and 28; $0.01 > p > 0.001$ and $p < 0.001$ respectively). The *Mmp-9* expression level in the synovial membranes was upregulated in the 1 mg MIA group, with a significant increase on days 2 ($p < 0.05$), 7 ($p < 0.001$) and 28 ($p < 0.001$) post-MIA treatment. In the 2 mg MIA group, the *Mmp-9* level increased; however, group variances were too substantial to reach statistical significance. In the 3 mg MIA group, *Mmp-9* expression also increased throughout the entire experimental period, with a significant elevation observed on experimental days 7 ($p < 0.001$) and 21 ($0.01 > p > 0.001$). In the cartilage samples, a significant increase in *Mmp-9* levels was observed at the advanced OA stages (on day 21 in the 1 mg MIA group; $0.01 > p > 0.001$, on day 28 in the 2 mg MIA group; $p < 0.05$; and on days 7 and 21 in the 3 mg MIA group; $p < 0.05$). Nevertheless, a trend toward increasing expression was observed throughout the entire experiment, particularly in the synovial membrane samples. For *Mmp-13* gene expression, in the 1 mg MIA group, an elevation was observed only at the beginning of the experiment (day 2) in both tissue types (Synovial membrane; $p < 0.001$; Cartilage;

TABLE 1 | Transcript abundance levels of selected genes in synovial membrane and cartilage samples from osteoarthritic rats.

Tissue	Gene	MIA [mg]	Days post-MIA injection				
			Ctrl	2	7	21	28
Synovial membrane	<i>Mmp-2</i>	1	1.1 ± 0.2	1.7 ± 0.4	2,9** ± 0,5	1.7 ± 0.1	2,6** ± 0,1
		2		.9 ± 0.1	6,4*** ± 0,8	4,1** ± 1,0	2.5 ± 0.9
		3		1.1 ± 0.1	7,2*** ± 0,5	3,5*** ± 0,3	4,4*** ± 0,3
	<i>Mmp-3</i>	1	1.0 ± 0.1	6,4** ± 2,1	9,8** ± 2,0	2.3 ± 0.2	2.5 ± 0.6
		2		7.0 ± 1.1	5.9 ± 1.0	10,7* ± 5,3	2.9 ± 0.4
		3		7,3** ± 1,6	6,7** ± 1,2	7,4** ± 0,9	14,0*** ± 1,9
	<i>Mmp-9</i>	1	1,1 ± 0,2	9,6* ± 1,6	30,7*** ± 1,8	4,6 ± 2,1	35,8*** ± 4,9
		2		27.9 ± 4.4	26.5 ± 6.0	40.4 ± 15.4	36.9 ± 33.4
		3		28.6 ± 4.6	94,5*** ± 18,3	75,1** ± 25,8	20.6 ± 1.8
	<i>Mmp-13</i>	1	1,5 ± 0,7	179,2*** ± 55,6	53,2 ± 11,9	3,1 ± 1,3	3,2 ± 0,9
		2		163.8 ± 67.9	42.1 ± 5.2	137.1 ± 103.4	12.0 ± 5.4
		3		53.3 ± 17.2	65,0* ± 9,5	80,5* ± 28,8	67,8* ± 24,3
Cartilage	<i>Mmp-2</i>	1	1.1 ± 0.3	2.3 ± 0.7	1.8 ± 0.7	4,6** ± 1,1	3,9* ± 0,8
		2		1.1 ± 0.3	2,7* ± 0,8	3,3** ± 0,5	3,5** ± 0,5
		3		1.3 ± 0.2	1.1 ± 0.4	8,1*** ± 0,8	4,3*** ± 0,7
	<i>Mmp-3</i>	1	1.1 ± 0.2	4,4* ± 1,3	2.8 ± 0.4	2.2 ± 0.9	1.7 ± 0.6
		2		2.2 ± 0.7	3,5* ± 0,8	2.3 ± 0.7	5,2*** ± 1,0
		3		2.3 ± 0.8	2.2 ± 0.3	4,2** ± 1,0	5,5*** ± 0,8
	<i>Mmp-9</i>	1	1.0 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1,9** ± 0,3	1.3 ± 0.2
		2		1.1 ± 0.1	1.5 ± 0.2	1.6 ± 0.2	2,6* ± 0,9
		3		1.1 ± 0.1	1,8* ± 0,2	1,8* ± 0,3	1.6 ± 0.2
	<i>Mmp-13</i>	1	1.2 ± 0.3	2,7* ± 0,5	0.9 ± 0.2	1.1 ± 0.3	1.8 ± 0.7
		2		1.1 ± 0.3	0.8 ± 0.2	1.2 ± 0.2	1.3 ± 0.5
		3		1.4 ± 0.3	0.6 ± 0.3	2,7** ± 0,4	1.1 ± 0.3

The results were assessed by quantitative PCR (qPCR). Total RNA samples were collected 2, 7, 21 or 28 days after OA induction. The results are presented as the mean group fold change ± SEM in comparison to the control group (healthy animals), n = 3–6 samples per group. Data were analyzed with one-way ANOVA followed by Dunnett's post-hoc test.

* denotes $p < 0.05$.

** denotes $p < 0.01$.

*** denotes $p < 0.001$ vs. intact animals (Ctrl).

Statistically significant values are shown in bold.

$p < 0.05$). In the 2 mg MIA group, no results reached statistical significance. In the 3 mg MIA group, in the synovial membrane, an increase was observed throughout nearly the entire experiment (days 7, 21 and 28; $p < 0.05$); in cartilage, elevated expression of *Mmp-13* was observed solely on day 21 post-MIA treatment ($0.01 > p > 0.001$). The results of this gene expression analysis are shown in **Table 1**. *Comp* expression in cartilage showed a decrease in the initial phases of OA in the 1 mg MIA group (day 2 and 7; $0.01 > p > 0.001$) and a subsequent increase in the 2 ($p < 0.05$) and 3 mg ($0.01 > p > 0.001$) MIA groups on day 21 (see **Supplementary Table S1**). In summary, the qPCR test showed a dose- and time-dependent changes in mRNA levels of *Mmps*.

MMP Protein Levels During OA Development

MMP-2 protein secretion was elevated in advanced OA stages in all experimental groups. In cartilage samples, a significant increase was observed solely on day 28 of the experiment at all MIA doses (MIA 1 mg; $p < 0.05$; MIA 2 mg; $0.01 > p > 0.001$; MIA 3 mg; $p < 0.001$; **Figures 6A–C**). In synovial membrane samples, MMP-2 protein levels were increased starting on day 7 (1 and 2 mg MIA groups; $p < 0.001$ and $p < 0.05$) or day 21 (3 mg MIA group; $p < 0.001$) and remained elevated until the end of the experiment (**Figures 6D–F**). In the synovial fluid of OA rats, MMP-2 was increased at the later stages of

the disease, on day 28 (1 mg MIA group; $0.01 > p > 0.001$) or days 21 and 28 (2 and 3 mg MIA groups; $p < 0.001$; **Figures 6G–I**).

MMP-3 protein levels, in cartilage samples in the 1 mg MIA group, showed an early increase (on day 2 post-MIA treatment; $p < 0.05$; **Figure 7A**). A similar trend was observed in the 2 mg MIA group; however, these results did not reach statistical significance. In the 2 and 3 mg MIA groups, a significant increase in MMP-3 levels was observed in the later OA stages (days 28; $0.01 > p > 0.001$; or days 21; $p < 0.05$; and 28; $p < 0.001$; respectively; **Figures 7B,C**). In the synovial membrane samples, the lowest MIA dose did not significantly alter MMP-3 protein production (**Figure 7D**). In the 2 mg MIA group, an increase was observed only on day 21 ($p < 0.05$), whereas in the 3 mg MIA group, a significant increase was observed on days 21 and 28 post-MIA injection ($p < 0.05$ and $0.01 > p > 0.001$; **Figures 7E,F**).

In the synovial fluid samples, an increase in MMP-3 protein levels was detected in the early stages of OA. The lowest dose of MIA (1 mg) resulted in an early release of MMP-3 into the synovial fluid; a significant elevation was observed on days 2, 7 and 28, with a peak on day 2 ($p < 0.001$ and $0.01 > p > 0.001$; $p < 0.05$ respectively; **Figure 7G**). In the 2 mg MIA group, a trend toward increased expression was observed from the beginning of the experiment; however, only the results on day 28 reached statistical significance ($p < 0.05$; **Figure 7H**). Similar results were observed in the 3 mg MIA

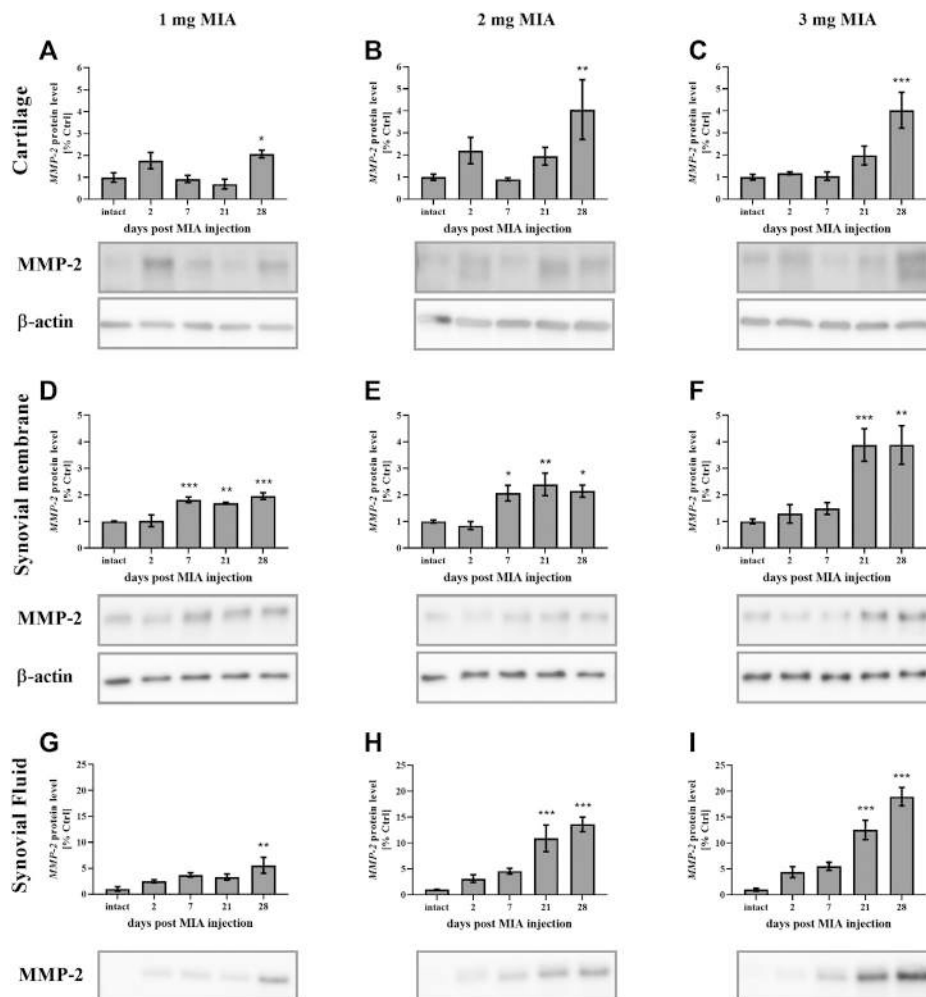


FIGURE 6 | Changes in MMP-2 protein levels in cartilage (A–C), synovial membrane (D–F) and synovial fluid (G–I) samples of osteoarthritic rats as measured by Western blot assay. Tissues were collected 2, 7, 21 or 28 days after OA induction. The results are presented as the mean group fold change \pm SEM in comparison to the control group (healthy animals), $n = 4$ –5 samples per group. Data were analyzed with one-way ANOVA followed by Dunnett's post-hoc test. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

group, with a significant increase in MMP-3 levels on days 7 and 28 ($p < 0.05$; **Figure 7I**).

MMP-9 protein levels in cartilage samples at low MIA doses (1 and 2 mg MIA groups) showed no significant changes (**Figures 8A,B**). However, in the 3 mg MIA group, MMP-9 protein levels increased significantly on day 28 ($p < 0.05$; **Figure 8C**). In synovial membrane samples, in all tested MIA doses, MMP-9 levels increased exclusively in the early OA stages (day 2 post MIA treatment; MIA 1 mg; $0.01 > p > 0.001$; MIA 2 mg; $p < 0.05$; MIA 3 mg; $p < 0.001$), subsequently returning to baseline in the following experimental days (**Figures 8D–F**). Similar observations were noted for the synovial fluid samples – an early (day 2) increase in MMP-9 protein levels was detected at all MIA doses (MIA 1 mg; $0.01 > p > 0.001$; MIA 2 mg; $0.01 > p > 0.001$; MIA 3 mg; $p < 0.05$), followed by a return nearly to baseline and a final increase on the last experimental day (a significant increase was observed only in the 3 mg MIA group ($0.01 > p > 0.001$). However, a trend toward

increased expression was also observed in the 2 mg MIA group; **Figures 8G–I**).

Regarding MMP-13, lower MIA doses (1 and 2 mg) were associated with an early increase (day 2) in MMP-13 production in cartilage ($p < 0.05$; both groups). However, in the 2 mg MIA group, an increase was also observed in the later OA stages (day 28; $p < 0.05$, with a trend toward increased expression on day 21; **Figures 9A,B**). In the 3 mg MIA group, there was a robust increase in MMP-13 levels on day 28 ($p < 0.001$; **Figure 9C**). In the synovial membrane, ambiguous results were obtained, with a significant increase identified only on day 2 in the 1 mg MIA group ($p < 0.05$; **Figures 9D–F**). In the synovial fluid samples, an increase in Mmp-13 levels was observed for almost the entire experimental period. In the 1 and 3 mg MIA groups, a significant increase was detected on days 2, 7 and 28 post-MIA treatment, whereas in the 2 mg MIA group, Mmp-13 levels significantly increased on days 2, 21 and 28 (**Figures 9G–I**).

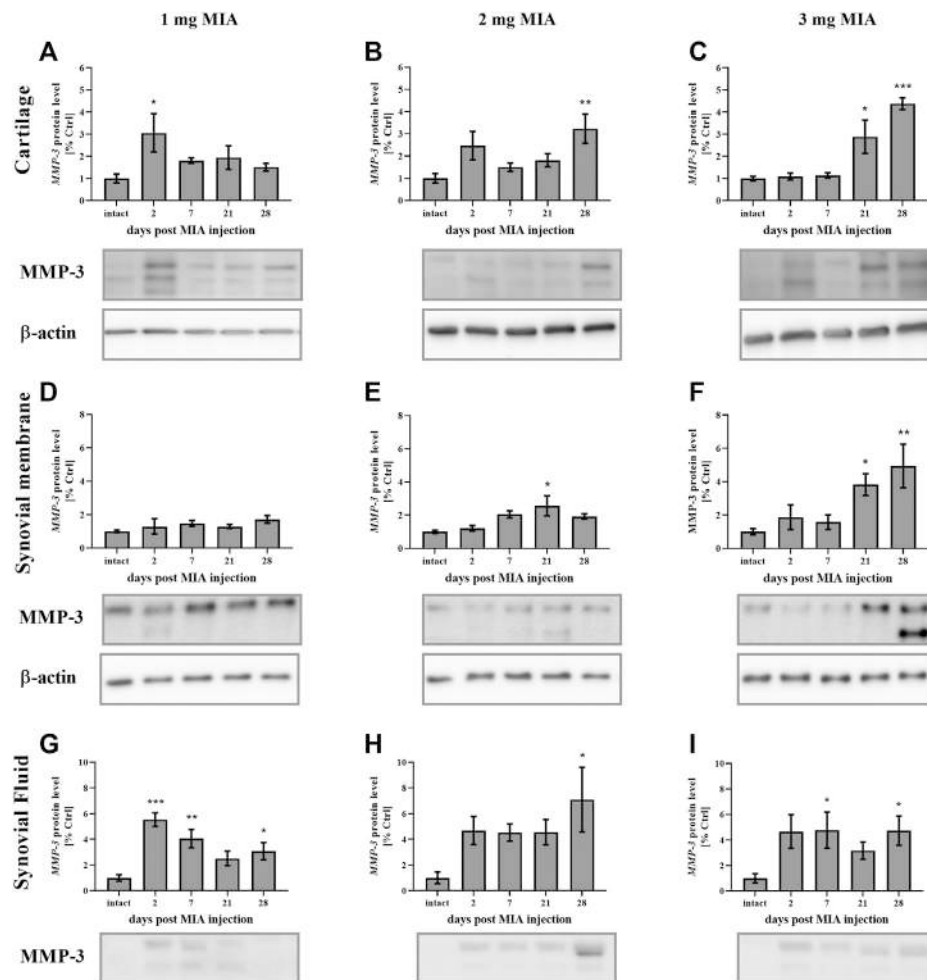


FIGURE 7 | Changes in MMP-3 protein levels in cartilage (A–C), synovial membrane (D–F) and synovial fluid (G–I) samples of osteoarthritic rats as measured by Western blot assay. Tissues were collected 2, 7, 21 or 28 days after OA induction. The results are presented as the mean group fold change \pm SEM in comparison to the control group (healthy animals), $n = 4$ –5 samples per group. Data were analyzed with one-way ANOVA followed by Dunnett's post-hoc test. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

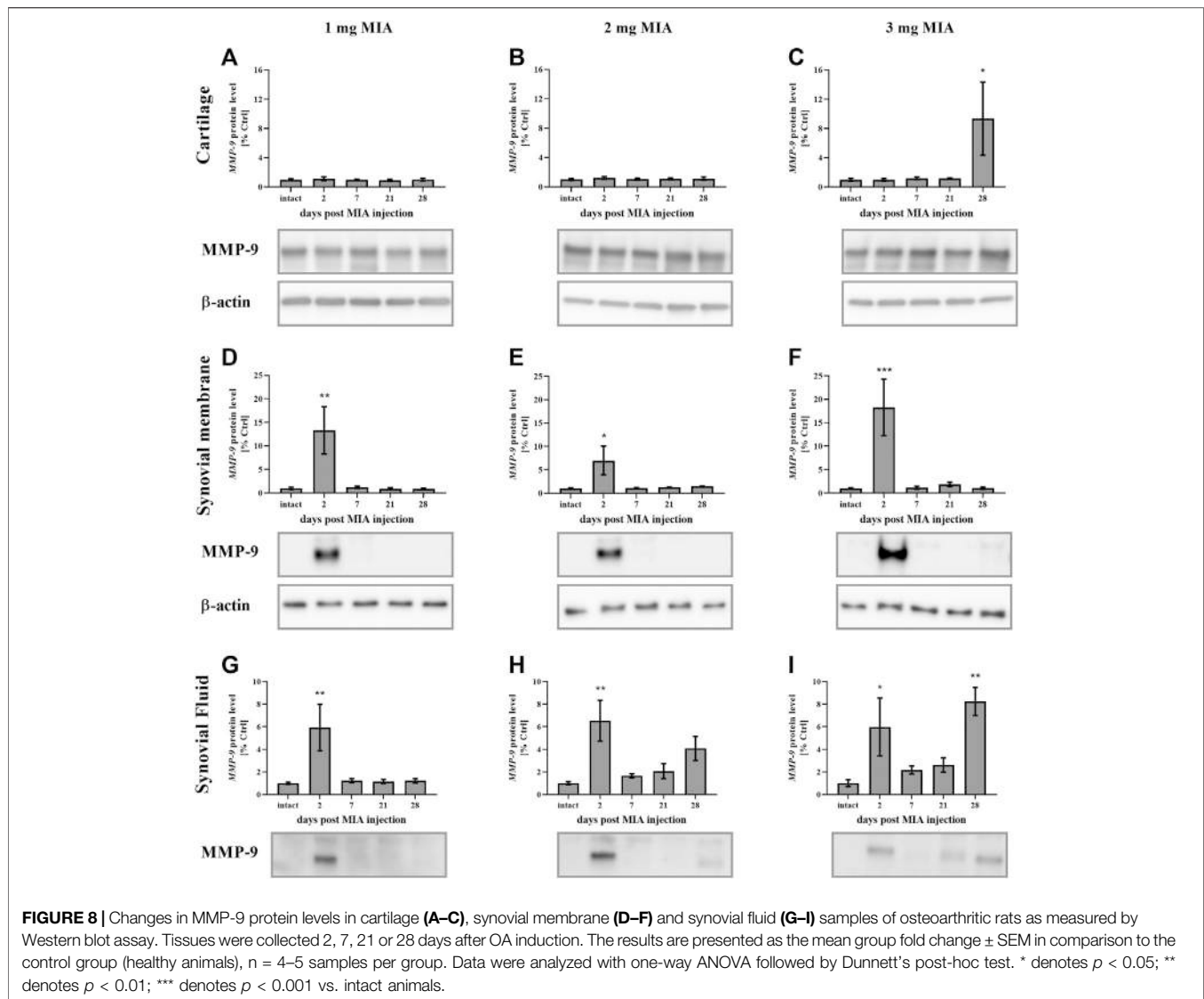
In summary, protein levels for investigated MMPs changed significantly in a dose- and time-dependent manner. All MIA doses significantly changed protein concentration in the synovial fluid, whereas in cartilage and synovial membrane significant changes were observed mostly in 2 and 3 mg of MIA groups.

DISCUSSION

In the current study, behavioral and biochemical OA-related changes were investigated. The molecular alterations in MMP and inflammation-related factor expression were measured during 28 days of OA progression across three MIA dose groups: 1, 2 or 3 mg of MIA i.a. In the behavioral study, both investigated MIA doses (1 and 3 mg) caused significant allodynia and disturbed weight bearing patterns starting on the second day

of the experiment. Although a stronger effect was observed in the 3 mg MIA group, both doses were sufficient to trigger OA-like pain behavior in rats. Furthermore, at the molecular level, significant differences between the investigated doses were observed.

OA progression is associated with an inflammatory state (Golding and Otero, 2011). OA-related knee pain correlates with joint synovitis, subarticular bone attrition, bone marrow lesions and meniscal tears (Torres et al., 2006; Baker et al., 2010). Moreover, synovitis and effusion are associated with cartilage degeneration and loss in human patients (Roemer et al., 2011). There are several pathways that reportedly play an important role in synovitis progression. Activated macrophages promote catabolic mediator production and Toll-like receptor (TLR) and NF κ B pathway activation, which leads to proinflammatory chemokine and cytokine production (e.g., IL-1 β , IL-2, IL-6, IL-8, IL-15, TNF α , CCL2, CCL5, CCL19) (Scanzello and Goldring,

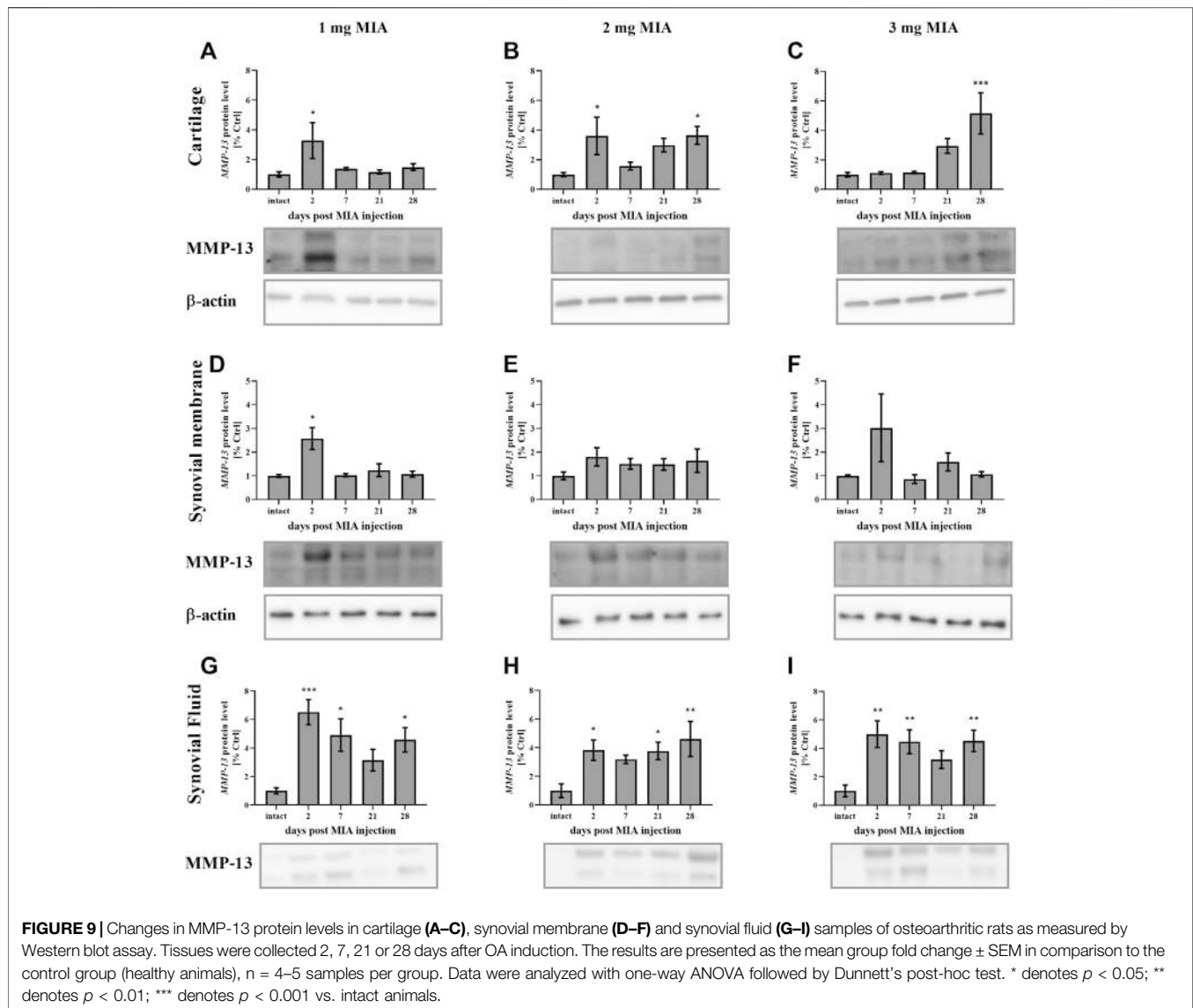


2012). Malek et al. previously observed an early increase in pain-related behavior in MIA-treated rats associated with transient inflammation triggered by i.a. MIA injection (Malek et al., 2015). Here, we observed an early peak (on day 2 post-MIA injection) in the gene expression of inflammation-related factors (*Ccl2*, *Cxcl-1*, and *Il-6*) in synovial membrane samples across all MIA doses (see **Supplementary Table S1**) and in protein levels in synovial fluid (CCL2, CXCL1, and IL-1 β).

One of the most promising molecular markers of OA is COMP, which regulates and stabilizes the collagen network in cartilaginous tissue (Živanović et al., 2011). Upregulation of this protein in OA patient serum has been correlated with disease progression (Jung et al., 2006). In this study, the COMP levels in the synovial membrane were elevated in the early OA stages across all MIA doses; however, on day 28, upregulation was observed only in the 2 and 3 mg MIA groups (3- and 4-fold vs. the control group). In the synovial fluid, COMP levels were

increased at the end of the experimental period (day 28 in all MIA doses).

In addition to inflammation-related factors, four MMPs were further investigated in this study. MMPs and their regulators (tissue inhibitors of metalloproteinases; TIMPs) are considered biological markers of OA (DeGroot et al., 2002; Rousseau and Garner, 2012). Here, the levels of MMP-2, -3, -9, and -13 were determined. The gelatinases MMP-2 and MMP-9 are important factors in the pathogenesis of several diseases, including cancer, liver fibrosis, cardiovascular diseases and rheumatoid arthritis (Kurzepa et al., 2014; Radenkovic et al., 2014; Zhou et al., 2014; Radosinska et al., 2017). They are also reportedly involved in inflammation and inflammatory cell migration (Cui et al., 2017; Hannocks et al., 2019; Kim et al., 2012). Gelatinases also play an important role in the inflammatory response during the course of OA. In synoviocyte and anterior cruciate ligament fibroblast cell cultures, TNF α stimulation increases MMP-2 and MMP-3 levels



(Wang et al., 2019). In rheumatoid arthritis (RA) synovial fibroblasts, MMP-2 and MMP-9 contribute to cell survival, proliferation and migration. MMP-9 has been shown to stimulate RA synovial fibroblast-mediated inflammation, whereas MMP-2 exhibited the opposite effect (Xue et al., 2014). Moreover, MMP-9 expression and activation are reportedly increased in septic native knee arthritis patients in comparison to aseptic knee arthritis patients (Fotopoulos et al., 2012). In cartilage and synovial fluid of OA patients, gelatinase protein levels are increased (Alunno et al., 2017; Duerr et al., 2004; Kim et al., 2011; Lipari and Gerbino, 2013); however, there may be differences between Asian and Caucasian patients (Zeng et al., 2015). In the present study, the gelatinase MMP-2 showed a gradual dose- and time-dependent increase in both gene expression and protein production. A significant increase was observed in the advanced phases of the disease at all MIA doses (from day 7 in qPCR and from day 21 in Western blot). This

indicates a role for MMP-2 in joint tissue degeneration and inflammation in the later stages of OA in a rat model. As described above, early inflammation connected to MIA i.e. injection may explain the early increase in MMP-9 protein secretion by synoviocytes into the synovial fluid observed in almost all experimental groups in the current study. A similar increase at the end of the experiment, observed in the 3 mg MIA dose group, may indicate the role of MMP-9 in ECM degradation in the advanced stages of the disease in the highest MIA dose group, in which the OA lesions are the most severe. Lower MIA doses (1 and 2 mg) may not be sufficient to trigger such an effect in a 4-week period. However, it remains possible that lower MIA doses could trigger such an effect if the experimental period were longer. Further experiments are needed to clarify this effect, although most studies report an experimental period of no longer than 4 weeks. Regarding the gene expression levels of *Mmp-9*, we

observed that synovial membrane samples provided a more substantial response than cartilage samples. The synovial membrane is a more secretory tissue compared to cartilage and is responsible for synovial fluid production, joint lubrication and maintaining homeostasis of the joint (Orr et al., 2017). In turn, cartilage does not play a primary secretory role in the joint and in fact degenerates over the course of the disease, further reducing its reactivity (Bryk et al., 2020).

MMP-3 (stromelysin 1) is also an important factor in osteoarthritis pathogenesis. In human synovial membrane cell culture and TNF- α -stimulated human cartilage experiments, MMP-3 levels are reportedly elevated (Sun et al., 2014). Additionally, in RA patients, MMP-3 serum levels are upregulated (Mahmoud et al., 2005) and can be reduced following anti-inflammatory (anti-TNF α) treatment (Sun et al., 2014). In OA patients, an elevated level of MMP-3 was also shown (Li et al., 2012). Moreover, the serum level of MMP-3 was higher in patients with OA changes in two or more locations (hands, hips, knees, spine, feet) than in people with only one location (knee joints) affected (Georgiev et al., 2018). In a reversible osteoarthritis rabbit model, MMP-3 and COMP levels were elevated in serum and synovial fluid samples from OA animals, which was correlated with OA severity (Chu et al., 2015). MMP-3 levels are also correlated with leptin concentrations in the synovial fluid of OA patients (Koskinen et al., 2011). In our study, at a low MIA dose (1 mg), MMP-3 gene expression and protein production increased in the initial stages of the experiment. At higher MIA doses (2 and 3 mg), we observed either late or constant upregulation of MMP-3 at both the gene and protein levels. This result may indicate an important role for MMP-3 in advanced OA (in the later experimental days at higher MIA doses). At the low MIA dose (1 mg), MMP-3 might be involved in the early inflammatory state; however, the OA grade in this group was lower than that in the 2 or 3 mg MIA groups; therefore, the contribution of MMP-3 to OA development may not be significant.

The final investigated matrix metalloproteinase, collagenase MMP-13, is an enzyme that plays a pivotal role in OA progression (Neuhold et al., 2001). Its knockout in mice results in deceleration of OA progression (Wang et al., 2013) and reduction of arthritis-evoked inflammation and cartilage erosion (Singh et al., 2013). In experimental equine OA, MMP-13 was elevated in synovial fluid samples (Ma et al., 2017). MMP-13 inhibition reduces cartilage erosion in animal models of RA (Jüngel et al., 2010) and blocks type II collagen degradation in bovine explants and human OA cartilage (Piecha et al., 2010). Moreover, in the synovial fluid of OA and RA patients, the level of MMP-13 is reportedly increased (Andereya et al., 2006; Kim et al., 2011; Özler et al., 2016). Li et al. demonstrated that in human OA cartilage, MMP-13 is elevated and suppresses cell proliferation (Li et al., 2019). MMP-13 also promotes cartilage degeneration via histone deacetylase (HDAC), and HDAC7 inhibition diminishes MMP-13 expression in chondrocytes (Higashiyama et al., 2010). MMP-13 inhibition may therefore also show therapeutic potential for OA treatment (Li et al., 2011). In the current study, MMP-13 protein levels in synovial fluid were elevated throughout the entire experimental period in all groups. In joint tissues, an early increase was observed at a lower MIA dose, whereas

3 mg MIA treatment resulted in MMP-13 elevation in cartilage samples in the later OA stages. *Mmp-13* gene expression was more elevated in synovial membrane samples than in cartilage, with a similar pattern of changes (an early increase at lower MIA doses and a prolonged increase at the highest dose). As noted above, the synovial membrane plays the primary secretory role in the joint and is responsible for synovial fluid production. This may explain the more significant effects observed in the synovial membrane than cartilage in *Mmp-13* gene expression, as well as its protein abundance in synovial fluid. Our results suggest that MMP-13 plays an important role in OA development, since its protein level (functionally more important than gene expression changes) was elevated across all groups. In the 3 mg MIA group, MMP-13 levels were also increased in cartilage at the later stages of OA, indicating that the highest MIA dose causes severe OA-like lesions in a rat model.

It should be noted that OA triggers not only local changes but also broader changes in the nervous system (Murphy et al., 2012; Clauw and Hassett, 2017). Thakur et al. investigated the influence of 1 or 2 mg MIA treatment on changes in dorsal root ganglion cells in OA animals, demonstrating that ATF-3 (a sensitive marker of peripheral neuron stress/injury) signaling was increased in 2 mg MIA-treated animals than in a 1 mg MIA treatment group. 2 mg MIA injection also reduced intraepidermal nerve fiber density in plantar hind paw skin and produced spinal cord dorsal and ventral horn microgliosis, which was not observed in a 1 mg MIA group (Thakur et al., 2012). These data are consistent with our results and suggest that 2 mg MIA, in addition to cartilage degeneration, evokes significant biochemical changes not only locally in the joint but also in the nervous system, although 1 mg MIA is not sufficient to trigger such changes.

CONCLUSION

In conclusion, at the behavioral level, both 1 and 3 mg MIA treatment triggered similar effects. However, at the biochemical level, 2 and 3 mg MIA treatment showed comparable effects, while 1 mg appeared insufficient to trigger substantive OA-like changes at the molecular level in a rat OA model. In turn, 3 mg MIA treatment provided the most severe response with respect to both inflammatory factor and MMP release. Although many global investigators use an MIA animal model to study the therapeutic potential of various compounds, the MIA-induced OA model in rats has not previously been fully described in the literature. Our results indicate that 2 mg MIA may represent the “gold standard” treatment, as the lowest possible dose that effectively triggers OA but does not induce overly high inflammation. Our study fills in critical missing information regarding OA development and progression in a rat model.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Bioethics Committee of the Maj Institute of Pharmacology (Cracow, Poland) (approval numbers: 938/2012 and 125/2018).

AUTHOR CONTRIBUTIONS

Conceptualization, KS, MB, JC, and JM; methodology, MB, JC, JM, and MK; formal analysis, JC, MB, and MK; investigation, MB, JC, MK, and JM; writing—original draft preparation, MB; writing—review and editing, JC, KS, MB, and MK; visualization, MB and MK; supervision, KS; project administration, KS; funding acquisition, KS. All authors

have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2021.643605/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Alterations in Anandamide Synthesis and Degradation during Osteoarthritis Progression in an Animal Model

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Abstract: Osteoarthritis (OA) is a degenerative joint disease manifested by movement limitations and chronic pain. Endocannabinoid system (ECS) may modulate nociception via cannabinoid and TRPV1 receptors. The purpose of our study was to examine alterations in the spinal and joint endocannabinoid system during pain development in an animal model of OA. Wistar rats received intra-articular injection of 3mg of sodium monoiodoacetate (MIA) into the knee joint. Animals were sacrificed on day 2, 7, 14, 21, 28 after injection and lumbar spinal cord, cartilage and synovium were collected. Changes in the transcription levels of the ECS elements were measured. At the spinal level, gene expression levels of the cannabinoid and TRPV1 receptors as well as enzymes involved in anandamide synthesis and degradation were elevated in the advanced OA phase. In the joint, an important role of the synovium was demonstrated, since cartilage degeneration resulted in attenuation of the changes in the gene expression. Enzymes responsible for anandamide synthesis and degradation were upregulated particularly in the early stages of OA, presumably in response to early local joint inflammation. The presented study provides missing information about the MIA-induced OA model and encourages the development of a therapy focused on the molecular role of ECS.

Keywords: osteoarthritis; endocannabinoid system; endocannabinoids; pain; chronic pain; TRPV1; animal model; cartilage; synovial membrane; spinal cord

1. Introduction

Osteoarthritis (OA) is a whole joint disease characterized by cartilage destruction, subchondral bone alterations and synovial membrane inflammation. Currently, OA is a leading cause of chronic pain and disability worldwide [1]. Current treatment is very limited and is mainly based on symptomatic pain relief by nonsteroidal anti-inflammatory drugs (NSAIDs) offering insufficient pain relief [2,3]. Despite the research progress, OA remains among the most challenging joint diseases due to the lack of self-healing capacity of articular cartilage and there is no cure for it. Therefore, an extensive search for new treatment options is needed. Preclinical studies suggest, that endocannabinoids have a potential to become novel molecular targets for drug development in chronic pain, including pain resulting from osteoarthritis [4].

The endocannabinoid system (ECS) is composed of cannabinoid receptors, CB1 and CB2, their endogenous ligands and their synthetic and metabolic enzymes. Anandamide (AEA) is the first identified

and best characterized endogenous ligand of cannabinoid receptors [5]. Levels of AEA are altered in the spinal cord of neuropathic and osteoarthritic animals [6,7], which emphasizes the importance of AEA in nociceptive processing in chronic pain. Valastro et al. demonstrated that AEA is present in the synovial fluid of arthritic knees in dogs [8]. Moreover, an increase in the level of AEA was detected in the synovial fluid collected from the patients with OA compared with that in the healthy controls [9]. Unlike conventional neurotransmitters, endocannabinoids are not stored but are locally synthesized on-demand in the areas of cellular stress. The main AEA synthesis pathway includes phospholipid precursor *N*-arachidonoyl phosphatidylethanolamine (NAPE), which is hydrolyzed by *N*-arachidonoyl phosphatidylethanolamine phospholipase D (NAPE-PLD) in the presence of Ca^{2+} [10]. However, there are two additional well-characterized pathways associated with AEA production in a Ca^{2+} -independent manner. The phospholipase C (PLC) pathway includes PLC and two other concurrent enzymes, protein tyrosine phosphatase non-receptor type 22 (PTPN22) and phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (INPP5D) [11,12]. Another alternative synthesis pathway involves secreted phospholipase A2 (sPLA2), β hydrolase domain-containing protein 4 (ABHD4) and glycerophosphodiester phosphodiesterase 1 (GDE1) [13]. AEA is also characterized by a short half-life particularly due to an effective enzymatic degradation by three enzymatic pathways, including fatty acid amide hydrolase (FAAH) generating arachidonic acid (AA) and ethanolamine (ETA); cyclooxygenase 2 (COX-2) generating prostaglandins (PG) and prostamides (PM); and arachidonate lipoxygenases 12 and 15 (LOX-12/15) generating 12/15 hydroxyeicosatetraenoylethanolamide (12/15-HETE-EA), respectively (Figure 1) [7,10,14].

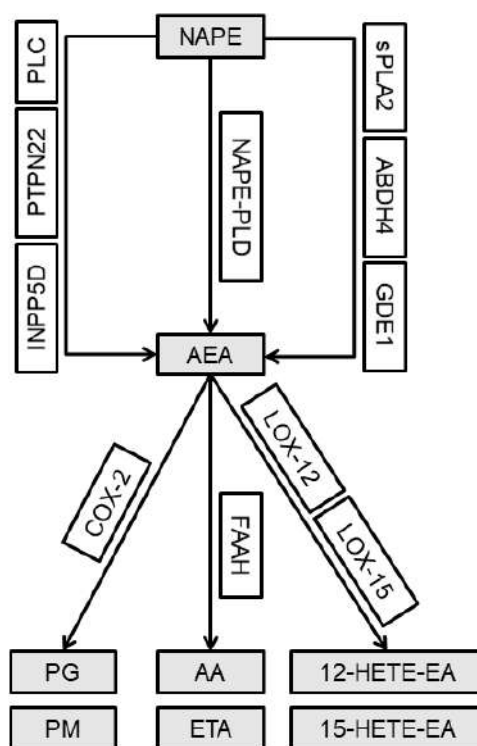


Figure 1. Simplified diagram of anandamide synthesis and degradation pathways. See text for details. Abbreviations: NAPE—*N*-arachidonoyl phosphatidylethanolamine; NAPE-PLD—*N*-arachidonoyl phosphatidylethanolamine phospholipase D; AEA—anandamide; PLC—phospholipase C; PTPN22—protein tyrosine phosphatase non-receptor type 22; INPP5D—phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1; sPLA2—secreted phospholipase A2; ABHD4— β hydrolase domain-containing protein 4; GDE1—glycerophosphodiester phosphodiesterase 1; FAAH—fatty acid amide hydrolase; AA—arachidonic acid; ETA—ethanolamine; COX-2—cyclooxygenase 2; PG—prostaglandins; PM—prostamides; LOX-12/15—arachidonate lipoxygenases 12 and 15, respectively; 12/15-HETE-EA—12/15 hydroxyeicosatetraenoylethanolamide, respectively.

AEA degradation reduces its use for analgesic purposes; therefore, targeting (by pharmacological or genetic inhibition) its degrading enzymes is a promising strategy to treat the pain syndromes. However, AEA may interact and activate other targets, such as the transient receptor potential vanilloid type 1 (TRPV1) [15]. TRPV1 was originally reported to be expressed on the peripheral nociceptive afferents and is present in the spinal and supraspinal structures [16]; TRPV1 is also expressed in multiple cell types of the joint [17–19]. Interestingly, a variant of the TRPV1 gene is associated with the risk of the development of symptomatic OA [20]. Other studies indicate that inflammation and neuronal injury alter the localization of TRPV1 and sensitize the channel to respond to stimulus modalities beyond the classical thermal profile [21]. TRPV1 contains multiple phosphorylation sites; hence, activation by kinases, such as Ca²⁺/calmodulin-dependent protein kinase (CaMKII), is one of the proposed mechanism of activation and sensitization of TRPV1 [22,23].

Unrelenting pain can induce changes in the spinal cord, which play an essential role in the integration and modulation of nociceptive signals from the peripheral tissues to the higher centers of the brain. An emerging concept is that the expression of CB1 and TRPV1 in the same or in neighboring cells, especially in the case of activation by the same mediator, such as AEA, enables a cross-talk with variable impact on pain perception. Thus, we aimed to improve the understanding of a possible cross-talk between the endocannabinoid and endovanilloid systems to assist with fine-tuning of analgesic strategies particularly for patients suffering from chronic pain. The degree of pain does not always correlate with the extent of joint damage or presence of active inflammation, we investigated the occurrence of a central component of OA pain by correlating OA pain behavior of the animals reported in the previous studies [24] with the assessment of ATF-3, a marker of spinal neuronal activity. Involvement of mitogen-activated protein kinases 3 and 14 (MAPK3 and MAPK14, respectively) in the chronic pain formation has been shown in preclinical studies [25,26]. Furthermore, the present investigation aimed to examine the spinal and joint endocannabinoid systems in combination with TRPV1 and its sensitization factors during pain development in a rat sodium monoiodoacetate (MIA) model of OA. We examined changes in the transcription of the ECS system elements, including cannabinoid receptors and multiple AEA synthesis and degradation enzymes, in the lumbar spinal cord, cartilage and synovial membrane of osteoarthritic rats. Inflammation and tissue hypoxia associated with OA lead to a decrease in local tissue pH resulting in TRPV1 sensitization [22,27]. This decrease may contribute to pain development; however, the downstream signaling of this pathway has been insufficiently characterized. Thus, we also investigated changes in the mRNA expression of TRPV1 receptor and CaMKII, which is involved in TRPV1 sensitization during OA development. In summary, we aimed to increase our understanding of the role of the endocannabinoid system in the pathogenesis of OA and to propose new molecular targets and therapeutic strategies for OA treatment to increase the efficacy of OA pain management.

2. Results

During OA development, a significant elevation of mRNA level of a neuronal marker of nerve injury *Atf3* in the dorsal lumbar spinal cord was detected 28 days after MIA injection (Figure 2A). MAP kinases p38 (*Mapk14*) and ERK1 (*Mapk3*) were detected in the L4–L6 spinal cord segments after MIA injection. Analysis of mRNA abundance revealed a significant increase in *Mapk3* exclusively 28 days after MIA treatment (Figure 2B). *Mapk14* gene was strongly upregulated on days 21 and 28 (Figure 2C). In the cartilage OA samples, no significant changes in the *Mapk3* and *Mapk14* gene expression levels were detected (Figure 2D,E). In the synovial membrane samples collected from OA rats, the levels of *Mapk3* and *Mapk14* were elevated only 7 days after MIA treatment (Figure 2F,G).

2.1. Changes in the *Cnr1*, *Cnr2* and *Trpv1* Gene Expression in the Dorsal Lumbar Spinal Cord and Joint Tissue of Osteoarthritic Rats

In rats with developed OA, a different pattern of *Cnr1* and *Cnr2* gene expression (encoding CB1 or CB2 receptors, respectively) was observed in the dorsal lumbar L4–L6 spinal cord segments during

the development of OA pain. A significant increase in the *Cnr1* gene expression was detected on day 28 after MIA injection in the ipsilateral part of the spinal cord (Figure 3A). An increase in the *Cnr2* transcript was observed on day 7 and the levels of the transcript were decreased at later time points (Figure 3B). The *Trpv1* mRNA level was significantly elevated exclusively on day 28 (Figure 3C).

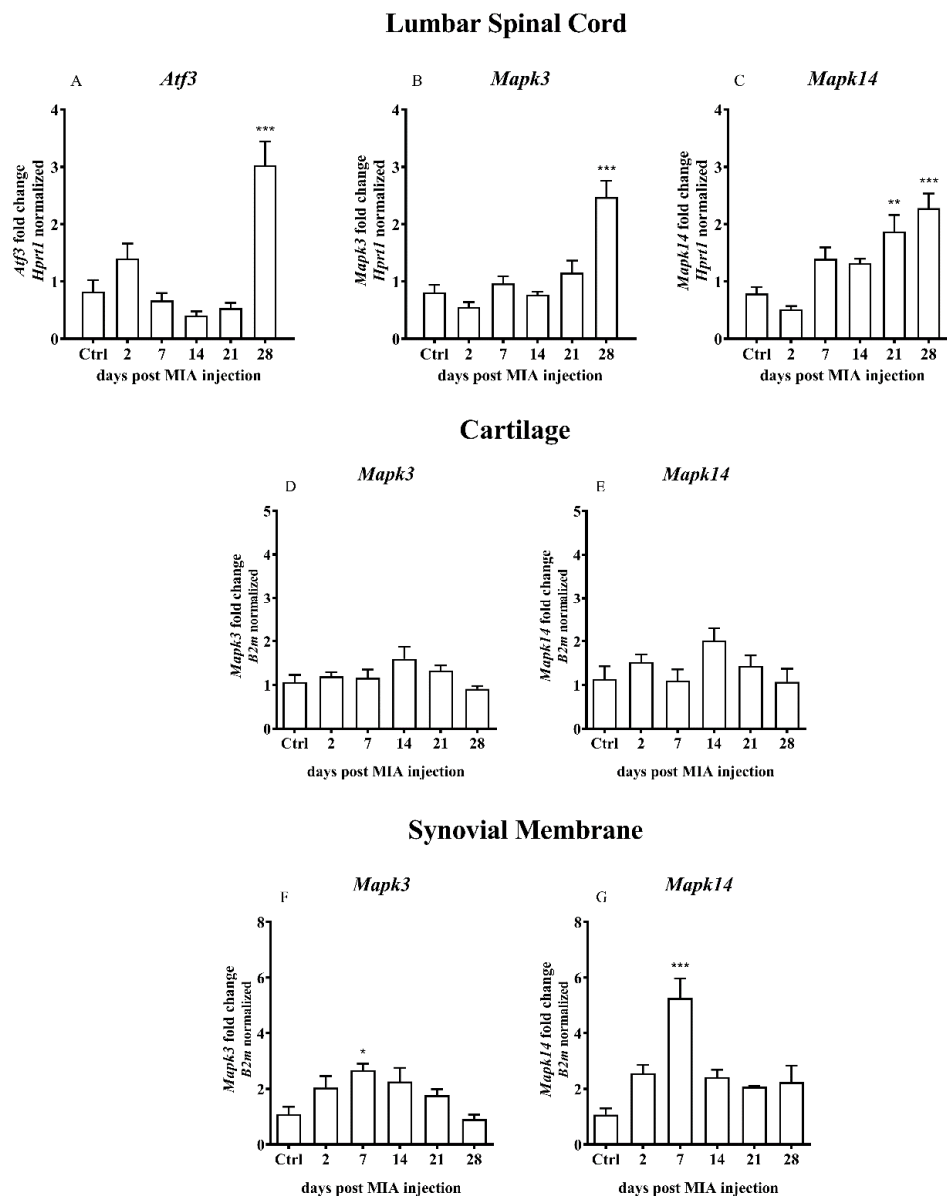


Figure 2. Transcript levels of cyclic AMP-dependent transcription factor (*Atf3*), mitogen-activated protein kinase 3 (*Mapk3*) and mitogen-activated protein kinase 14 (*Mapk14*) genes in the (A–C) lumbar L4–L6 spinal cord segments, (D,E) cartilage and (F,G) synovial membrane of rats after 3 mg MIA injection during OA development. The samples were collected 2, 7, 14, 21 and 28 days after MIA injection; the control group did not receive any treatment. Groups contained 6–9 samples (for the spinal cord analysis) or 3–6 (for the cartilage/synovial membrane analysis). Data are presented as the mean \pm SEM of fold changes of the group average normalized versus the reference gene (*Hprt1* or *B2m*). Statistical analysis was performed using one-way ANOVA followed by Dunnett post hoc-test (intact animals treated as a control group). * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

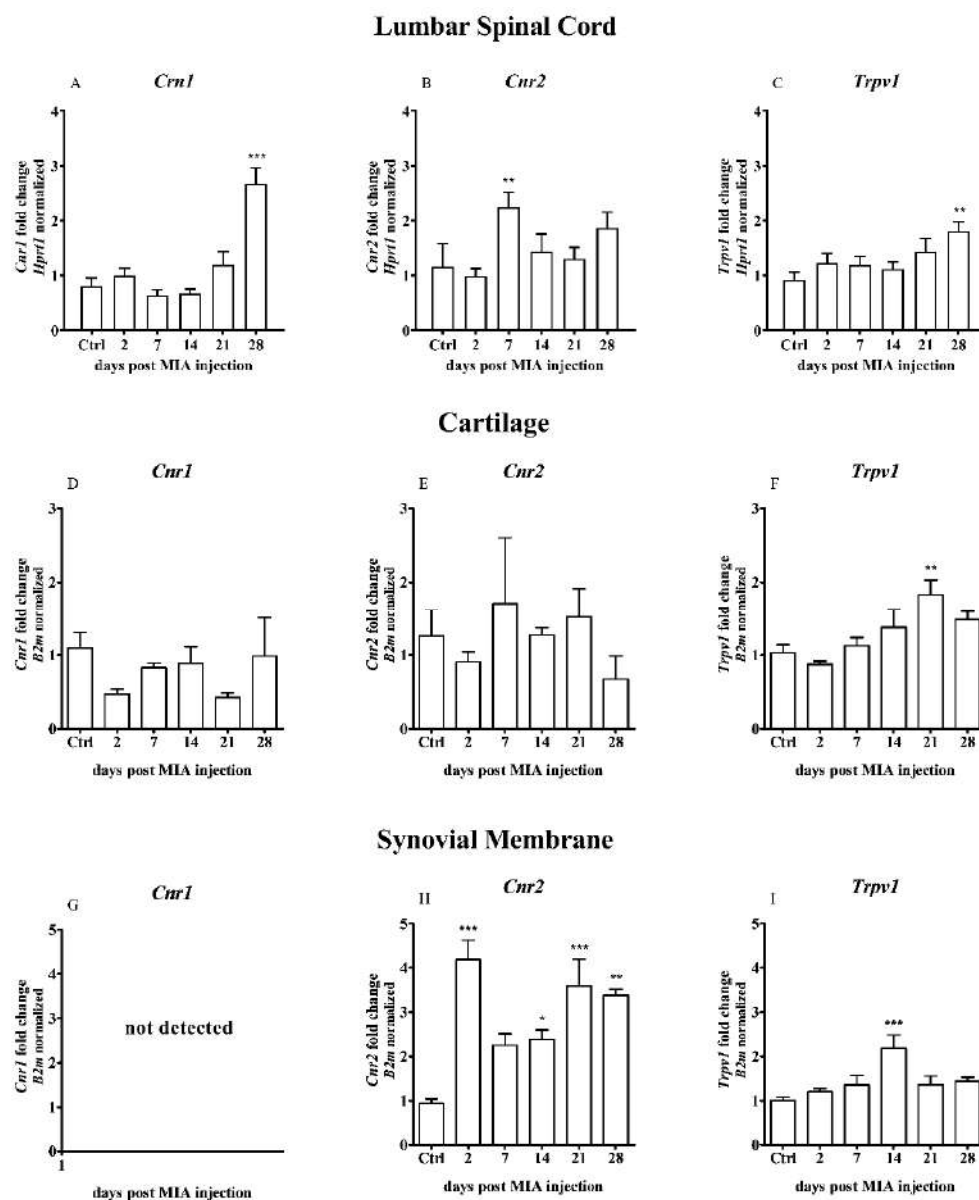


Figure 3. Transcript levels of the cannabinoid receptor type 1 and 2 (*Cnr1* and *Cnr2*) and transient receptor potential cation channel subfamily V member 1 (*Trpv1*) genes in the (A–C) lumbar L4–L6 spinal cord segments, (D–F) cartilage and (G–I) synovial membrane of rats after 3 mg MIA injection during OA development. Samples were collected 2, 7, 14, 21 and 28 days after MIA injection; the control group did not receive any treatment. Groups contained 6–9 samples (for the spinal cord analysis) or 3–6 samples (for the cartilage/synovial membrane analysis). Data are presented as the mean \pm SEM of fold changes of the group average normalized versus the reference gene (*Hprt1* or *B2m*). Statistical analysis was performed using one-way ANOVA followed by Dunnett post hoc-test (intact animals treated as a control group). * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

In the OA cartilage samples, no significant changes were detected in the *Cnr1* and *Cnr2* gene expression (Figure 3D,E), whereas a significant elevation in the *Trpv1* gene expression was observed on day 21 after MIA injection (Figure 3F).

In the synovial membrane collected from OA rats, the *Cnr1* gene expression was below the detection limit (Figure 3G); however, the *Cnr2* gene expression was increased starting from day 2 after MIA injection and was significantly elevated starting from day 14 till the end of the experiment (Figure 3H). The expression level of *Trpv1* has increased only 14 days after MIA injection (Figure 3I).

2.2. Expression of the Main Enzymes of AEA Synthesis and Degradation in the Dorsal Lumbar Spinal Cord and Joint Tissue of MIA-Treated Rats

The analysis of the transcript levels of the enzymes of main AEA synthesis and degradation, including *Nape-pld* and *Faah*, in the dorsal lumbar spinal cord during OA progression indicated an incremental increase along with the disease progression. No significant changes were observed in the *Nape-pld* expression on days 2, 7, 14 and 21 after MIA injection. Substantial *Nape-pld* upregulation was detected only on day 28 (Figure 4A). The *Faah* expression showed a trend to gradually increase from day 7 and was significantly elevated on days 21 and 28 (Figure 4B).

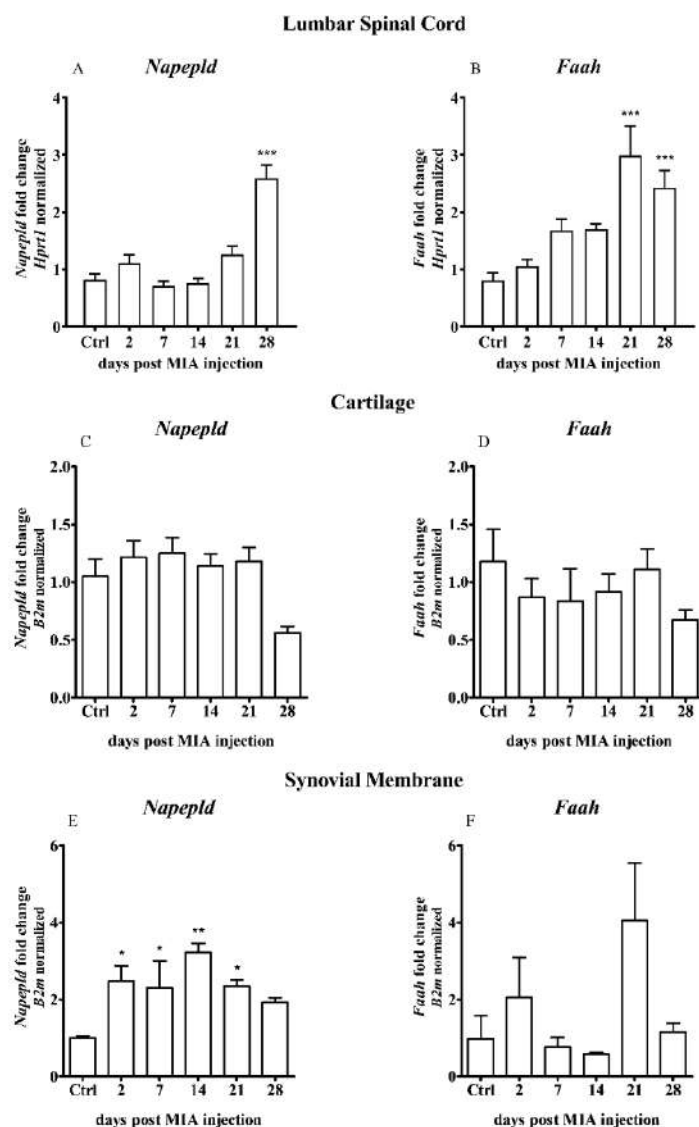


Figure 4. Transcript levels of the main enzymes of AEA synthesis and degradation, including N-acyl phosphatidylethanolamine-specific phospholipase D (*Nape-pld*) and fatty acid amide hydrolase (*Faah*) genes, in the (A,B) lumbar L4–L6 spinal cord segments, (C,D) cartilage and (E,F) synovial membrane of rats after 3 mg MIA injection during OA development. The samples were collected 2, 7, 14, 21 and 28 days after MIA injection; the control group did not receive any treatment. The groups contained 6–9 samples (for the spinal cord analysis) or 3–6 samples (for the cartilage/synovial membrane analysis). Data are presented as the mean \pm SEM of fold changes of the group average normalized versus the reference gene (*Hprt1* or *B2m*). Statistical analysis was performed using one-way ANOVA followed by Dunnett post hoc-test (intact animals treated as a control group). * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

No significant changes were detected in the cartilage of MIA-treated rats (Figure 4C,D).

An increase in the *Nape-pld* gene expression in the synovial membrane samples was observed two days after MIA injection and persisted until the end of the experiment (Figure 4E). A rising trend in the *Faah* gene expression was observed on day 21; however, the results did not reach statistical significance (Figure 4F).

2.3. Alterations in the Gene Expression of the Alternative AEA Synthesis and Degradation Pathways in the Lumbar Spinal Cord and Joint Tissue of Rats after MIA Injection

OA caused by intra-articular (i.a.) 3 mg MIA injection leads to the changes of the levels of mRNA encoding the enzymes of the alternative AEA synthesis and degradation pathway in the dorsal lumbar L4-L6 spinal cord segments, cartilage and synovial membrane. In the lumbar spinal cord, the gene expression levels of phospholipase C (*Plc*) and protein tyrosine phosphatase non-receptor type 22 (*Ptpn22*) tended to increase in response to MIA injection; the highest significant expression was detected 28 days after OA induction in the spinal cord (Figure 5A,B). Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (*Inpp5d*) was increased on day 7 and the increase persisted till day 28 (Figure 5C). Phospholipase A2 (*sPla2*) mRNA level was upregulated starting from day 7 after MIA injection; however, the highest expression was observed on days 14 and 21 (Figure 5D). The level of β hydrolase domain-containing protein 4 (*Abdh4*) mRNA was elevated only on day 28 (Figure 5E). The expression of the glycerophosphodiester phosphodiesterase 1 (*Gde1*) transcript was significantly increased 2 and 28 days after MIA injection (Figure 5F). MIA injection increased the levels of cyclooxygenase 2 (COX-2 encoded by the *Ptgs2* gene) and arachidonate lipoxygenases 12 (*Alox12*) transcripts on day 28 (Figure 5G,H). The expression of arachidonate lipoxygenases 15 (*Alox15*) had a trend to increase at the last two time points; however, no significant changes were observed (Figure 5I).

In the cartilage samples, an increase in the gene expression was observed only for *Plc* on day 14 (Figure 6A), *Ptpn22* on day 7 (Figure 6B) and *Ptgs2* on days 2–21 with a significant increase on day 14 (Figure 6G). A time-dependent decrease in the *sPla2* gene expression was observed (Figure 6D).

OA caused by MIA injection resulted in certain changes in the expression of the synovial membrane genes involved in the alternative AEA synthesis and degradation pathways. Expression of *Ptpn22* gene was significantly increased starting from day 2 after MIA treatment and remained increased until the end of the experiment (Figure 7B). The *Inpp5d* gene expression was elevated shortly after MIA injection (days 2 and 7) and was gradually decreased till day 28, when it almost reached a baseline (Figure 7C). A significant decrease in the *sPla2* gene expression was detected throughout the entire experiment (Figure 7D). The *Abdh4* and *Gde1* gene expression levels were gradually increased from the beginning of the experiment, reaching the highest level in the middle (day 7 of 14 days, respectively). Subsequently, the level gradually decreased until the end of the experiment (Figure 7E,F). The level of the *Alox12* gene expression was elevated from day 7 to the end of the experiment (Figure 7H). Changes in other genes (*Plc*, *Ptgs2*, *Alox15*) did not reach statistical significance.

2.4. Overexpression of Calcium/Calmodulin-Dependent Protein Kinase II Delta (CaMKII) as a Result of MIA-Induced OA-like Changes

TRPV1 sensitization may be involved in chronic pain caused by osteoarthritis. Transcript of CaMKII, a kinase which is involved in TRPV1 activation, was detected in the L4 L6 spinal cord segments of the rats. Analysis of mRNA levels indicated a significant increase in *Camk2d* on days 7, 21 and 28 after MIA injection (Figure 8A). In the OA cartilage samples, a significant increase in the *Camk2d* gene expression was detected 14 and 21 days after MIA injection (Figure 8B). In the synovial membranes collected from OA rats, the mRNA level of *Camk2d* were increased in the middle of the experiment, 7 and 14 days after MIA treatment (Figure 8C).

Lumbar Spinal Cord

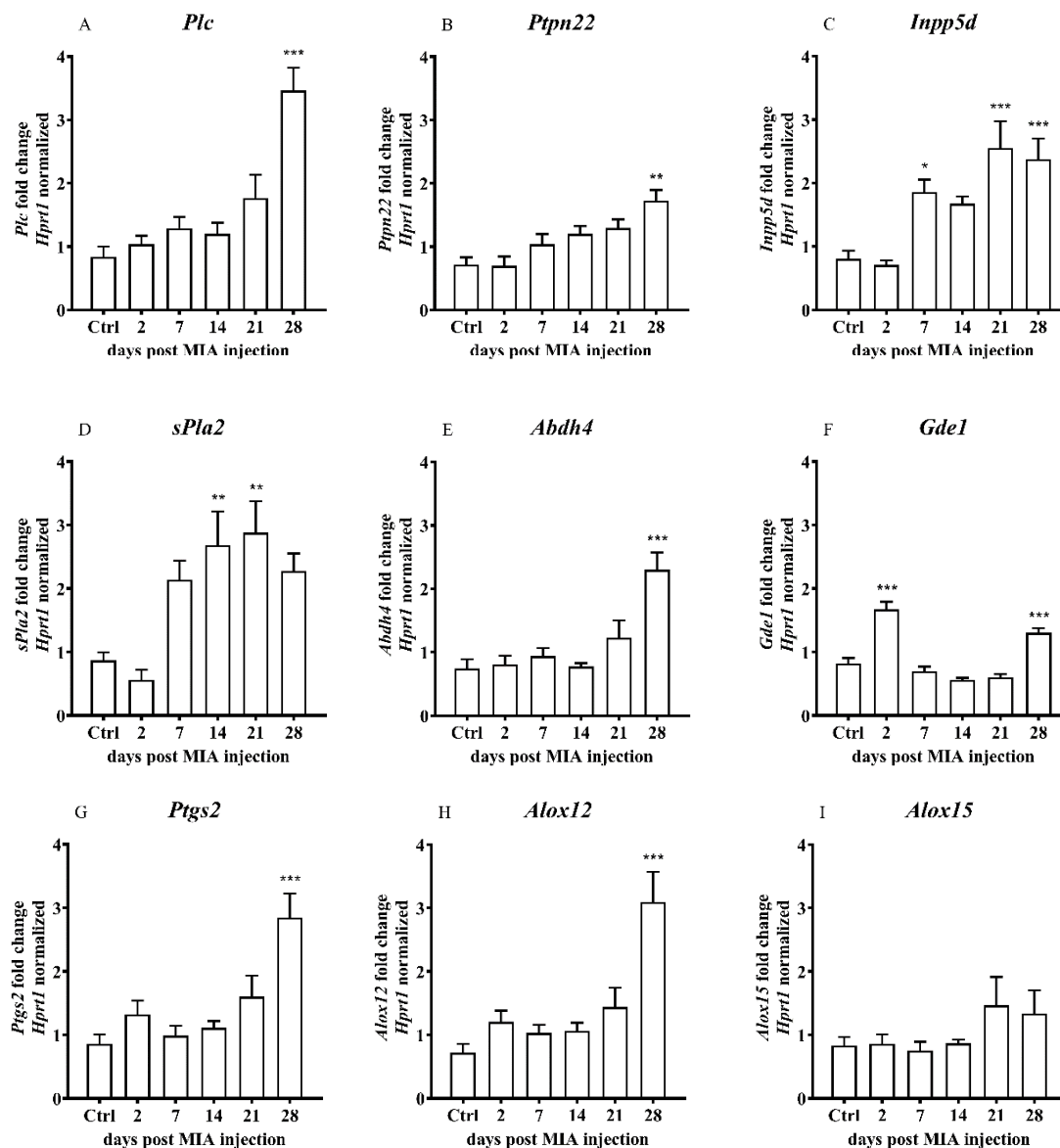


Figure 5. Transcript levels of the alternative AEA synthesis and degradation pathways (A–I), including phospholipase C (*Plc*), protein tyrosine phosphatase, non-receptor type 22 (*Ptpn22*), inositol polyphosphate-5-phosphatase D (*Inpp5d*), phospholipase A2 (*sPla2g2a*), β hydrolase domain containing protein 4 (*Abdh4*), glycerophosphodiester phosphodiesterase 1 (*Gde1*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), arachidonate 12-lipoxygenase (*Alox12*) and arachidonate 15-lipoxygenase (*Alox15*), in the lumbar L4–L6 spinal cord segments of rats after 3 mg MIA injection during OA development. Samples were collected 2, 7, 14, 21 and 28 days after MIA injection; the control group did not receive any treatment. The groups contained 6–9 samples. Data are presented as the mean \pm SEM of fold changes of the group average normalized versus the reference gene (*Hprt1*). Statistical analysis was performed using one-way ANOVA followed by Dunnett post hoc-test (intact animals treated as a control group). * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

Cartilage

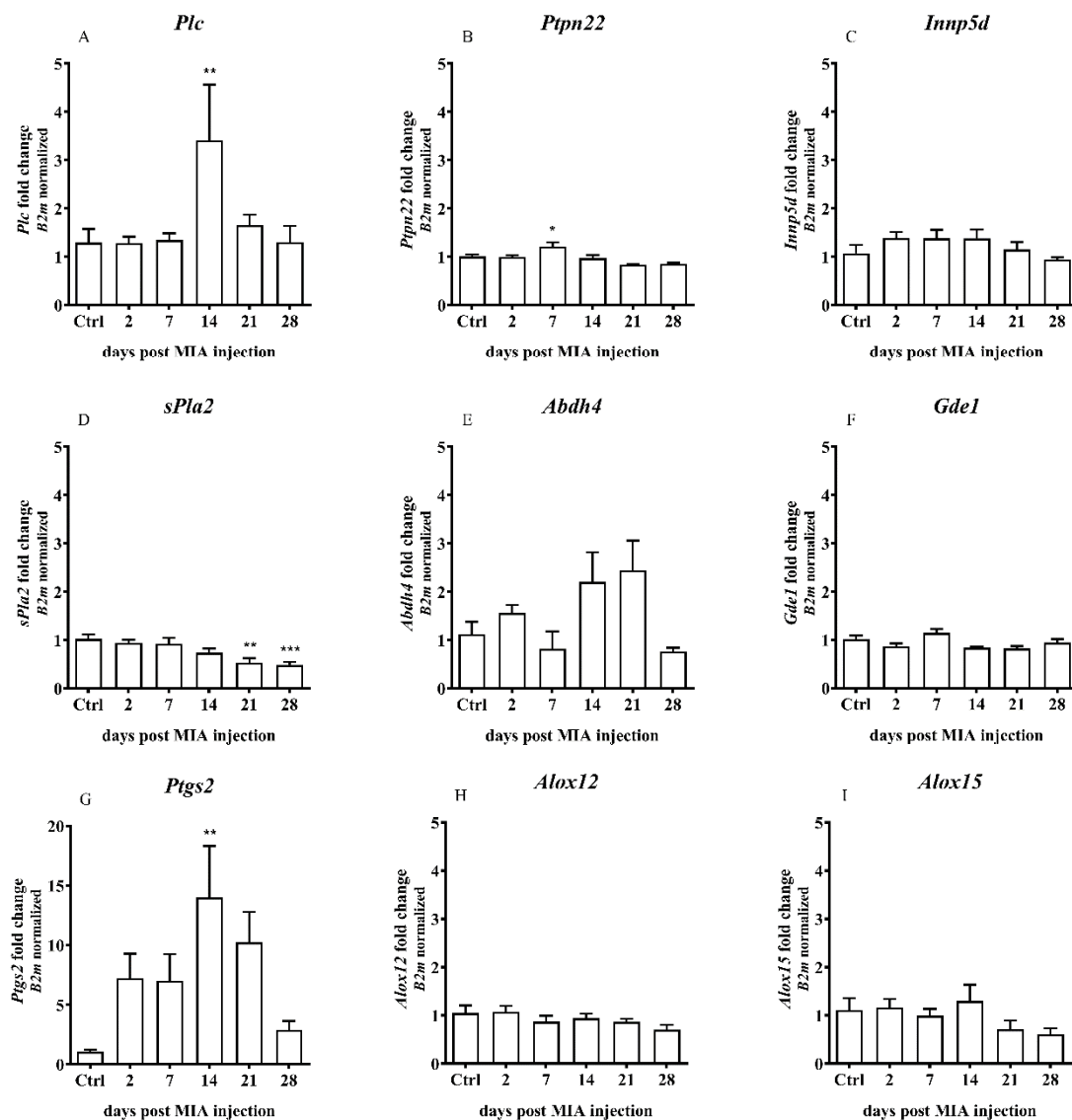


Figure 6. Transcript levels of the alternative AEA synthesis and degradation pathways (A–I), including phospholipase C (*Plc*), protein tyrosine phosphatase, non-receptor type 22 (*Ptpn22*), inositol polyphosphate- 5-phosphatase D (*Inpp5d*), phospholipase A2 (*sPla2g2a*), β hydrolase domain containing protein 4 (*Abdh4*), glycerophosphodiester phosphodiesterase 1 (*Gde1*), prostaglandin- endoperoxide synthase 2 (*Ptgs2*), arachidonate 12-lipoxygenase (*Alox12*) and arachidonate 15-lipoxygenase (*Alox15*), in the cartilage of rats after 3 mg MIA injection during OA development. The samples were collected 2, 7, 14, 21 and 28 days after MIA injection; the control group did not receive any treatment. The groups contained 3–6 samples. Data are presented as the mean \pm SEM of fold changes of the group average normalized versus the reference gene (*B2m*). Statistical analysis was performed using one-way ANOVA followed by Dunnett post hoc-test (intact animals treated as a control group). * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

Synovial Membrane

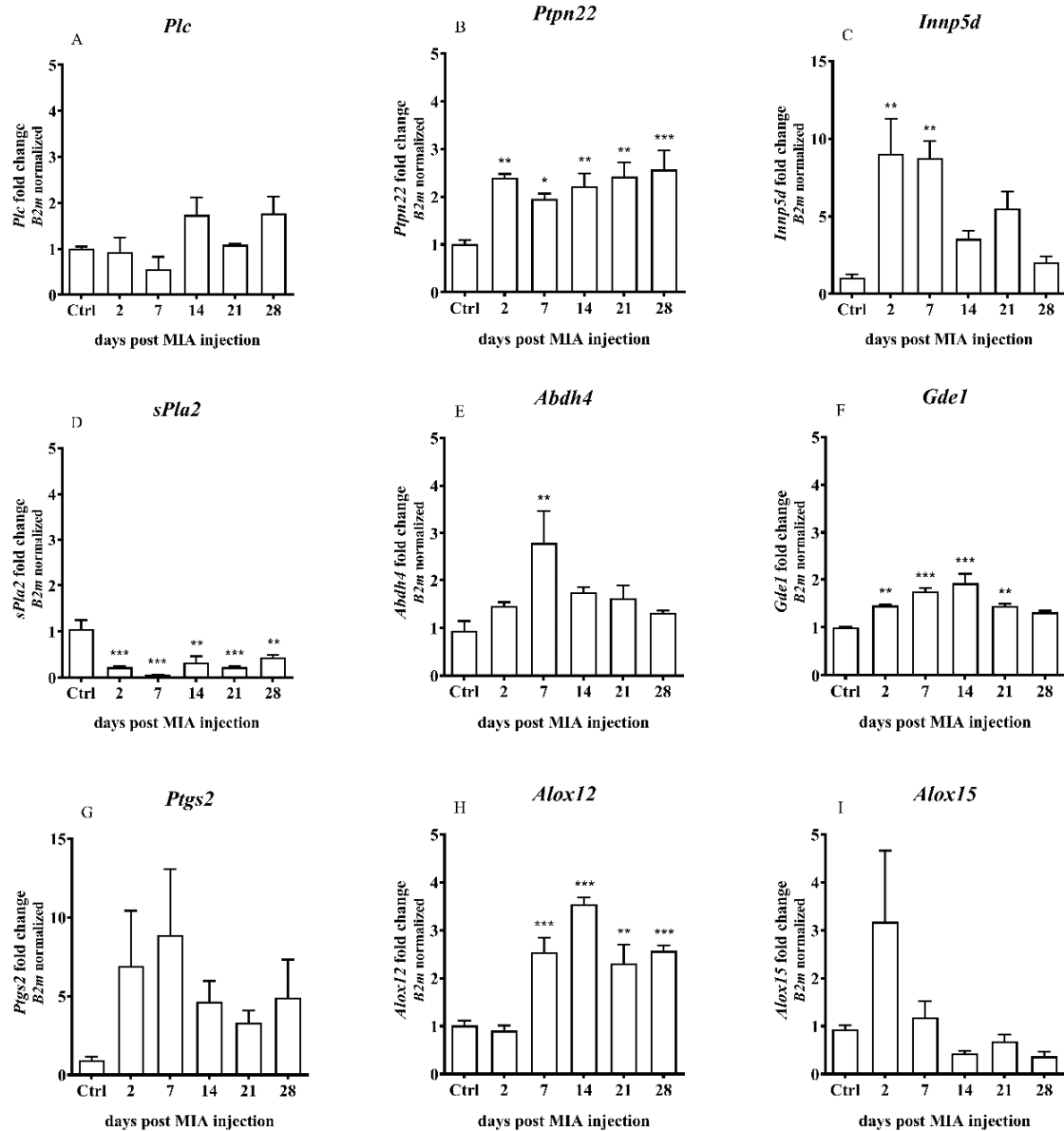


Figure 7. Transcript levels of the alternative AEA synthesis and degradation pathways (A–I), including phospholipase C (*Plc*), protein tyrosine phosphatase, non-receptor type 22 (*Ptpn22*), inositol polyphosphate-5-phosphatase D (*Inpp5d*), phospholipase A2 (*sPla2g2a*), β hydrolase domain containing protein 4 (*Abdh4*), glycerophosphodiester phosphodiesterase 1 (*Gde1*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), arachidonate 12-lipoxygenase (*Alox12*) and arachidonate 15-lipoxygenase (*Alox15*), in the synovial membrane of rats after 3 mg MIA injection during OA development. The samples were collected 2, 7, 14, 21 and 28 days after MIA injection; the control group did not receive any treatment. The groups contained 3–6 samples. Data are presented as the mean \pm SEM of fold changes of the group average normalized versus the reference gene (*B2m*). Statistical analysis was performed using one-way ANOVA followed by Dunnett post hoc-test (intact animals treated as a control group). * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

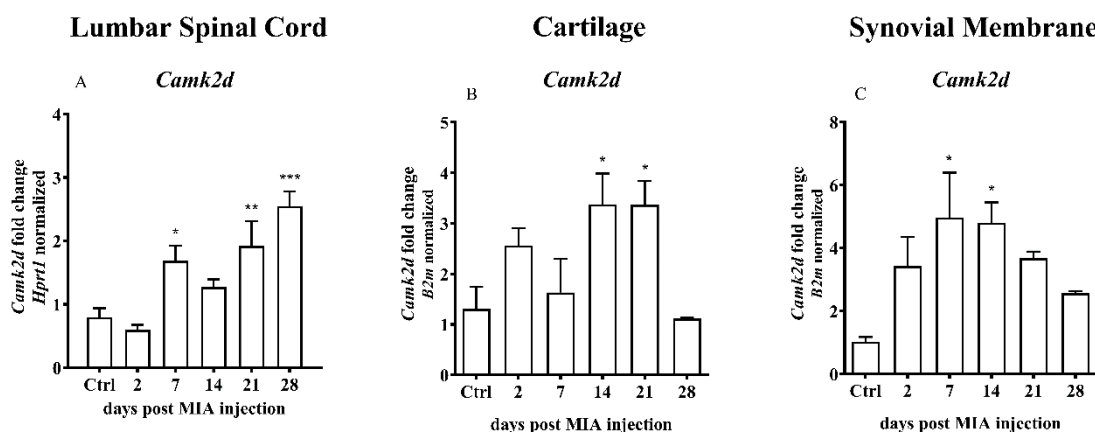


Figure 8. Transcript levels of calcium/calmodulin-dependent protein kinase II delta (*Camk2d*) in the (A) lumbar L4–L6 spinal cord segments, (B) cartilage and (C) synovial membrane of rats after 3 mg MIA injection during OA development. The samples were collected 2, 7, 14, 21 and 28 days after MIA injection; the control group did not receive any treatment. The groups contained 6–9 samples (for the spinal cord analysis) or 3–6 samples (for the cartilage/synovial membrane analysis). Data are presented as the mean \pm SEM of fold changes of the group average normalized versus the reference gene (*Hprt1* or *B2m*). Statistical analysis was performed using one-way ANOVA followed by Dunnett post hoc-test (intact animals treated as a control group). * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals.

3. Discussion

Changes associated with the late stages of OA have been extensively studied; however, the exact mechanism of OA development and the role of the ECS molecules are poorly understood. The present study confirms the changes in the expression of the ECS molecules during OA development. The data indicate that the mRNA levels of cannabinoid receptors (CB1 and CB2) are elevated in the spinal cord or synovial membrane, respectively, which may indicate a role of endocannabinoids in OA progression. Moreover, the changes in the expression of the genes encoding for the enzymes participating in various AEA metabolic pathways were detected, which may play a role in OA pain development.

In the current study, the connection between MIA-induced osteoarthritic pain and central nerve sensitization is demonstrated. The gene expression of *Atf3* (a neuronal marker of nerve injury) is proven to be altered in the dorsal root ganglia; however, the changes are also present in the spinal cord after nerve injury or in chronic pain [28–30]. *Atf3* gene was significantly increased in the advanced OA stage, indicating a neuronal alteration at the spinal level in the advanced OA stages. In the neuropathic pain model (ligation of the L5 spinal nerve), Jin et al. demonstrated that the lesions result in immediate phosphorylation of Mapk14 (p-p38 increase) in the ipsilateral spinal cord microglia and delayed activation of Mapk14 in the L5 DRG neurons [31]. Mapk14 is involved in TRPV1 sensitization in DRGs via inflammation-induced NGF upregulation. Under the inflammatory conditions, NGF in DRGs contributes to Mapk14 phosphorylation, which increases TRPV1 translation and transport to the peripheral terminals. This process entails an increase in the pain sensitivity [26]. Moreover, in the primary DRG culture, ERK (MAPK3) participated in the sensitization of TRPV1 during inflammation [32]. Our data indicate the importance of *Mapk3* and *Mapk14* genes at the later OA stage, when chronic pain is fully developed.

Preclinical and clinical studies have suggested that the endocannabinoid system may be useful to treat various diseases, including the diseases related to chronic pain. In the course of several diseases, an elevated level of the ECS components was reported in preclinical models [33] and in clinical trials [34,35]. Bishay et al. demonstrated that aged mice have lower AEA levels in particular brain structures compared with that in young mice, which resulted in stronger nociceptive development after spared nerve injury and weaker response for R-flurbiprofen [36]. In particular, an increase in

the level of ECS molecules was observed during OA progression at the local [9] and spinal levels [37]. A number of studies emphasized the role of ECS modulators in the alleviation of pain in animal OA models [38–41].

Moreover, on the spinal level, in the advanced phase of the disease, significant alterations in the expression levels of several investigated genes were detected. From the therapeutic point of view, enzymes of the AEA metabolic pathway are more important than receptors on which AEA acts. The main enzymes responsible for AEA synthesis and degradation (*Nape-pld* and *Faah*) showed a similar pattern of expression with an increase at the end stages of the experiment. Moreover, the enzymes involved in the alternative pathways of AEA synthesis and degradation (*Plc*, *Ptpn22*, *Inpp5d*, *sPla2*, *Abdh4*, *Gde1*, *Ptgs2*, *Alox12* and *Alox15*) showed a trend to increase after MIA injection, particularly at the later stages of OA. Interestingly, similar patterns of gene changes were observed in the previous studies. Malek et al. investigated changes in the genes in the DRGs and spinal cord of neuropathic rats. The results indicated significant upregulation of several ECS member genes after CCI induction, including *Crn1*, *Cnr2*, *Pla2g2a*, *Plcb1*, *Inpp5d*, *Faah*, *Ptgs2*, *Alox-12* and *Alox-15* [42]. *Alox-15* gene expression and protein synthesis in the lumbar spinal cord were confirmed in a CCI model already 7 days after the surgery [7]. Guo et al. showed an increase in CAMKII, TRPV1 and pERK1/2 protein levels in the spinal dorsal horn of animals suffering from chronic pain. Moreover, silencing of the TRPV1 gene attenuated mechanical and thermal hyperalgesia and CaMKII and ERK2 phosphorylation [25]. On the other hand, CaMKII is required for TRPV1 ligand binding. Only phosphorylated TRPV1 can be activated by capsaicin and mutations in the TRPV1 at CaMKII sites fail to elicit capsaicin-sensitive currents [23]. In our study, a significant enhancement of the *Camk2d* gene expression in the lumbar spinal cord was observed, particularly in the advanced phase of OA, which may be associated with TRPV1 sensitization in the advanced OA stage.

The data of the present study indicate that NAPE-PLD (the main enzyme responsible for AEA synthesis) plays a role during chronic pain formation; additionally, several alternative factors are important for nerve sensitization in OA progression. Similarly, there is more than a single AEA degradation pathway. Our study revealed that alternative enzymes involved in AEA degradation are upregulated under the OA conditions on the spinal level. This result provides insight into the spinal nociceptive processing and central sensitization during OA development. Important, the majority of the genes were upregulated on the spinal level in the advanced phase of the disease (14 days after MIA injection). This result indicates that ECS molecules may modulate spinal pain processing during the advanced phase of the disease. MIA-induced OA model requires several days to develop the changes, which cause chronic pain, in the cartilage, synovium and subchondral bone. Studies using animal OA models demonstrate constant animal pain responses several days after MIA-injection. In the previous studies, a precise behavioral description of the changes in pain threshold and molecular alterations in DRGs after 3 mg MIA injection was provided. A biphasic pain response was observed in the early inflammatory stage (associated with i.a. injection) and advanced stage beginning on day 14 after MIA treatment [24]. Thus, in most experiments, days 21 or 28 after i.a. MIA injection are used for drug testing. Nevertheless, spinal OA-related alterations arise from the chronic pain impulses transmitted from the animal joints to the nervous system. Sagar et al. demonstrated that in advanced OA phase (28–31 days after OA induction), the response of the wide dynamic range (WDR) dorsal horn neurons induced by innocuous and noxious mechanical stimulation of the hind paw was significantly increased in MIA treated animals compared with that in the control group [37]. Thus, ECS molecules may play an important role especially in the advanced phase of OA on the spinal level.

In addition to the central sensitization, measured at the level of the lumbar spinal cord, local changes in ECS molecules were investigated in our study. Cartilage is the main joint tissue, which covers bone surfaces, prevents bone abrasion and facilitates joint movements. During OA development, degenerated cartilage fragments released into the joint space sensitize the synovial membrane. Thus, the secretion of proinflammatory factors and local inflammation (synovitis) are induced. The exact mechanism of this process remains unknown (especially for idiopathic OA); however, disease progression represents

a self-perpetuating cycle of inflammation and extracellular matrix degradation, in which the main role is played by cartilage, synovial membrane and subchondral bone [43]. The main degenerating tissue during OA is cartilage; however, the malfunctioning of the synovial membrane also has very serious consequences. The synovial membrane is responsible for the production of the synovial fluid and joint lubrication; hence, it plays a primary secretory role in the joint. Thus, the synovial membrane can be more sensitive to gene expression alterations during OA. In the present study, higher variability in the gene expression in the synovial membrane was detected compared with that in the cartilage of rats after MIA treatment.

In the cartilage samples, our results indicate a less significant role of cartilage in ECS functioning during OA progression as the advanced OA phase was not accompanied by significant ECS alterations. However, the cartilage tissue is the most vulnerable tissue to degeneration during the disease progression. Histological staining demonstrated that in 1 mg MIA-induced OA model after 14 days, cartilage is significantly degraded; however, starting from 21 day after MIA injection, the cartilage is essentially completely destroyed (Figure S1). Based on the 3Rs approach to animal testing, testing of the 3 mg dose was not performed when the advanced cartilage changes were observed after administration of 1 mg of MIA. This phenomenon may explain less significant role of cartilage in ECS functioning during OA progression. Alternative pathways (including PLC, PTPN22 and COX-2) may be involved in the early stage of the disease and transition from the early to advanced stage, since alterations in the expression of these genes were observed. However, a more thorough study is needed to explain and clarify these changes.

The role of the synovial membrane was investigated in detail. A significant increase in the CB2 receptor gene expression was detected during almost the entire experimental period. The mRNA of the TRPV1 receptor reached a maximum on day 14 suggesting the main role of the CB2 receptor, rather than TRPV1, in chronic pain associated with OA, with marginal role of CB1 (not detectable, in agreement with [44]). The results of the analysis of the genes involved in TRPV1 sensitization confirm more important role of CB2 rather than TRPV1 in advanced OA phase, including an increase in the mRNA of the *Camk2d* gene in the middle of the study, which returned to the baseline at the end of the experimental period. This result indicates that TRPV1 did not play a very important role in advanced OA in an animal model. The presented study is restricted in targeting of this type of receptor during clinical trials in patients with advanced stage OA. Several clinical studies with TRPV1 inhibitors had been conducted; however, the results have been poor [45].

Furthermore, based on our results an important role of the main AEA synthesis pathway in the synovial membrane at the early stages of OA is suggested. This result is consistent with the data of the literature that elevated level of AEA was detected in the synovial tissue and fluid of patients suffering from OA [9]. Our results indicate that degrading enzymes apparently do not play a key role in OA progression. Nevertheless, these genes cannot be completely skipped when planning the treatment strategies. Similarly to already discussed spinal cord and cartilage, significant increase in synovial's expression levels of genes involved in the alternative AEA synthesis and degradation pathways (*Ptpn22*, *Alox12*) was observed at the latter stages of OA. *Inpp5d*, *Abdh4* and *Gde1* gene expression was augmented in the initial phase, while *sPla2* was diminished since the 2nd day of the study similarly to the pattern observed in the cartilage samples. This result indicates that separate sets of genes may be important in particular OA stages. Considerable changes have been observed in the present study; however, additional investigation is required to draw firm conclusions about the role of the ECS molecules on the local level (in cartilage and synovial membrane) during the progression of OA.

In the current study we proved the AEA synthesis and degradation enzyme upregulation, during the course of OA. Increase in the AEA synthesis enzymes lead to an increase in the AEA level, which is a desired result, because of the AEA's analgesic effect. In turn, AEA degradation enzymes' enhancement increase the level of AEA metabolites (arachidonic acid, ethanolamine, prostaglandins, prostamides, 12-/15-HETE-EA) and reduce the level of AEA. The latter AEA's metabolites, derived on the main

and alternative pathways can be involved in the inflammatory process. Therefore an effective approach to omit problem might be dual-acting substances, for example, FAAH/COX-2 inhibitors, that target both enzymes. Dual-acting drugs offer an analgesic effect by elevating the endogenously produced endocannabinoids (by inhibiting FAAH) and lower the production of pro-inflammatory prostaglandins (by inhibiting COX-2) [46]. In turn, LOX-12/15 metabolites may act in an analgesic way. Indeed, in an animal model of neuropathic pain (chronic constriction injury, CCI, to the sciatic nerve), FAAH inhibitor URB597, diminished thermal and tactile allodynia but also decreased the spinal AEA level and increased LOX-15 level at the same time. This may lead to the TRPV1-mediated analgesia in CCI rats, via 15-hydroxy-AEA, together with oleoylethanolamide and palmitoylethanolamide [7]. AEA metabolites can also be important in several other pathologies. Turcotte et al. widely summarizes the regulatory role of AEA metabolites in various diseases [47]. Nevertheless, to confirm this hypothesis the levels of metabolites should be measured in the animals' tissues, what is a proper direction for the future research.

Considering the analgesic and anti-inflammatory effects of cannabinoids in the pre-clinical studies, cannabinoid therapy seems to be a promising target for the treatment of several diseases. In the arthritis animal models, phytocannabinoid cannabidiol (CBD) reduced inflammation and analgesia in a rat model [48,49]. CBD may also preferentially target inflammatory-activated fibroblasts and reduce its viability, therefore may have an anti-arthritic activity [50]. Synthetic CB2 receptor agonists were also proven to exert an anti-inflammatory response in several arthritis animal models. JWH-015 inhibited inflammation in the rheumatoid arthritis synovial fibroblasts cell cultures and in the arthritis rat model [51]. JWH133 suppressed collagen-induced arthritis in mice, acted anti-inflammatory by repolarizing macrophages from the M1 to M2 phenotype and reduced pro-inflammatory cytokine expression [52]. CB2 agonist 4Q3C showed an anti-inflammatory effect in rheumatoid arthritis mouse model (reduced bone erosion, inhibited formation of osteoclasts and lowered the level of TNF α , IL-1 β , COX-2 and inducible NO synthase) [53].

4. Materials and Methods

4.1. Animals

All experiments were approved by the Local Bioethics Committee of the Institute of Pharmacology (Cracow, Poland, approval numbers: 938/2012; 125/2018). Male Wistar rats (Charles River, Hamburg, Germany) initially weighing 225–250 g were used for all experiments. Animals were housed 5 per cage under a 12/12-h light/dark cycle with food and water available *ad libitum*. All experiments were performed in the morning hours (between 9:00 and 12:00). Experimental groups consisted of $n = 9$ animals (for spinal cord isolation) or $n = 6$ animals (for cartilage/synovium isolation). In rare cases, individual samples had to be excluded from the analysis due to abnormalities during isolation or sample contamination and groups were indicated with an appropriate n number.

Bearing in mind the 3R rule for the ethical use of animals in testing, we decided not to repeat the behavioral experiments performed previously by our group. In the previous studies, a behavioral pattern of changes occurring during OA progression was precisely described [24,54]. Both papers characterized OA-related pain behavior by Pressure Application Measurement test (Ugo Basile, Italy) and Dynamic Weight Bearing test (Bioseb, France). Additionally, these results were supported by microtomography-based 3-dimensional visualizations of rat knees in the consecutive days of the experiment [24], confirming permanent and irreversible changes within the studied subchondral bones of OA rats, correlated with disease progression.

4.2. Induction of Osteoarthritis

Rats were briefly anesthetized with 5% isoflurane (Forane®, Baxter Healthcare Corporation, USA) in 100% O₂ (3 L/min). Joint damage was induced by a single intra-articular injection of sodium monoiodoacetate (MIA; 3 mg/50 μ L; Sigma-Aldrich, Poznan, Poland) in 0.9% saline into the rear right

knee. All surgical procedures were performed under sterile conditions in an animal procedure room away from animal holding areas.

4.3. RNA Extraction, cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction

On days 2, 7, 14, 21 or 28 after MIA injection, the animals were sacrificed and a group of intact animals was used as a control. Control animals were sacrificed in various days (1 or 2 animals every experimental day) to minimize the differences associated with the duration of the experiment. No difference in the intact group in biochemical analysis was observed. The lumbar spinal cord (L4–L6 segments), cartilage and synovial membrane samples from the ipsilateral side were collected in RNA-later (Invitrogen) solution in individual tubes and stored at -80°C until RNA isolation. Then, the samples were homogenized in 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and RNA isolation was performed according to the manufacturer's protocol. The total RNA was quantified using a Nanodrop spectrophotometer (ND-1000, Nanodrop; Labtech International, UK). The samples were adjusted to a concentration of $1\text{ }\mu\text{g }\mu\text{L}^{-1}$ and reverse transcribed to cDNA using iScript reverse transcription supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The qPCR reactions were carried out by iTaq universal probe supermix (Bio-Rad) and TaqMan assays (Thermo Fisher, Applied Biosystems, Waltham, MA, USA). The following assays were performed: Rn01527840_m1 (*Hprt1*), Rn00560865_m1 (*B2m*), Rn02758689_s1 (*Cnr1*), Rn04342831_s1 (*Cnr2*), Rn00583117_m1 (*Trpv1*), Rn01786262_m1 (*Nape-pld*), Rn00577086_m1 (*Faah*), Rn00668379_g1 (*Pla2g2a*), Rn01488539_m1 (*Abdh4*), Rn00583529_m1 (*Gde1*), Rn01514511_m1 (*Plcb1*), Rn01533758_m1 (*Ptpn22*), Rn01400935_m1 (*Inpp5d*), Rn01483828_m1 (*Ptgs2*), Rn01461082_m1 (*Alox12*), Rn00696151_m1 (*Alox15*), Rn00560913 (*Camk2d*), Rn00578842_m1 (*Mapk14*), Rn00820922_g1 (*Mapk3*) and Rn00563784_m (*Atf3*). Reactions were run on a real-time PCR CFX96 touch system (Bio-Rad). Expression levels were assessed using the housekeeping genes *Hprt1* (lumbar spinal cord) or *B2m* (cartilage and synovial membrane). Cycle threshold values were calculated automatically by the CFX Manager software. RNA abundance was calculated as $\text{ddCT} \times 2^{-(\text{threshold cycle})}$ and normalized to the reference gene values (*Hprt1* or *B2m*).

4.4. Statistical Analysis

The analysis was performed using Statistica 13 (StatSoft Software, Tulsa, OK, USA); graphs were prepared using Prism V.5 (GraphPad Software, La Jolla, CA, USA). All data are presented as the mean \pm SEM. The results of RT-qPCR were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett post hoc test. The groups included 39 animals. A value of $p < 0.05$ was considered to be statistically significant (* denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ vs. intact animals).

5. Conclusions

In the present study, a detailed description of the role of ECS in the OA pathogenesis and progression has been provided. The data indicated that during OA progression, AEA synthesis and degradation enzymes were altered on the spinal and local levels. In addition to the main pathway, changes in the enzyme gene expression of two additional alternative pathways of AEA synthesis and degradation were observed. This result may indicate a role of ECS in OA development. Recent ECS-based therapies provide an expanded perspective on the development of an effective OA treatment; however, the detailed mechanism needs to be investigated. The presented study provides the missing information about the MIA-induced OA model and assists in the progress towards more adequate therapy in the future.

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/1422-0067/21/19/7381/s1>.

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Conflicts of Interest: Katarzyna Starowicz, the author of this manuscript, is on the Advisory Board for Phytects and consults on how endogenous cannabinoids function in the central nervous system. Phytects had no financial contribution to the current work. The other authors have no conflicts of interest to declare.

Abbreviations

12-HETE-EA	12-Hydroxyeicosatetraenoyl-ethanolamide
15-HETE-EA	15-Hydroxyeicosatetraenoyl-ethanolamide
AA	Arachidonic acid
ABHD4	α/β Hydrolase domain-containing protein 4
AEA	Anandamide
ALOX-12	Arachidonate 12-lipoxygenase
ALOX-15	Arachidonate 15-lipoxygenase
CaMK2	Ca ²⁺ /Calmodulin-dependent protein kinase
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
COX-2	Cyclooxygenase 2
ECS	Endocannabinoid system
ETA	Ethanolamine
FAAH	Fatty acid amide hydrolase
INPP5D	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1
MAPK3	Mitogen activated protein kinase 3 (ERK1)
MAPK14	Mitogen-activated protein kinase (p38)
NAPE-PLD	N-Arachidonoyl phosphatidylethanolamine phospholipase D
OA	Osteoarthritis
PG	Prostaglandins
PLC	Phospholipase C
PM	Prostamides
PRKCA (PKA)	Protein kinase A
PRKCG (PKC)	Protein kinase C (isoform gamma)
sPLA2	Secreted phospholipase A2
PTPN22	Protein tyrosine phosphatase nonreceptor type 22
TRPV1	Transient receptor potential vanilloid 1

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Inhibition of anandamide breakdown reduces pain and restores LTP and monoamine levels in the rat hippocampus via the CB₁ receptor following osteoarthritis

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ABSTRACT

Chronic pain is a persistent, complex condition that contributes to impaired mood, anxiety and emotional problems. Osteoarthritis (OA) is one of the major causes of chronic pain in adults and elderly people. A substantial body of evidence demonstrate that hippocampal neural circuits, especially monoamine dopamine and serotonin levels, contributes to negative affect and avoidance motivation experienced during pain. Current pharmacological strategies for OA patients are unsatisfying and the endocannabinoid system modulation might represent an alternative for the treatment of OA-related pain. In the present study, we used a rat model of osteoarthritis induced by intra-articular injection of sodium monoiodoacetate to assess, 28 days post-induction, the contribution of endocannabinoid system on the possible alteration in pain perception and affective behavior, in LTP and monoamine levels in the lateral entorhinal cortex-dentate gyrus pathway. The results show that OA-related chronic pain induces working memory impairment and depressive-like behavior appearance, diminishes LTP, decreases dopamine levels and increases serotonin levels in the rat dentate gyrus. URB597 administration (i. p., 1 mg/kg) reduces hyperalgesia and mechanical allodynia, improves recognition memory and depressive-like behavior, restores LTP and normalizes monoamine levels in the hippocampus. The effect was observed 60–120 min post-treatment and was blocked by AM251, which proves the action of URB597 via the CB₁ receptor. Therefore, our study confirms the role of anandamide in OA-related chronic pain management at the behavioral and hippocampal levels.

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1. Introduction

Chronic pain is a persistent, complex condition with no biological value that exists longer than the expected time of healing. It was estimated that in 2019 in the US, more than 20% of adults suffered from chronic pain, and for 7.4%, this condition limited the patient's life or work activities (Zelaya et al., 2020). One of the major causes of chronic pain in adults is osteoarthritis (OA) – a degenerative joint disease, for which the primary cause is abnormal joint loading (e.g., obesity, excessive sport) and elderly age (the "wear and tear" mechanism). In the US, it is estimated that 14 million people have symptomatic knee OA,

with advanced OA comprising over half of those cases (Deshpande et al., 2016). OA is characterized by progressive cartilage and subchondral bone degeneration, local inflammation of the synovial membrane (synovitis), osteophyte formation and chronic pain (Hunter and Bierma-Zeinstra, 2019). Due to movement difficulties and persistent pain, OA is a major cause of disability in working-age people (Palmer, 2016). Chronic pain patients are at high risk of emotional and cognitive disturbances, manifested by mood swings and anxiety (IsHak et al., 2018; Li, 2015) and impaired memory and attention (the relationship is influenced by age) (Moriarty et al., 2017). Neuropathic pain induces anxiety-like and/or depressive-like behavior in rats (Alba-Delgado et al., 2013), and chronic pain is also accompanied by memory impairment in

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Abbreviations

2-AG –	2-arachidonolglycerol
5-HT –	serotonin
AEA –	anandamide
CB ₁ –	cannabinoid receptor type 1
CB ₂ –	cannabinoid receptor type 2
CCI –	chronic constriction injury
CNS –	central nervous system
DA –	dopamine
DG –	dentate gyrus of the hippocampus
FAAH –	fatty-acid amide hydrolase
i.a. –	intra-articular injection
i.p. –	intraperitoneal injection
LEC –	lateral entorhinal cortex
LTP –	long term potentiation
OA –	osteoarthritis
MIA –	sodium monoiodoacetate
PAM –	pressure application measurement
PEA –	palmitoylethanolamide
SNI –	spared nerve injury

mice (Zhang et al., 2021). This might be because chronic pain is a major stressor that impacts neuronal circuits within the brain. Altered hippocampal neurogenesis found in chronic pain animal models could be the reason for accompanying mood disorders and cognitive dysfunction (Apkarian et al., 2016). It was also proven that anxiety, depressive-like behavior and cognitive impairment co-occur with decreased survival of newly generated cells in the dentate gyrus of the hippocampus (Somelar et al., 2021), while in the ventral striatum, chronic back pain alters dopamine neurotransmission (Martikainen et al., 2015). Neuropathic pain impairs hippocampal long-term potentiation (LTP) (Kodama et al., 2007) and decreases neurogenesis and short-term synaptic plasticity (Mutso et al., 2012) in animal models. OA patients are also more likely to have comorbidities, including depression (12.4 vs. 6.4%), anxiety (6.6 vs. 3.5%), and sleep disorders (11.9 vs. 4.2%), than healthy controls (Gore et al., 2011). A study from 2017 showed that more than 50% of OA patients suffer from mental health disorders (Nazarinasab et al., 2017). In a rat model, OA pain decreased dopamine metabolism in the nucleus accumbens, striatum and hippocampus and noradrenaline levels in the frontal cortex and hippocampus (Mlost et al., 2018). Therefore, there is evidence for the association of OA-related pain and cognitive disturbances. Current therapeutic possibilities for OA patients are limited and focus mostly on pain relief via nonsteroidal anti-inflammatory drugs and opioids. However, opioids, in addition to analgesic and antinociceptive action, also possess strong rewarding effects, which may lead to addiction and a phenomenon called opioid-induced hyperalgesia (Lee et al., 2011). An alternative for the treatment of OA-related pain is the endocannabinoid system (ECS), which consists of cannabinoid receptors CB₁ and CB₂, endocannabinoids anandamide (AEA) and 2-arachidonolglycerol (2-AG), and enzymes responsible for its synthesis and degradation. There are growing reports that confirm the role of ECS in the pathogenesis of OA, as well as the role of ECS modulation in the alleviation of OA-related pain (Bryk and Starowicz, 2021; Burston et al., 2013; O'Brien and McDougall, 2018), however more research is needed to conclusively confirm this relationship. OA progression elevated the levels of AEA synthesis and degradation enzymes in the spinal cord, synovial membrane and cartilage in a rat model (Bryk et al., 2020). Fatty-acid amide hydrolase (FAAH) is an enzyme that catalyzes intracellular hydrolysis of AEA. Its blockade was proven to attenuate the development of collagen-induced arthritis and related thermal hyperalgesia in a mouse model (Kinsey et al., 2011). In chronic constriction injury (CCI)-induced chronic pain, the FAAH inhibitor URB597 also

reduced depressive-like behavior (immobility in the forced swimming test) and latency in the novelty-suppressed feeding test (Jiang et al., 2019). URB597 administered bilaterally into the ventral hippocampus enhanced analgesia in fear-conditioned rats (blocked by the CB₁ receptor antagonist rimonabant) (Ford et al., 2011). Therefore, ECS modulation may lead to not only pain reduction but also to normalization of neuronal circuit changes within the hippocampus, altered by chronic pain. In a spared nerve injury model, chronic pain reduced spatial memory, hippocampal LTP, and postsynaptic density volume in the dentate gyrus (DG) (Boccella et al., 2019). Therefore, the aim of this study was to examine changes in LTP and monoamine levels in the lateral entorhinal cortex (LEC)-DG pathway of the hippocampus of animals suffering from chronic pain associated with OA and to determine the role of the FAAH inhibitor URB597 in the reversal of the observed changes.

2. Material and methods

2.1. Animal model of OA

Male Wistar rats (Charles River, Hamburg, Germany), initially weighing 225–250 g, were housed 5–6 per cage in a 12/12 light/dark cycle with food and water *ad libitum*. Experiments were approved by the Local Bioethics Committee of the Maj Institute of Pharmacology (Cracow, Poland) and University of Campania “Luigi Vanvitelli” (Naples, Italy), approval numbers: 217/2019 and 30/2021-PR, respectively. Separate sets of animals were used for behavioral and biochemical studies to avoid the effects of stress associated with behavioral tests on tissue changes. OA was induced in the animal's rear right knee by intra-articular (i.a.) injection on 1 mg of sodium monoiodoacetate (MIA; Sigma–Aldrich, Saint Louis, United States). The dose was selected based on literature data and our previous experience (Bryk et al., 2021). Animals were briefly anesthetized with 5% isoflurane (Aerrane, Baxter, United States) in air (3.5 L/min). The rear right knee skin surface was shaved and swabbed with 75% ethanol. One milligram of MIA dissolved in 50 µl of physiological saline was injected into the joint capsule via a 30 G × 1/2" needle. All surgical procedures were performed under sterile conditions. After OA induction, animals were moved back into their home cages and observed until full recovery from anesthesia. Sham-operated animals were administered 50 µl of physiological saline instead of MIA solution, and intact animals did not receive any treatment. Experimental groups contained N = 7–8 animals each. Behavioral experiments were performed 28 days post-OA induction between 9:00 and 12:00 a.m. Day 28 post-MIA injection was selected as a time point when OA was fully developed and decrease in pain threshold was observed in behavioral tests in this study, as well as based in the previous studies which described in details the development of OA in MIA model (Bryk et al., 2021, 2020; Malek et al., 2015; Pajak et al., 2017). As shown in our previous publications, MIA-induced OA leads to irreversible changes in joint histology. Progressive cartilage degradation is followed by time-dependent decrease in pain threshold which reaches a stable value about 21–28 days after MIA administration. Histological analysis of animal's knee joint show, that 28 days post-OA induction cartilage undergoes significant degradation, which may be quantified and described with a significant increase in the OARSI score, which is an established tool for the histological evaluation of cartilage degeneration in OA (see Supplementary Figure 1 in: (Bryk et al., 2020).

2.2. Drugs and reagents

The FAAH inhibitor URB597 was obtained from Tocris Bioscience (United Kingdom). The CB₁ receptor antagonist AM251 was obtained from Sigma–Aldrich (United States). For behavioral studies, compounds were administered intraperitoneally (i.p.). URB597 was administered at a dose of 1 mg/kg, and AM251 was administered 30 min before URB597, also at a dose of 1 mg/kg. Compounds were dissolved in a vehicle

solution containing 5% DMSO, 5% Kolliphor® EL and 5% ethanol in 0.9% saline. Organic solvents (DMSO and ethanol) were used to dissolve cannabinoids, and Kolliphor® EL stabilized the emulsion in aqueous solution. Total administration volume for i.p. administration was 2 mL/kg. For electrophysiological studies, URB597 was administered intraperitoneally (i.p.) at 1 mg/kg (5% DMSO, 5% Kolliphor® EL and 5% ethanol in 0.9% saline) and AM251 (DMSO 0.1% and saline 0.9%) was microinjected in the LEC at 1 nmol/600 nl.

2.3. Von Frey's test

Calibrated von Frey monofilaments (Bioseb, France) were used to assess mechanical allodynia. Rats were placed in Plexiglas cages with a wire net floor 5 min before the experiment. Von Frey filaments were applied to the mid plantar surface of the ipsilateral hind paw according to the ascending method (Deuis et al., 2017). Each filament was applied five times for an approximately 2–3 s period. The monofilament that evoked at least two responses was noted as the paw withdrawal latency. The strength of the von Frey monofilament bending forces was as follows: 0.6; 1.0; 1.4; 2; 4; 6; 8; 10; 15 and 26 g as a cutoff for response.

2.4. Pressure application measurement

A pressure application measurement (PAM) device (PAM; Ugo-Basile, Italy) has been used for the assessment of joint hyperalgesia (Barton et al., 2007). The animals were held lightly, and the operator placed a thumb with a force transducer mounted unit on one side of the animal's knee joint. A gradually increasing squeeze force was applied across the joint at a rate of approximately 30 g/s with a maximum test duration of 15 s or applied 500 g force. The test was stopped when the animal showed a sign of pain and distress, such as limb withdrawal, wriggling or vocalizing, and the peak gram force (gf) applied immediately before limb withdrawal was noted. The baseline measurements were obtained directly before i.p. drug administration.

2.5. Novel object recognition (NOR) test

The object recognition was performed to assess different components of episodic memory. The task began with a period of habituation, during which rats could freely explore the apparatus, which consisted of a rectangular open box (70 × 70 × 9 cm) made of grey polyvinyl chloride (PVC) illuminated by a dim light, for 1 h. The next day, each rat was allowed to explore two identical objects positioned in the back left and right corners for 5 min (acquisition). A camera recorded the time the rat spent exploring each object. In the test trial (5min), which was carried out after 2 h after acquisition, one of the two objects were replaced with a new object. Time spent with an object was defined by the amount of time the rat spent with its nose directed to and within 1 cm from the object. Data are expressed as an index of the percentage of recognition (RI%), which was calculated as the percentage of time the rat spent exploring the novel object/time spent exploring the novel object + time spent exploring the familiar object × 100.

2.6. Porsolt forced swim test (FST)

For the FST, rats were placed individually into a glass cylinder measuring 20.32 cm diameter X 40.64 cm height containing water (25 °C ± 1.0 °C). Cylinder water was deep enough (30 cm) to ensure that the rats' hind-paws could not touch the cylinder's bottom. The swimming sessions consisted of a 15-min pretest, followed 24 h later by a 5-min test session, based on the original protocol (Porsolt RD et al., 1977). The FST sessions were recorded by a video camera from the side of the cylinder and the animals' escape directed behaviors (swimming and climbing) and immobility were later manually scored. An experimenter was always present in the room during the swim sessions. After successful completion of the FST, each rat was dried with a towel, placed in a warm

enclosure, and then placed in a dry transport cage (20 × 43 × 20 cm) equipped with bedding, food, and water.

2.7. In vivo field potential recordings at LEC-DG pathway

Long term potentiation (LTP) at LEC-DG pathway was induced in Sham and MIA rats treated with vehicle, URB597 alone or in combination with AM251, 28 days post-induction. Briefly, Wistar rats (200–250 g, Envigo Laboratories) were first anesthetized with urethane (1.5 g/kg, i.p.) and fixed in a stereotaxic device (David Kopf Instruments, Tujunga, CA, USA). Body temperature was maintained at 37 °C with a temperature-controlled heating pad (Harvard Apparatus Limited, Edenbridge, Kent). In all surgical preparations, the scalp was incised, and holes were drilled in the skull overlying the site of recording, DG (AP = −3.5 mm from bregma, ML = 2.25 and DV = 2.5 mm below dura) and stimulation, LEC (AP = −7.2 from bregma, ML = 4.0 and DV = 2.3 mm below the dura) according to the coordinates from the atlas of Paxinos and Watson (1986) and contralateral with respect to the injured knee joint. The stimulating electrode was custom-designed for simultaneously stimulating and administering drugs into the LEC. The stimulating and recording electrodes were lowered slowly in the LEC and DG, respectively, until a field excitatory postsynaptic potential (fEPSP) induced by test pulses (0.2 ms in duration delivered at the frequency of 0.033 Hz) was felt. Although increased electrode penetration would result in better electrode positioning for electrophysiological recording, it would induce brain tissue trauma, resulting in trauma-induced neurogenesis. To prevent this from occurring, electrode positioning was limited to four penetrations. After a stabilization of the responses, a baseline was recorded for 30 min, and a theta burst stimulation protocol (TBS), consisting of 6 trains, 6 bursts, 6 pulses at 400 Hz, interburst interval: 200 ms, intertrain interval: 20 s, was applied in the LEC to stimulate the perforant path (PP) fibers to induce LTP. This protocol is able to show that patterns of neuronal firing (complex spikes) that occurred spontaneously during behavior and the optimal repetition rate corresponds to the frequency of the hippocampal theta rhythm. LTP was considered an increase in the amplitude and slope of the fEPSPs that exceeded the baseline by 20% and lasted for at least 30 min from the TBS (Bortolotto et al., 2011; Wu et al., 2005). After TBS, the recording of the fEPSPs was continued for 90–120 min. Field recordings were performed with a tungsten microelectrode (1–5 Mohm), and signals were acquired and analyzed with WinLTP software. Vehicle or drugs were administered in the LEC by connecting the custom-designed stimulating electrode to a polyethylene tube associated with an SGE 1 µl syringe. Volumes of 600 nl of vehicle or drug solution were injected into the LEC over a period of 60 s.

2.8. In vivo microdialysis and chromatographic assessment of DA and 5-HT levels

Neurotransmitter collection was performed in the DG in Sham and MIA rats treated with vehicle, URB597 alone or in combination with AM251, 28 days post-induction. One day before *in vivo* microdialysis, rats were anesthetized with isoflurane and stereotaxically implanted with concentric microdialysis probes into the DG, according to the following coordinates: AP = −3.2 mm posterior to bregma; ML = 1.6 mm lateral to the midline and contralateral to the arthritic knee joint; DV = 2.5 ventral to the dura mater, from the Paxinos and Watson atlas (Paxinos and Watson, 1986). Probes were constructed using a stainless-steel tubing guide of 25 G (0.3 mm inner diameter, 0.5 mm outer diameter, A-M Systems). Inlet and outlet cannulae consisted of fused silica tubing (0.04 mm inner diameter, 0.14 mm outer diameter, Scientific Glass Engineering) positioned inside the guide connected with polyethylene tubes in the upper part and coated by a tubular dialysis membrane (Enka AG, Wuppertal, Germany) in the lower part. They were allowed to advance from the guide and rise to the area of interest. After 24 h of recovery, *in vivo* microdialysis was performed in awake and

freely moving mice. Dialysis was obtained through aCSF (NaCl 147 mM, CaCl₂ 2.2, KCl 4 mM; pH 7.2) infusion at a rate flow of 1 μ L/min by a Harvard Apparatus micropump. After 60 min of equilibration, 12 consecutive dialysate samples were collected, including 6 samples that preceded pharmacological challenge and 6 samples with post-administration of URB597 (1 mg/kg), AM251 + URB597 (1 mg/kg) or Veh (saline 0.9%). Finally, brains were dissected and fixed in 4% paraformaldehyde. After 48 h, each brain was cut into 40 μ m thick slices and observed under a light microscope to check for probe tip localization. Dopamine (DA) and serotonin (5-HT) levels were assessed by HPLC consisting of a Model 590 pump (Waters Associates, Milford, USA) and BIORAD mod electrochemical detector 1640 with an Ag/AgCl reference electrode. Detection potential was set at 0.55 V. A C-18 reversed-phase analytical column (Discovery, 5 μ m, 4.6 mm i.d. x 150 mm; SUPELCO, USA) was maintained at a fixed temperature of 40 °C and eluted by a mobile phase composed of 12.5% methanol, 0.15 mM NaH₂PO₄, 0.01 mM octanosulfonic acid, and 0.5 mM EDTA (pH 3.8). The delivery flow rate was 1 ml/min. Volume injection was 10 μ L. Finally, DA and 5-HT levels were calculated by CDM software, comparing peak areas of samples with areas of standards containing known concentrations of each neurotransmitter. Concentrations of monoamines were expressed as % variation normalized to basal levels of sham rats for each time point (time course analysis) or as the mean monoamine levels [fmol/10 μ L] of

consecutive samples pre- and postdrug administration. Finally, the mean \pm SEM of 5 rats per group was used for statistical analysis (Boccella et al., 2020, 2021).

2.9. Statistical analysis

Data were expressed as numbers or percentages for categorical variables. Continuous data were expressed as the mean \pm SEM. Normal distribution was tested using the Kolmogorov–Smirnov test with Lilliefors correction. Electrophysiological data were analyzed by using two-way ANOVA followed by Tukey's post-hoc test for comparisons between groups. One-way ANOVA followed by Sidak's multiple comparison test was applied for pre- and postdrug comparisons within groups. Statistical analysis of monoamine levels was carried out through point-by-point comparisons among groups (time course % variation normalized to the naïve group) and pre-versus postdrug comparisons of monoamine levels within groups (fmol/10 μ L of dialysate). Repeated measures-2-way ANOVA with Greenhouse–Geisser correction followed by Tukey's multiple comparison test or Sidak's post-hoc test was used. For the behavioral studies, the results were analyzed by two-way ANOVA (or mixed model) with Tukey's HSD post hoc test. * denotes $p < 0.05$; ** denotes $p < 0.01$; *** denotes $p < 0.001$ at each time point vs. the control group. Analysis and graphs were prepared using

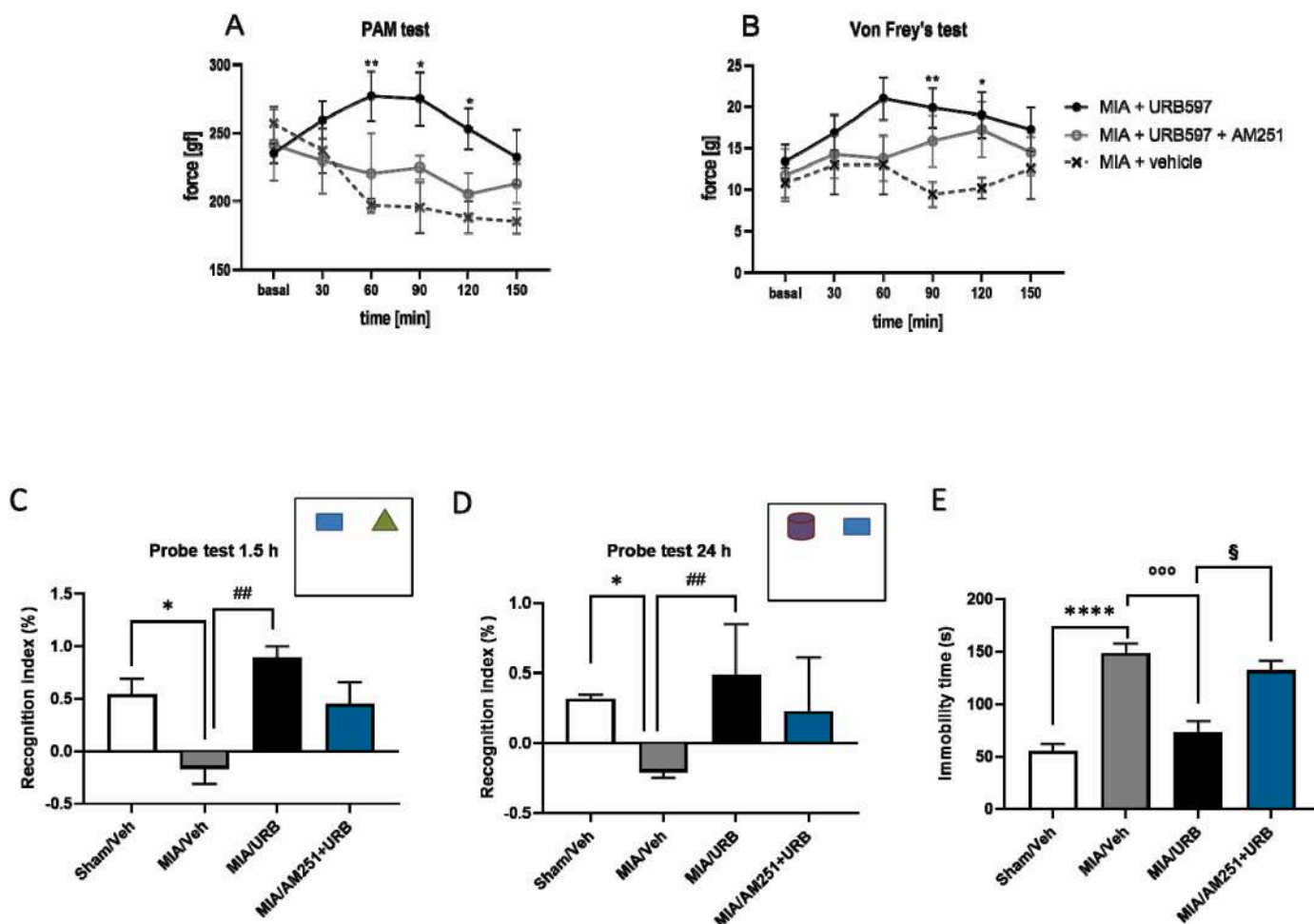


Fig. 1. Hyperalgesia (A) and mechanical allodynia (B) after URB597 treatment in osteoarthritis rats. Paw sensitivity was measured by PAM and Von Frey's tests. Basal measurements were performed directly before drug administration, and subsequent measurements were taken every 30 min up to 150 min posttreatment. Two-way ANOVA (or mixed model) and Tukey's HSD post hoc testing were performed, and groups contained N = 7–8 animals. * denotes $p < 0.05$; ** denotes $p < 0.01$ of the MIA + URB597 group at each time point vs. the control group (MIA + vehicle). (C and D) Recognition index (%) in NOR test and (E) Immobility time (s) in Porsolt forced swimming test, in MIA rats treated with URB597 alone or in combination with AM251. Ordinary one-way ANOVA followed by Sidak's multiple comparisons test was performed (n = 6 per group). * $p < 0.05$ vs. Sham/Veh, ° $p < 0.05$ vs. MIA/Veh and § $p < 0.05$ vs. MIA/URB597.

GraphPad Prism 9 software (La Jolla, CA, USA).

3. Results

3.1. URB597 diminishes pain and allodynia in MIA-treated rats

Directly before drug administration, both PAM and Von Frey's test results were comparable in all animals ($p > 0.05$, Fig. 1 A, B). Significant pain attenuation was observed 60–120 min after URB597 treatment in comparison to vehicle-treated osteoarthritic animals in the PAM test ($p = 0.0095$ at the 60 min time point, $p = 0.0323$ at the 90 min time point, and $p = 0.0146$, at the 120 min time point, Fig. 1A). This effect was blocked by the CB₁ receptor antagonist AM251. A decrease in pain threshold in the MIA/Veh and MIA/URB597 + AM251 groups was observed (Fig. 1A), probably associated with repeated PAM tests. Nevertheless, URB597-treated animals were protected from these effects. Mechanical allodynia measured by Von Frey's test was significantly reduced 90–120 min post-URB597 treatment ($p = 0.0095$ at the 90 min time point and $p = 0.0412$ at the 120 min time point, Fig. 1B). AM251 did not significantly alter the pain threshold in Von Frey's test in comparison to vehicle-treated animals in all time points ($p > 0.05$, Fig. 1B).

3.2. URB597 improves short- and long-term recognition memory in MIA-treated rats

It has been previously shown that osteoarthritis pain is frequently accompanied by co-morbid affective manifestations, such as cognitive alterations including memory dysfunction, which contribute to an overall impairment of the quality of life (Moriarty et al., 2011; Moriarty and Finn, 2014). In this study, we assessed learning and memory in the novel object recognition test and we found that exchanging one of the objects by a novel (dissimilar) object induced a significant side preference towards the novel object in the 1.5 and 24h-delay test phases (1.5h: 0.54 ± 0.15 ; 24h: 0.31 ± 0.0032) in Sham rats (Fig. 1 C and D). On the contrary, MIA rats showed a significant reduced discrimination index at both 1.5h and 24h (1.5h: 0.17 ± 0.13 ; 24h: 0.20 ± 0.017), as compared to Sham/Veh animals (Fig. 1 C and D). Single administration of URB597 was able to normalize NOR index in MIA rats at both 1.5h and 24h (1.5h: 0.89 ± 0.1 ; 24h: 0.49 ± 0.17), as compared to MIA/Veh group (Fig. 1 C and D). Co-administration of URB597 with AM251 showed a trend in decrease of NOR index rats at both 1.5h and 24h (1.5h: 0.45 ± 0.21 ; 24h: 0.22 ± 0.19), reflecting the partial involvement of CB₁ receptor in URB-induced pro-cognitive effect in osteoarthritis rat model (Fig. 1C and D).

3.3. URB597 reduces depressive-like behavior in MIA-treated rats

Prior studies have identified that OA pain severity may contribute to

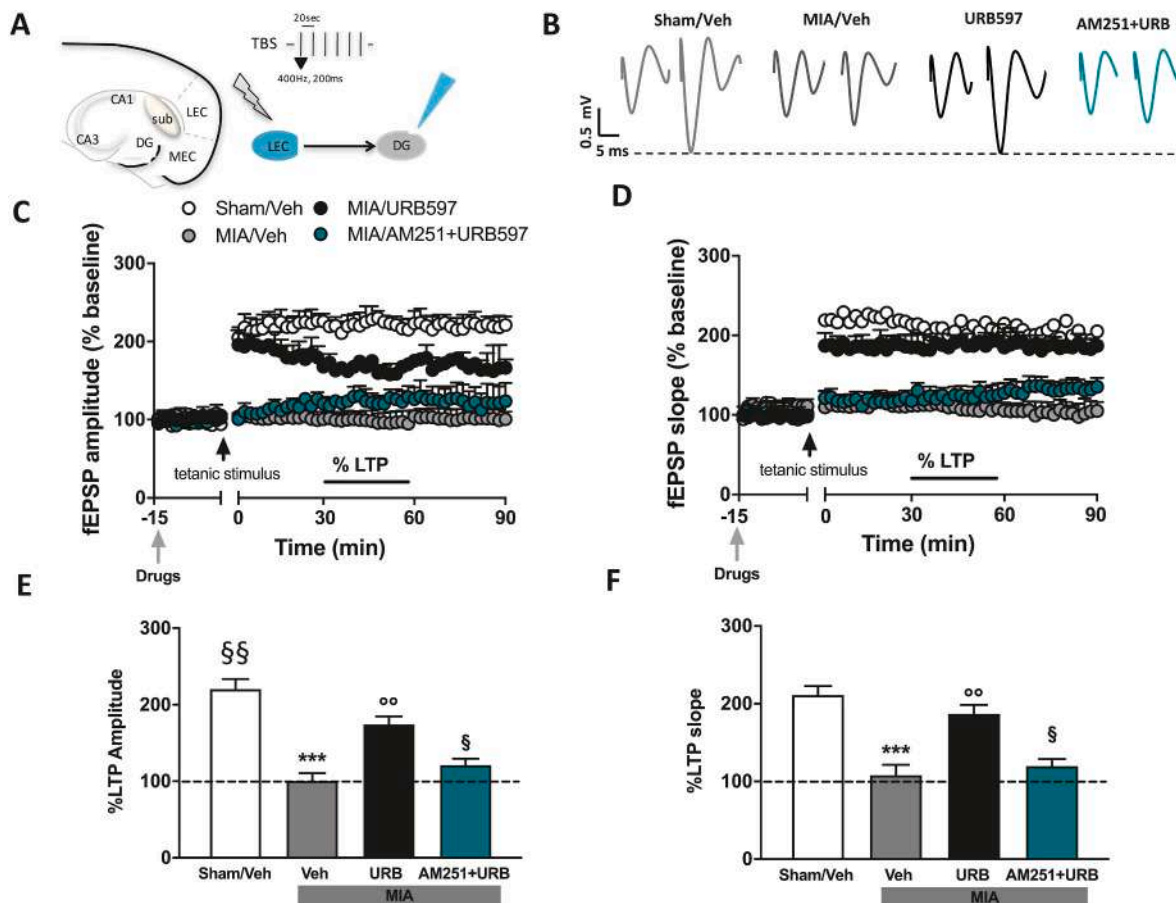


Fig. 2. MIA-mediated impairment of plasticity of LEC-DG via CB₁ signaling. (a) Graphical representation of the recording electrode and stimulating electrode in the dentate gyrus and in the lateral entorhinal cortex, respectively. (b) Representative traces recorded in Sham/Veh, MIA/Veh, MIA/URB597 and MIA/AM251 + URB597 before and after TBS. (c and d) Plot of the fEPSP amplitude and slope recorded before and after the induction of LTP (time 0) in the DG of sham/veh, MIA/veh, MIA/URB597 and MIA/AM251 + URB597. The extent of LTP was calculated as a percentage of the baseline between 30 and 60 min of recording. (e and f) Bar graphs of LTP in the sham/veh, MIA/veh, MIA/URB597 and MIA/AM251 + URB597 groups, $n = 5$. One-way ANOVA followed by Sidak's multiple comparisons was performed. * $p < 0.05$ vs. Sham/Veh, ° $p < 0.05$ vs. MIA/Veh and §inscnp < 0.05 vs. MIA/URB597.

the development and worsening of depressive symptoms (Kroenke K et al., 2011). Here, the time of immobility in porsolt forced swimming test was significantly higher in MIA rats as compared to Sham animals at 28 days post-induction (MIA: 148.5 ± 9.33 s; Sham: 55.25 ± 6.94 s) (Fig. 1 E). URB597 administration reverted depressive-like behaviour in MIA rats (132.3 ± 8.98 s) and this effect was completely blocked by AM251 (Fig. 1E).

3.4. Effect of intra-LEC AM251 microinjection on LTP at the LEC-DG pathway in arthritic rats treated with URB597

The theta burst stimulation protocol applied at the LEC induced long-term potentiation by increasing both the amplitude (30–60 min: $220.29 \pm 13.42\%$) ($p = 0.0021$) and slope (30–60 min: $211.02 \pm 11.53\%$) ($p = 0.0046$) of the fEPSPs in the DG compared to basal values in sham mice treated with vehicle (Fig. 2B–F). Intriguingly, we found impaired LTP after TBS application at LEC synapses in arthritic rats at 28 days post-MIA injection, corresponding to the peak of mechanical allodynia. Indeed, MIA rats showed post-TBS amplitude (30–60 min: $100.70 \pm 9.98\%$) ($p = 0.0001$) and slope (30–60 min: $108.01 \pm 13.3\%$) ($p = 0.0007$) of the fEPSPs in the DG similar to the basal values (Fig. 2B–F).

Strikingly, single administration of URB597 (1 mg/kg, i.p.) induced a functional recovery of LTP magnitude compared to vehicle-treated MIA rats. Indeed, both the amplitude (30–60 min: $174.35 \pm 10.5\%$) ($p = 0.0039$) and slope (30–60 min: $186.82 \pm 11.7\%$) ($p = 0.0040$) of the fEPSPs in the DG significantly increased after TBS application in MIA rats (Fig. 2B–F). The recovery of LTP induced by URB597 was completely prevented by intra-LEC microinjection of AM251 (1 nmol/600 nl) in MIA rats. In fact, both the amplitude (30–60 min: $121.15 \pm 8.57\%$) ($p = 0.0024$) and slope (30–60 min: $119.54 \pm 9.39\%$) ($p = 0.01$) of the DG fEPSPs did not change compared to MIA/URB597 rats. Ordinary one-way ANOVA for repeated measures followed by Sidak post hoc test revealed significant differences in the amplitude ($F_{3,8} = 25.8$, $P = 0.0002$) and in the slope ($F_{3,8} = 18.95$, $P = 0.0005$) between the treatments.

3.5. URB597 restores decreased dopamine and serotonin levels in the hippocampal dentate gyrus of MIA-treated rats

Monoamine levels have been reported both as time-course percentage variation from baseline values, comparing difference among groups (Fig. 3A and C) and as pre-versus post-drug levels within each group

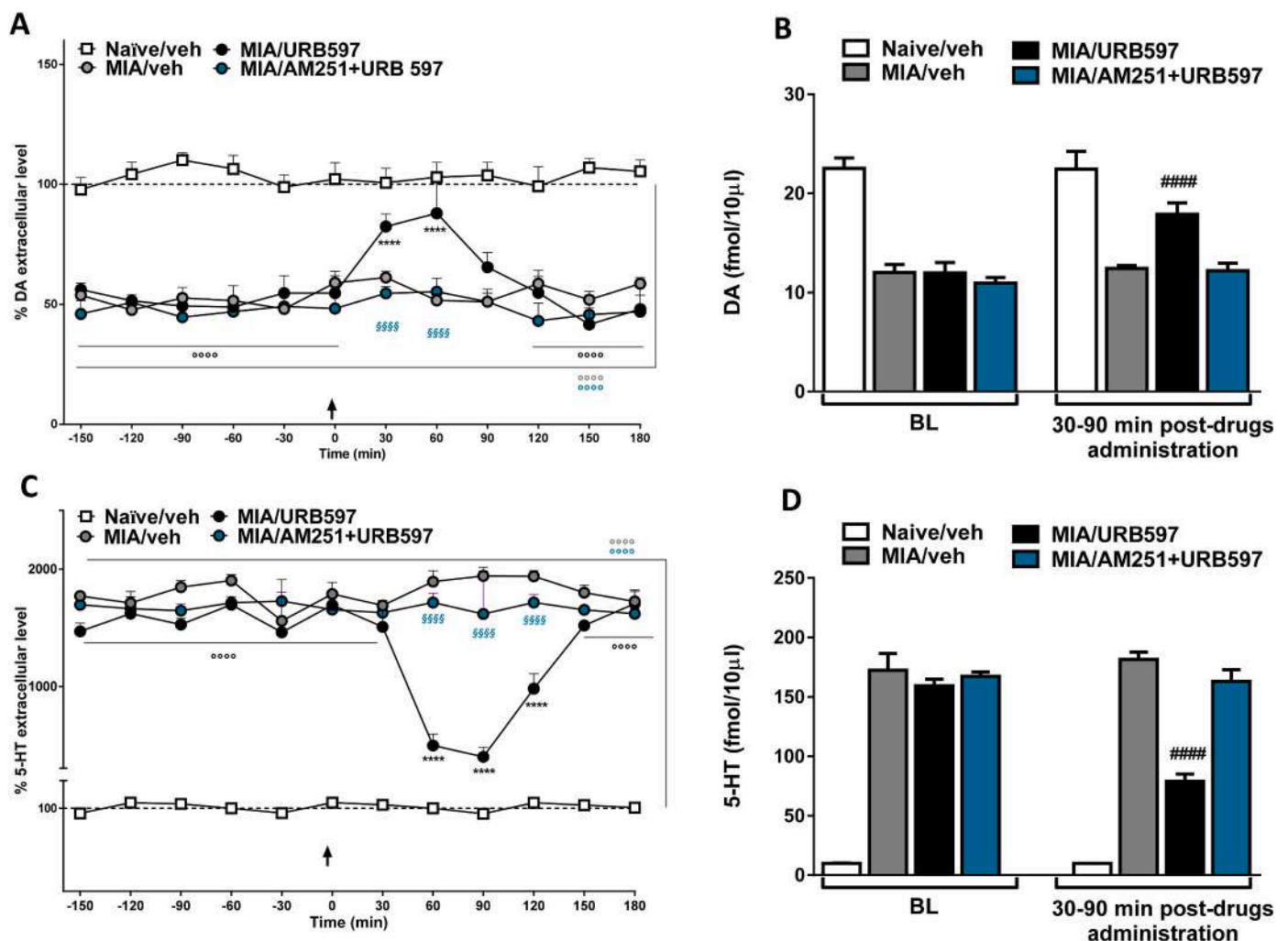


Fig. 3. (A, C) Point-by-point time course of extracellular levels of dopamine (DA) and serotonin (5-HT) in the DG in naive or MIA rats treated with a single administration of URB597 (1 mg/kg i.p.), AM251 + URB597 (1 mg/kg) or vehicle (saline 0.9%). The arrow indicates drug administration, and it corresponds to time 0. Extracellular levels of monoamines are expressed as the percentage variation normalized to the basal levels of the naive rats. Each point represents the mean \pm S.E. M of 5 animals per group. (B, D) Pre- and postdrug administration extracellular levels of DA and 5-HT. Predrug measurements are indicated as baseline (BL). For each rat, the mean of three consecutive predrug values and the mean of three consecutive postdrug values (30-60-90 min) were considered for the analysis. Finally, each bar represents mean \pm S.E. M of 5 animals per group. ° vs. Naive/veh, * vs. MIA/veh, § vs. MIA/URB597. RM-2 way ANOVA followed by Tukey's (A,C) or Sidak's (B, D) post hoc multiple comparisons test was applied. $P < 0.05$ was considered to be statistically significant.

(Fig. 3B and D). A $58.99 \pm 4.47\%$ decrease of basal DA levels in MIA/veh group compared to naïve rats ($p < 0.001$) was found. In contrast, serotonin levels were importantly increased in MIA/veh rats ($+1787.78 \pm 98.53\%$; $p < 0.001$). A single administration of URB597 (1 mg/kg i.p.) in MIA-treated rats transiently normalized both DA (60 min post-drug = $+36.32 \pm 15.35\%$; $p < 0.001$) and 5-HT levels (60 min postdrug = $-1398 \pm 98.56\%$ vs.; $P < 0.0001$). Moreover, the preceding administration of AM251 (1 mg/kg i.p.) blocked URB597 effect (Fig. 3A and C). Data were analyzed by repeated measures-2 way ANOVA, followed by Tukey's multiple comparisons post-hoc test (DA levels: $F_{(3,192)} = 219.1$, $p < 0.0001$; 5-HT levels: $F_{(3,192)} = 4.34$, $p < 0.0001$). Pre- and post-drug monoamine extracellular concentrations (fmol/10 μ L) analysis revealed a significant post-drug increase of DA in MIA/URB597 (30–90 min: 17.87 ± 2.60 fmol/10 μ L) compared to pre-drug levels (11.39 ± 2.32 fmol/10 μ L). Conversely, post-drug 5-HT was decreased in MIA/URB597 (30–90 min 78.94 ± 6.18 fmol/10 μ L compared to pre-drug levels (172.4 ± 14.2 fmol/10 μ L, $p < 0.0001$). No significant changes between pre- and post-drug administration were observed in naïve group, and MIA rats receiving vehicle or AM251 + URB597 (Fig. 3B and D). Data were analyzed by repeated measures-2-way ANOVA followed by Sidak's multiple comparisons post-hoc test (DA levels: $F_{(3,16)} = 53.13$, $p < 0.0001$; 5-HT- $_{(3,16)} = 164.2$, $p < 0.0001$).

4. Discussion

A large number of studies confirm the involvement of endocannabinoids in OA pathophysiology and the possible therapeutic effect for both the treatment of pain and related inflammation (Mlost et al., 2022; Philpott et al., 2017; Rzeczycki et al., 2021). CB₁ and CB₂ receptors are both present in the synovial membrane of patients with OA; in addition, the levels of AEA and 2-AG, the two main endocannabinoids, are elevated in the synovial fluid of patients compared to healthy controls (Richardson et al., 2008). The level of 2-AG in CSF and synovial fluid has also been correlated with the severity of postoperative pain after total knee arthroplasty in patients with OA (Azim et al., 2015). What we highlighted in this study is that the analgesic effect associated with the increase in extracellular levels of AEA by the FAAH inhibitor URB597 was achieved 28 days after the induction of OA. This time period corresponds to the time when both allodynia and cartilage degeneration were fully developed, largely recapitulating the advanced stage of OA in humans and in rodents (Han et al., 2021; Haywood et al., 2018). Blocking of the metabolism of acylethanolamines (i.e., AEA, PEA) with URB597 in addition to exerting a significant antiallodynic effect 60–120 min after a single administration, it also avoided the development of hypersensitivity associated with repeated PAM tests. The PAM test could cause increased pain in the animal's knee joint after repeated measurements, as its specificity requires the experimenter to apply an external force to the animal's knee, which could sensitize the already painful knee joint because of advanced OA. This effect was observed in vehicle-treated animals and in animals that received URB597 and AM251 but not in animals with OA treated with URB597. The analgesic effect observed is consistent with the evidence that FAAH inhibition-mediated analgesia is primarily CB₁-dependent (Kwilasz et al., 2014; Schuelert et al., 2011) and that URB597 is a FAAH inhibitor that penetrates the central nervous system (CNS) (Clapper et al., 2010), although its effect via CB₁ activation on peripheral nerve terminals can also be important for relieving OA symptoms.

Since URB597 passes into the CNS (Clapper et al., 2010), it became intriguing for us to see if the brain structures have a role in the integration of pain. In this study, we investigated the hippocampus and OA-induced neurochemical and functional variations at that level and whether URB597 could have some restorative effect on those changes.

In the present study, animals suffering from chronic pain had significantly diminished LTP in the LEC-DG pathway of the hippocampus (as measured by both amplitude and slope of fEPSP). After treatment with URB597 (AEA increase via FAAH inhibition) an increase in LTP was

observed in OA animals. The results confirming the role of CB₁ receptors in this neural pathway for the induction of neuroplasticity were recently obtained in the SNI model, in which neuropathic pain reduced LTP in the LEC-DG pathway, spatial memory and postsynaptic density, volume and arborization of DG dendrites and increased the level of 2-AG in the LEC (Boccella et al., 2019). Furthermore, the increase in AEA by chronic treatment with URB597 partially restored age-related LTP deficiencies in the hippocampal dentate gyrus of rats and reduced proinflammatory cytokine levels in elderly animals (Murphy et al., 2012). Treatment with chronic synaptamide (DHEA; structural analog of AEA) restored LTP, and hippocampal neurogenesis was inhibited by activation of LPS-exposed microglia (Tyrtysnaia et al., 2020). Chronic palmitoylethanolamide (PEA), an AEA analog, administered to neuropathic mice not only exhibited antiallodynic and antihyperalgesic activity but also recovered memory deficits and restored hippocampal LTP induction (Boccella et al., 2019). It has already been shown previously, that within another important brain area of the pain matrix, the prefrontal cortex, 2-AG is increased in a mouse model of OA, while the anxious component of OA was increased in CB₁ receptor knockout animals and absent in CB₂ receptor knockout animals (La Porta et al., 2015). Furthermore, 2-AG-CB₁ receptor signaling in the ventral hippocampus has been shown to have an anti-adverse effect, which is abolished by persistent pain (Rea et al., 2014). An interesting new aspect that has recently emerged is that cannabinoids can facilitate neural plasticity of the hippocampus in some specific pathways. In this regard, it has been observed that specific projections of the lateral perforating pathway toward the dentate gyrus use the CB₁ receptor for memory formation (Wang et al., 2018). In this pathway, the reduced level of 2-AG helped to decrease LTP, while the blockade of 2-AG degradation by JZL184 led to an improvement in LTP. However, URB597 did not alter LTP in the lateral perforating pathway, indicating that AEA, or its other congeners, does not play a crucial role in this particular neural pathway (Wang et al., 2016).

In this study, we measured DA and 5-HT levels in the contralateral hippocampus, specifically in the DG. The dysregulation of these two fundamental monoaminergic neurotransmitters is often associated with various affective-cognitive disorders, including depressive-like behavior described in subjects with chronic inflammatory and neuropathic pain (Dunlop and Nemeroff, 2007; Mlost et al., 2018; Sindrup et al., 2005). There is evidence that clinical and preclinical data have described a functional maladjustment of the dopaminergic system in depressive syndromes of various origins (Dunlop and Nemeroff, 2007). Spared nerve injury-induced neuropathic pain disrupted working memory and hippocampal spike activity, which was reversed by systemic administration of the dopamine D2/D3 agonist quinpirole (Cardoso-Cruz et al., 2014). The dopaminergic system in the hippocampus also plays an important role in orofacial pain (Reisi et al., 2014), inflammatory pain (Torkamand et al., 2021) or migraine (Kim et al., 2021). Several studies indicate an influence of ECS modulation on DA level. Intravenous administration of AEA induced an increase in DA in nucleus accumbens shell of rats, this effect was magnified and prolonged by URB597 (Solinas et al., 2006). In non-human primate Parkinson Disease model, URB597 increased plasma levels of the FAAH substrates: AEA, N-oleoyl ethanolamide (OEA), and N-palmitoyl ethanolamide (PEA) 4 h after administration (Johnston et al., 2010). Local (intraplantar) injection of URB597 in a carrageenan-induced inflammatory pain increased levels of AEA and 2-AG, but not PEA or OEA (Jhaveri et al., 2008). However, in the same model of inflammatory pain, Okine et al. showed, that i.p. injection of URB597 did not alter AEA, PEA or OEA level in animal's hind paw, but increased AEA, PEA and OEA level in the spinal cord and midbrain, but only after single administration, not chronic treatment. This might indicate that sustained pharmacological inhibition of FAAH results in an adaptation in the synthesis of the endocannabinoids, and related N-acylethanolamines, or their catabolism by alternative pathways (not dependent upon FAAH) (Okine et al., 2012). In the current OA-induced pain model, we observed a massive decrease in the baseline level of extracellular DA in the DG hippocampus. Conversely, the 5-HT

levels in the DG were instead increased in the same group. At this point, it is important to point out that 5-HT is involved in the storage of both positive and negative or aversive information in DG and CA3 hippocampal structures through different circuits involving serotonergic neurons of the medial raphe (MRR) (Ohmura et al., 2020). Interestingly, this limbic structure also plays a role in the storage of negative memories, such as the typical experience of chronic pain (Commons, 2016; Hayashi et al., 2015). Furthermore, a population of glutamatergic neurons innervates the medial nucleus of the raphe and, through lateral habenula involvement, may induce aversion and anxiety (Szönyi et al., 2019). Very high levels of 5-HT in the hippocampus justify the memory of the painful and persistent negative affective component associated with it. Furthermore, it has recently been shown that high levels of 5-HT in the hippocampus negatively affect neurogenesis, leading to maladaptive synaptic plasticity. This concept fits with our electrophysiological findings, picturing the possible development of affective-cognitive disorders related to chronicization of malaise states, including pain (Song et al., 2016).

This study was performed on male rats only, which is a limitation of the research. In human patients, the risk of prevalence and incidence of OA is higher in women than in men (Laitner et al., 2021; Mal-eki-Fischbach and Jordan, 2010), also OA in women is often more advanced and causes more pain than in men (Logerstedt et al., 2014). However, although studies investigating the role of sex hormones in OA pathogenesis and development indicate the role of estrogen and estrogen receptor in joint health, a strong conclusion regarding its influence on OA development cannot be drawn from these studies (de Klerk et al., 2009; Hussain et al., 2018; Migliorini et al., 2022; Tanamas et al., 2011). Otterness and Eckstein hypothesized that higher risk of OA in women results from smaller subchondral bone area, cartilage thickness and volume as compared to men (even after adjustment for patient's height and weight), however estimated tibial and patellar pressures are similar between sexes, which cannot explain differences in OA prevalence between sexes (Otterness and Eckstein, 2007). A bioinformatics analysis revealed 278 genes differentially expressed in men and women after menopause (e.g. EGFR, ERBB2, CDC42, and STAT1), suggesting that pathways of PI3K-Akt, osteoclast differentiation, and focal adhesion may play important roles in the development of OA (Wang et al., 2019). However, more studies are needed to confirm this result. Therefore, the effect of sex on the development of OA would be an additional unknown factor in the study, which is already undertaking an explanation of a very complicated mechanism for the development of OA and the effect of ECS on this disease. Investigating sex differences in the development of OA and the effect of ECS modulation on cognitive changes induced by chronic pain is an aspect worth of further research, however is enough for a whole separate study. Therefore, in this article authors focused only on the role of ECS in OA pain and cognitive deficits reduction in male rats.

5. Conclusions

In the present study, the effect of OA-induced chronic pain on hippocampal LTP and monoamines level was investigated. We proved, that inhibition of fatty-acid amide hydrolase (an enzyme responsible for catalysis of intracellular hydrolysis of AEA) by URB597 not only reduces allodynic and hyperalgesic component of OA, but also restores maladaptive neuroplasticity at the level of LEC-DG pathway and decreases or increases the level of DA and 5-HT in the CA3 hippocampus, respectively. Interestingly, analgesic effects in PAM and Von Frey's test were observed 60–120 min post-URB597 treatment. Similarly, a significant elevation in DA was found 30–60 min post-URB597 treatment, while 5-HT level normalization was observed 60–120 min after URB597 administration. Thus, normalization observed in both the monoamine (DA and 5-HT) hippocampal levels was consistent with the onset of the analgesic effect. The endocannabinoid modulation of LTP may depend on the paradigm of its induction, as well as the site of stimulation or

tetanzation. A study conducted on hippocampal slices showed that endocannabinoids enhance strong θ burst-induced LTP but inhibit weak θ burst-induced LTP via the CB₁ receptor (Silva-Cruz et al., 2017). Therefore, the action of URB597 on LTP recovery or enhancement by CB₁ stimulation in relation to the specific hippocampal pathway confirmed some previous somewhat unexpected but interesting observations. Finally, it is worth noting that these electrophysiological data are consistent with previous evidence that URB597 reversed depressive-like behavior in chronically stressed mice (Lomazzo et al., 2015; Tejeda-Martínez et al., 2021).

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Discussion

4. Discussion

Considering the prevalence of OA, the paucity of currently available therapeutic options and the gaps in the description of the mechanism of the disease, the study of OA pathomechanism and possible treatment options represents a top priority. The current dissertation aimed to partially bridge the knowledge gaps about the mechanism of the disease, both in the context of inflammatory changes in the knee joint and disruption of the ECS during OA progression. Moreover, given the links between ECS and the progression of OA, the studies included in this thesis also tested whether drugs targeting the ECS would alleviate OA signs.

4.1. Characterization of biochemical changes during osteoarthritis progression in a rat model

A highly important aspect of the search for novel therapeutic strategies in basic science is a thorough understanding of the mechanism of the disease and an accurate characterization of the model. The MIA-induced OA rat model mimics the idiopathic form of OA, where no joint injury is involved in OA initiation. A gradual cartilage degradation accompanied by a local inflammatory state reflects degenerative changes observed in OA patients. MIA-induced model is widely used to study OA in rats and mice (Ogbonna *et al.*, 2013; Havelin *et al.*, 2016). However, the dose of MIA varies from 0.5 to 4.8 mg in rat studies (Allen *et al.*, 2017; Haywood *et al.*, 2018; Lee *et al.*, 2018; Piao *et al.*, 2020), therefore outcome possibilities might have a different efficacy when tested at various OA severity levels. Indeed in our studies, we have demonstrated that the amount (1, 2, or 3 mg) of MIA significantly affects the outcome of the experiment, as the inflammatory and degenerative changes observed in animals strongly depend on the administered dose of MIA (Bryk *et al.*, 2021). Different MIA doses might also reflect distinct OA stages: early OA can be modeled with a lower MIA dose (which exerts less severe effects) and late OA might be induced by a higher MIA dose. In human patients, the grade of OA joint changes (e.g. bone attrition, bone marrow lesions, synovitis, meniscal tears) is associated with the severity of joint pain (Torres *et al.*, 2006). Also, the level of osteopontin and cartilage oligomeric matrix protein (proteins responsible for osteoclasts and chondrocytes function regulation) in the OA patients' synovial fluid were elevated in OA samples in comparison to controls and correlated with the degree of OA (Li *et al.*, 2022). In a dog stifle OA, a group of pro-inflammatory factors was proven to be upregulated in OA animals compared to controls, and IL-1 β , IL-6, CXCL8, TNF α , PTGS2 (COX-2 encoding gene), and prostaglandin E receptor 4 (PTGER4) genes expression showed a positive correlation with the synovitis score (Yamazaki *et al.*, 2021). Proteoglycan syndecan-4 expression was positively

correlated with MMP-9 amount in the synovial fluid of OA patients (Bollmann *et al.*, 2021). In turn, TNF-like weak inducer of apoptosis (TWEAK) and MMP-1 proteins in the synovial fluid of OA patients were negatively correlated with the OA stage (Hwang *et al.*, 2018).

Our findings regarding the MMPs and pro-inflammatory factors levels increase along with OA development are in accordance with literature data indicating MMPs upregulation as a hallmark of OA progression (Kumar *et al.*, 2022). Time- and dose-dependent changes in the inflammatory factors and MMPs observed at various time points after MIA injection may model different OA stages and severity. An early increase in the inflammatory factors abundance (CCL2, CXCL1, IL-1 β , COMP) can be associated with a transient, local inflammatory state connected with i.a. MIA injection. In the later OA stages, we have demonstrated the upregulation of MMPs (e.g. MMP-2, MMP-3, MMP-9) secretion, which was the most abundant after the 3 mg MIA. Higher MIA dose also induced a greater decrease in nociceptive thresholds (allodynia and paws' weight-bearing differences) in tested rats. Therefore, we have concluded that this dose may induce severe OA lesions, which reflect a very advanced OA stage. Accordingly, it cannot be suitable for testing novel drugs intended to inhibit disease progression. Nevertheless, it is also worth remembering, that the latest research shows, that OA is not a homogenous disease and patients may be divided into several subtypes, which differences in gene expression (Cao *et al.*, 2022) and as such MIA model may fail to reflect changes observed in various OA types.

4.2. Endocannabinoid system fluctuations during osteoarthritis development

Studies investigating the role of ECS in OA pathogenesis during the course of its development are limited, however, the available research suggest the ECS disruption during OA progression. Our results indicating alterations in the levels of enzymes responsible for AEA synthesis and degradation characterize an important element of OA pathology description. ECS components are present in the joint tissues and play a role in bone development, remodeling and metabolism (Ehrenkranz & Levine, 2019). The CB2 receptor is involved in bone mass regulation and CB2 knockout mice show increased activity of trabecular osteoblasts and the number of osteoclasts, as well as a decreased number of diaphyseal osteoblast precursors (Ofek *et al.*, 2006). In turn, combined inhibition of CB1 and CB2 receptors may be beneficial in preventing age-related bone loss in mice, while blockade of individual receptors may be detrimental (Sophocleous *et al.*, 2017). Cannabinoid receptors have also been found on human osteoarthritic cartilage and chondrocyte cell lines (Dunn *et al.*, 2014, 2016). After joint injury, CB2 receptors are upregulated in the synovial membrane

and mediate anti-inflammatory response (Rzeczycki *et al.*, 2021). Besides joint tissue presence, CB2 receptors are also present on the immune cells, and as previously mentioned, the inflammatory reaction is an important factor in OA progression. MIA-treated rats show increased levels of 12-hydroxyeicosatetraenoic acid (12-HETE, a product of endocannabinoid degradation by LOXs, see Introduction Fig. 7) in their knee joints and 15-HETE in their DRGs (Wong *et al.*, 2014). After total knee arthroplasty, AEA level in serum was positively correlated with PEA and OEA levels, but no correlation was found for 2-AG and AEA, PEA, or OEA. However, levels of 2-AG in the CSF and synovial fluid were correlated with higher post-operative pain and opioid usage by patients (Azim *et al.*, 2015). An *in vitro* study of synovial fibroblasts showed, that AEA, PEA and OEA treatment diminished IL-6, IL-8 and MMP-3 in OA and RA synoviocytes cell cultures (Lowin *et al.*, 2015).

We have proven, that the levels of genes involved in AEA synthesis and degradation are disrupted in OA rats in comparison to control animals. The changes in enzymes of both main and alternative pathways (see Fig. 7) were altered in the joint tissues (cartilage and synovial membrane), as well as in the lumbar part of the spinal cord. These results provide a very important addition to the knowledge gap regarding the involvement of ECS in the OA progression, since show changes not only in the main pathways of AEA synthesis and degradation, but also in the alternative ones. It is worth remembering, that the degradation of endocannabinoids in alternative pathways can lead to the formation of pro-inflammatory factors (e.g., on the COX-2-dependent route). Many studies which aim to target ECS for OA treatment focus mostly on the inhibition of AEA degradation targeting the enzymes of main pathways, ignoring the fact that OA-related pain modifies also alternative pathways. Therefore, our results provide vastly important findings to fill the knowledge gap about ECS modulation in OA-related pain.

Our results are in accordance with previous reported studies showing that OA models lead to fluctuations in the ECS. For example, in the spinal cord of rats with MIA-induced OA, elevated levels of AEA and 2-AG, as well as NAPE-PLD and DAGL α proteins were found. Moreover, mechanically evoked responses of wide dynamic range neurons in the dorsal horn were facilitated in OA rats compared to the sham group, which indicates central sensitization and spinal ECS adaptive changes (Sagar *et al.*, 2010). What is more, 2-AG levels were augmented in prefrontal cortex and plasma obtained from a mouse OA model, as well as in the peripheral blood lymphocytes of OA patients (Porta *et al.*, 2015). FAAH knockout mice showed decreased severity of collagen-induced arthritis and associated thermal hyperalgesia

(Kinsey *et al.*, 2011). Also, a low dose of FAAH inhibitor URB597 decreased leukocyte rolling and adhesion, as well as inflammation-induced hyperaemia and improved limb weight bearing and allodynia in a mouse RA model (Krustev *et al.*, 2014). Understanding the changes in enzymes levels during the progression of OA can direct therapeutic targets to normalize these alterations and therefore inhibit disease progression. The final outcome of our research, which prove changes in the levels of enzymes involved in AEA pathways partially fill the knowledge gap about the role of ECS in OA development and may form the basis for further research targeting AEA for OA symptoms alleviation.

4.3. Inhibition of anandamide degradation as a strategy for osteoarthritis-related pain and mood impairment treatment

OA-related pain impairs patients' quality of life (Yokota *et al.*, 2023). Moreover, in many cases, there are no sufficient treatment options, since even after total knee replacement approximately 20% of patients still experience chronic pain (Wylde *et al.*, 2018). Therefore, identifying novel effective therapeutic strategies represents vastly important endeavor. ECS offers a promising targets for OA pain management, especially CB2 receptor modulators (Bryk & Starowicz, 2021), since due to mostly peripheral location of CB2 on the immune cells, its modulators are generally devoid of side effects associated with cannabis or CB1 receptor activation. Spinal CB2 receptor activation regulates central sensitization and pain responses during OA progression (Burston *et al.*, 2013). Moreover, CB2 receptors are expressed on the immune cells and their stimulation has been demonstrated to modulate inflammatory reactions (Cabral & Griffin-Thomas, 2009), which have important implications for OA progression.

OA-related chronic pain significantly deteriorates the quality of patients' life both in physical and mental domains (Wojcieszek *et al.*, 2022), increased OA pain can also lead to depression development (Jakobsson & Hallberg, 2002). Hip OA patients had significantly higher noradrenaline metabolite 3-methoxy-4-hydroxyphenylglycol (HMPG) in the cerebrospinal fluid than the control group. Also, increase in 5-hydroxyindoleacetic acid (5-HIAA, serotonin metabolite) and homovanillic acid (HVA, dopamine metabolite) correlated with more severe preoperative pain after total hip arthroplasty (Bjurström *et al.*, 2022). La Porta *et al.* demonstrated, that in the MIA-induced OA model, the affective manifestations of OA were enhanced in CB1 receptor knockout mice and absent in CB2 receptor knockout mice, and that CB1 and CB2 selective agonists ameliorated these respective alterations (Porta *et al.*, 2015). What is more, OA rats exhibited a decrease in dopamine (in the nucleus accumbens, striatum and hippocampus) and noradrenaline (in the frontal cortex and hippocampus) brain metabolism,

most of which were normalized by a FAAH inhibitor (URB597) (Mlost *et al.*, 2018). Our findings are consistent with this study (decreased dopamine level was found in the dentate gyrus of OA rats). Moreover, we have demonstrated that URB597 not only causes an analgesic effect, but also that it is correlated with reversal of dopamine and serotonin disruption in the hippocampus (both effects were observed 60-120 minutes post-treatment).

Our study found, that OA-related chronic nociception may lead to memory impairment, induce depressive-like behavior and disrupt monoamine levels in the dentate gyrus. Twenty-eight days after MIA treatment, OA animals showed not only signs of hyperalgesia, but also impaired working memory. These disturbances were reflected in the disruption of LTP in the lateral entorhinal cortex-dentate gyrus pathway and dopamine and serotonin levels alterations in the hippocampus. The FAAH inhibitor URB597 reversed both MIA-induced nociception and disruptions of LTP. Acute URB597 administration decreased mechanical allodynia and joint hyperalgesia and improved memory, which was reflected in increased LTP in the hippocampus. Moreover, monoamine levels normalization occurred and the time window of observed changes corresponded to the analgesic effect of URB597. The observed effect was CB1-dependent, because it was blocked by CB1 receptor antagonist AM251. Therefore, our study confirmed the efficacy of AEA increase via FAAH inhibition in the treatment of OA symptoms (both pain and related cognitive disruptions).

FAAH inhibition has been studied not only in animal models, but also in clinical trials. Despite promising pre-clinical results, a clinical study in OA patients investigating the FAAH inhibitor PF-04457845 failed to find analgesic effects in comparison to placebo (however positive control drug, naproxen, significantly reduced pain) (Huggins *et al.*, 2012). However, a phase 1b clinical trial testing BIA10-2474, a nonselective FAAH inhibitor, that ended in a tragedy with mild-to-severe neurological adverse effects in four healthy volunteers and the death of a fifth subject, temporarily halted all clinical studies investigating other FAAH inhibitors. It was found that BIA10-2474 inhibited several different lipases and high dose repeated administration led to neurotoxicity (Van Esbroeck *et al.*, 2017). Consequently, more detailed basic research is needed, especially in the area of ECS modulation in the treatment of OA and accompanying mood alterations, an area that has not been well studied to date. As of January 2023, only one phase 1 clinical study investigating URB597 (for schizophrenia treatment) can be found in the Clinical Trials database (clinicaltrials.gov), however, its status is unknown. Nevertheless, preclinical research demonstrates the efficacy of URB597 in pain and depressive-like symptoms reduction in rodents. A topical administration to the animal knee with induced OA

(early stage) resulted in a reduction of local inflammatory response and joint pain. Also, prophylactic URB597 administration prevented MIA-induced saphenous nerve demyelination and chronic pain (McDougall *et al.*, 2017). Systemic URB597 administration reduced hind limb incapacitation and afferent neurons firing rate by up to 56% in the rat OA model and by up to 69% in the guinea pig OA model. Moreover, local i.a. injection reduced mechanonociception and pain (the result was CB1 receptor mediated) (Schuelert *et al.*, 2011).

FAAH inhibition for depressive-like symptoms reduction is a relatively new approach and was not well studied to date, especially in mood disturbances triggered by OA-related chronic pain. FAAH inhibition has been shown to be an effective strategy in other animal models of mood impairment and depressive-like behavior. For example, in the early life stress rat model, URB597 reduced depressive-like symptoms (Tejeda-Martínez *et al.*, 2021) and reversed downregulation in specific microRNAs disrupted by stress (Portugalov *et al.*, 2022). However, the FAAH inhibition was beneficial only when URB597 was administered in late adolescence, but not in mid-adolescence (Alteba *et al.*, 2021). Besides the reduction of depressive-like behavior, URB597 was also found to improve social behavior, neuronal plasticity and hippocampal changes impaired by stress (Alteba *et al.*, 2020). In the CCI neuropathic pain model, rats exhibited signs of mood impairment measured by the forced swimming test and novelty-suppressed feeding test, which were reversed after URB597 treatment. Moreover, FAAH inhibition also increased the number of proliferating cells and the survival of new mature neurons in the hippocampus, which were reduced after the CCI procedure (Jiang *et al.*, 2019). In mice exposed to chronic stress and with induced hypernociception, URB597 reduced anxiety and thermal hyperalgesia, while the MAGL inhibitor JZL184 failed to mitigate long-lasting widespread hyperalgesia (Lomazzo *et al.*, 2015). Therefore, the results presented in this dissertation are consistent with the literature data on the anti-depressive properties of FAAH inhibition. Our results confirm that the administration of URB597 is also effective in reducing depressive-like behavior in the OA rat model.

5. Summary

The research presented in this dissertation supports the hypothesis about the role of ECS in OA pathogenesis. Our studies allowed for a description of local changes within the joint tissue (pro-inflammatory factors and MMPs), as well as AEA synthesis and degradation pathways alterations. The role of ECS modulation for OA-related chronic pain was widely discussed (i.e. antinociceptive and anti-depression-like effects). Finally, a novel area of OA research – the link between chronic OA pain, mood impairment and CNS changes in monoamines levels and LTP

was evaluated with a particular focus on AEA level modulation for combating these changes (with particular importance of reversal of impaired LTP). The role of ECS and its modulation in OA pathogenesis is still an understudied area, thus further research is needed to understand OA mechanisms and propose a novel, improved therapeutics.

5.1. Summary of the main research achievements

- 1) Characterization of progressive changes after injection of different dosages of MIA for the OA induction at the behavioral and molecular levels.
- 2) Characterization of the OA pathomechanism by a description of changes in the inflammatory factors (CCL2, CXCL1, IL-1 β) and MMPs (MMP-2, -3, -9, -13) during OA progression on the level of the cartilage, synovial membrane and synovial fluid, representing crucial trigger points for the current understanding of therapeutic implications.
- 3) Evaluation of the role of ECS in the spinal cord and joint tissue during OA development by characterization of the changes in the enzymes responsible for AEA synthesis and degradation, which might be important indicators of OA progression.
- 4) Linking the role of OA chronic pain in mood and memory impairment, which is associated with LTP reduction in the hippocampal lateral entorhinal cortex-dentate gyrus pathway and disruption of monoamines (dopamine and serotonin) levels.
- 5) Confirmation of the effectiveness of ECS modulation by URB597 (FAAH enzyme inhibitor) in OA-related chronic pain reduction, memory and mood impairment restoration and normalization of monoamines' brain changes in OA rats.

6. References

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Authors' statements

Oświadczenie

Oświadczam, że mój udział w niżej wymienionych publikacjach polegał na stworzeniu ogólnej koncepcji badań, wykonaniu części doświadczalnej, analizie uzyskanych wyników, przygotowaniu manuskryptów do druku.

1. Marta Bryk, Jakub Chwastek, Jakub Mlost, Magdalena Kostrzewa, **Katarzyna Starowicz**. Sodium Monoiodoacetate Dose-Dependent Changes in Matrix Metalloproteinases and Inflammatory Components as Prognostic Factors for the Progression of Osteoarthritis. *Frontiers in Pharmacology* 2021, 12:643605, doi: 10.3389/fphar.2021.643605
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Podpisany elektronicznie przez
Marta Kędziora
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Oświadczenie

Oświadczam, że udział mgr Marty Kędziory (wcześniej: Marty Bryk) w niżej wymienionych publikacjach polegał na stworzeniu ogólnej koncepcji badań, wykonaniu części doświadczalnej, analizie uzyskanych wyników, przygotowaniu manuskryptu do druku. Żadna z poniższych publikacji nie stanowi oraz nie będzie stanowiła dla mnie podstawy do uzyskania stopni naukowych. Mój udział w powstaniu niżej wymienionych prac polegał na współuczestnictwie w planowaniu badań, przygotowaniu manuskryptów oraz pozyskaniu finansowania na badania w ramach grantów Narodowego Centrum Nauki oraz działalności statutowej Instytutu Farmakologii im. Jerzego Maja Polskiej Akademii Nauk.

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Podpisany elektronicznie przez
Katarzyna Starowicz-Bubak
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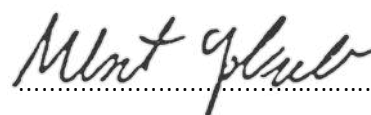
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Aleksandra Pędracka

Naples, February 14, 2023
Place, date

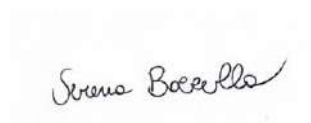
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Co-author's statement

I declare that my contribution in the preparation of the publication *Marta Kędziora, Serena Boccella, Ida Marabese, Jakub Mlost, Rosmara Infantino, Sabatino Maione, Katarzyna Starowicz Inhibition of anandamide breakdown reduces pain and restores LTP and monoamine levels in the rat hippocampus via the CB1 receptor following osteoarthritis Neuropharmacology 2023 Jan 1;222:109304*, which forms part of the doctoral dissertation of Mrs. Marta Kędziora consisted of conceptualization, study execution, writing: original draft preparation.

At the same time, I agree to submit the above-mentioned paper by Mrs. Marta Kędziora as a part of her doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.



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Co-author's statement

I declare that my contribution in the preparation of the publication *Marta Kędziora, Serena Boccella, Ida Marabese, Jakub Mlost, Rosmara Infantino, Sabatino Maione, Katarzyna Starowicz Inhibition of anandamide breakdown reduces pain and restores LTP and monoamine levels in the rat hippocampus via the CB1 receptor following osteoarthritis Neuropharmacology 2023 Jan 1;222:109304*, which forms part of the doctoral dissertation of Mrs. Marta Kędziora consisted of conceptualization, writing: review and editing.

At the same time, I agree to submit the above-mentioned paper by Mrs. Marta Kędziora as a part of her doctoral dissertation in the form of a thematically coherent series of papers published in scientific journals.



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Naples, February the 13rd, 2023

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