VNIVERSITAT DE VALÈNCIA

Doctoral Programme in Neuroscience

WHICH COMES FIRST, NEUROIMMUNITY OR MU-OPIOID RECEPTORS? A PIVOTAL RELATIONSHIP TO UNRAVEL INFLAMMATORY PAIN-INDUCED ALCOHOL RELAPSE

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Doctorado en Neurociencias

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CERTIFICAN:

Que el trabajo presentado por **Javier Cuitavi Martín,** Graduado en Biotecnología y con el Máster Euromediterráneo en Neurociencias y Biotecnología, titulado "**Which comes first, ¿neuroimmunity or Mu-Opioid receptors? A pivotal relationship to unravel inflammatory pain-induced alcohol relapse**", para obtener el grado de Doctor con mención internacional, ha sido realizado bajo nuestra dirección y asesoramiento.

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación de la Tesis, para que sea juzgada por el tribunal correspondiente. Para que así conste, firman la presente, en València a 04 mayo de 2023.

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COMPARE
CENTRE OF MEMBRANE PROTEINS AND RECEPTORS

Centre of Membrane Proteins and Receptors
Medical School
Queen's Medical Centre
Nottingham, NG7 2UH

26th October 2020

Re: Javier Cuitavi Martín visit

To Whom It May Concern:

This is to confirm that Javier Cuitavi Martin from the University of Valencia visited my laboratory at the University of Nottingham from the 1st of March 2020 until the 24th of August 2020. Javier's visit was funded by the EMBO Short-Term Fellowship 8602.

Sincerely,

Meritxell Canals, PhD

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Per a la realització d'aquesta Tesi Doctoral, l'autor ha estat beneficiari d'un contracte per a la formació de personal investigador de caràcter predoctoral, en el marc del subprograma Atracció de Talent de la Universitat de València (Convocatòria de 8 de juny de 2020) segons la resolució de 21 de maig de 2021 del Rectorat de la Universitat de València (UV-INV-PREDOC-1327981). A més a més, l'autor ha obtingut finançament per a la realització d'estades de tres mesos a la Universitat de Nottingham en dues ocasions (EMBO Short-Term Fellowship 8602 i IBRO Exchange Fellowship)

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A la abuela Maruja, el abuelo Teo, el yayo Juan, y la yaya Consuelo.

ALAS DE MARIPOSA

Ráfaga de luz y grana
mostraba ya en el oriente
el crepúsculo esplendente
precursor de la mañana.

En los cálices silvestres
de recién nacidas flores
lucían sus mil colores
las mariposas campestres.

Un niño las perseguía
y arrancándoles las alas,
todas sus brillantes galas
en una mano escondía.

Mostró el Sol sus rayos de oro
y el niño alegre y ufano
abrió su cerrada mano
para mirar su tesoro.

- ¡¿Qué es esto?!- Exclama al momento
el incauto simplecillo
viendo un ligero polvillo
que se disipa en el viento.

- ¿De qué te asombras, mi amor?
-clama su madre querida-,
si es polvo la humana vida,
polvo la planta y la flor.

Ese despojo que vuela
y que a tus ojos esconde,
mejor que yo te responde
y el triste fin revela.

Calló la madre amorosa,
y él en edad tan temprana
vio escrita la ley tirana
con alas de mariposa.

Domingo Ramón Hernández

"Observar sin pensar es tan peligroso como pensar sin observar."

Santiago Ramón y Cajal

Non-included publications related to the thesis

Research papers and reviews (Last impact factor available)

Lorente, J. D., **Cuitavi, J.**, Campos-Jurado, Y., & Hipólito, L. (2022). Pain-induced alterations in the dynorphinergic system within the mesocorticolimbic pathway: Implication for alcohol addiction. *Journal of Neuroscience Research*, *100*(1), 165-182. DOI: 10.1002/jnr.24703. IF: 4.433. Q2 (Neurosciences).

Lorente, J. D., **Cuitavi, J.**, Campos-Jurado, Y., Montón-Molina, R., González-Romero, J. L., & Hipólito, L. (2022). Kappa opioid receptor blockade in the nucleus accumbens shell prevents sex-dependent alcohol deprivation effect induced by inflammatory pain. *Pain*, *163*(1), e137-e147. DOI: 10.1097/j.pain.0000000000002332. IF: 7.926. Q1 (Anesthesiology), Q1(Clinical neurology) and Q1 (Neurosciences).

Lorente, J. D., **Cuitavi, J.**, Rullo, L., Candeletti, S., Romualdi, P., & Hipólito, L. (Under review) Sex-dependent effect of inflammatory pain on negative affective states is prevented by kappa opioid receptors blockade in the nucleus accumbens shell. Submitted to *British Journal of Pharmacology.* IF: 9.473. Q1 (Pharmacology and Pharmacy)

Book chapter

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Abbreviations

aCSF – Artificial cerebrospinal fluid

AD – Alzheimer's disease

ADE – Alcohol deprivation effect

AKT – Protein kinase B

AMY – Amygdala

AP1 – Activator protein 1

AUD – Alcohol use disorder

AUDIT – AUD identification test

BBB – Blood brain barrier

BCA1 – B cell-attracting chemokine 1

BDNF – Brain-derived neurotrophic factor

BLA – Basolateral amygdala

BRET – Bioluminescence resonance energy transfer

C5a – Complement component 5a

cAMP – Cyclic adenosine monophosphate

cDNA – Complementary DNA

CeA – Central amygdala

CFA – Complete Freund's adjuvant

CgL – Cingulate cortex

CGRP – Calcitonin gene-related peptide

CNS – Central nervous system

COX2 – Cyclooxygenase 2

CPP – Conditioned place preference

CREB – cAMP-response element binding protein

CSF1 – Colony stimulating factor 1

D2 – Dopamine 2 receptor

DA – Dopamine

DAMGO – [D-Ala2, N-MePhe4, Gly-ol]-enkephalin

DAMP – Damage-associated molecular pattern

DAPI – 4',6-diamidino-2-phenylindole

DLPT – Dorsolateral pontine tegmentum

DMEM/F12 – Dulbecco's modified Eagle's medium with Nutrient Mixture F12

DNA – Deoxyribonucleic acid

DOR – Delta-opioid receptor

DRG – Dorsal root ganglia

EDADES – Encuesta sobre alcohol y otras drogas en España

ERK – Extracellular signal-regulated kinases

GABA – γ-Aminobutyric acid

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

G-CSF – Granulocyte colony-stimulating factor

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GPCR – G protein-coupled receptor

GRK – G protein receptor kinases

HIV – Human immunodeficiency viruse

HPA – Hypothalamic–pituitary–adrenal

HRP – Horseradish peroxidase

i.p. – Intraperitoneal

I309 – Small inducible cytokine A1

IA – Intermitent access

IBA1 – Ionized calcium binding adapter molecule 1

IKK – IKappaB Kinase

IL – Infralimbic cortex

IL(n) – Interleukin(n)

IL1ra – Interleukin-1 receptor antagonist

INFγ – Interferon γ

iNOS – Inducible nitric oxide sysntase

IP10 – Interferon γ-induced protein 10

I-TAC – Interferon-inducible T cell alpha chemoattractant

JNK – c-Jun N-terminal kinases

KC – Keratinocyte-derived cytokine

KOR – Kappa-opioid receptor

LPS – Lipopolysaccharide

M1 – Primary motor cortex

MAC – Minimum alveolar concentration

MAPK – Mitogen-activated protein kinase

MCLS – Mesocorticolimbic system

MCP1 – Monocyte chemoattractant protein 1

MCP5 – Monocyte chemoattractant protein 5

M-CSF – Macrophage colony-stimulating factor

MGI – Mean grey intensity

MIG – Monokine induced by gamma interferon

MIP1α – Macrophage inflammatory protein 1α

MIP1β – Macrophage inflammatory protein 1β

MIP2 – Macrophage inflammatory protein 2

miRNA – MicroRNA

MOR – Mu-opioid receptor

mRNA – Messenger RNA

NAc – Nucleus accumbens

NAcC – Nucleus accumbens core

NAcS – Nucleus accumbens shell

NFκB – Nuclear factor κ-light-chain-enhancer of activated B cells

NLR – NOD-like receptor

NLuc – Nano luciferase

NOD – Nucleotide-binding oligomerisation domain

NRK1 – Neurokinin receptor 1

OIH – Opioid-induced hyperalgesia

OR – Opioid receptor

ORL1 – Opioid receptor-like 1

OUD – Opioid use disorder

PAG – Periaqueductal gray

PB – Phosphate buffer

pcDNA – Plasmid cloning DNA

PDL – Poly-D-Lysine

PFC – Prefrontal cortex

PKC – Protein kinase C

PrL – Prelimbic cortex

PRR – Pattern recognition receptor

PTM – Post-translational modification

RANTES – Regulated on activation, normal T cell expressed and secreted

RLuc8 – Renilla luciferase 8

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT-qPCR – Real time quantitative polymerase chain reaction

RVM – Rostral ventromedial medulla

s.c. – Subcutaneous

SAL – Saline

SDF1 – Stromal cell-derived factor 1

SEM – Standard error of the mean

siCAM1 – Soluble intercellular adhesion molecule-1

siRNA – Small interfering RNA

SNP – Single-nucleotide polymorphism

TARC – Thymus- and activation-regulated chemokine

TGFβ – Transforming growth factor β

TIMP1 – Tissue inhibitor of metalloprotease 1

TLR – Toll-like receptor

TM – Transmembrane domain

TNFα – Tumour necrosis factor α

TREM1 – Triggering receptor expressed on myeloid cells 1

UTR – Untranslated region

Vh – Vehicle

VTA – Ventral tegmental area

β2AR – β-2 Adrenergic Receptor

βArr2 – β-arrestin 2

βFNA – β-Funaltrexamine

Content index

CHAPTER 1

Background: Alcohol Use Disorder, Pain and Neuroimmunity

1.1. Alcohol Use Disorder: A complex behavioural disease

According to the American National Institute on Alcohol Abuse and Alcoholism "Alcohol use disorder (AUD) is a medical condition characterised by an impaired ability to stop or control alcohol use despite adverse social, occupational, or health consequences." This term includes alcohol-related affections such as alcoholism and alcohol addiction, dependence, and abuse (National Institute on Alcohol Abuse and Alcoholism, 2021). AUDs are so complex that their diagnosis is based on 11 symptoms comprised in several grouping criteria, namely pharmacological, impaired control over substance use, social impairment, and risky use (Guha, 2014). The number of symptoms shown by the patient is directly related to the severity of the AUD: mild (2-3 symptoms), moderate (4-5 symptoms), and severe (>6 symptoms), with addiction being the term used for the most severe grade of the AUD.

The total number of people affected by an AUD has not stopped increasing since 283 million people were diagnosed with an AUD in 2016 compared to the 76 million diagnosed in 2008 (World Health organisation, 2008; 2018). AUD is also more prevalent in Europe and in America, specifically in wealthier countries (World Health organisation, 2018). In fact, the latest "Encuesta sobre alcohol y otras drogas en España" (EDADES; Survey about alcohol and other drugs in Spain) shows that although the alcohol consumption rate has not changed in the last few years, it remains the most consumed drug of abuse in Spain with a prevalence of consumption at some point during people's lifespans of 82.1% in men and 70.8% in women of between 15 and 64 years old (Ministerio de Sanidad de España, 2023). Moreover, EDADES also uses the AUD identification test (AUDIT) to assess intense alcohol consumption. The latest EDADES shows an increase in the AUDIT score in the

last couple of years with a current score of 7.2 in men and a 4.8 in women, which is close to risky alcohol consumption according to the test (8 in men and 6 in women).

It is also important to notice that excessive alcohol consumption accounts for many chronic and harming physiological and psychological comorbidities, such as brain and liver damage, cancer, depression, and social isolation. Altogether, these conditions can eventually lead to deadly situations. In fact, according to the World Health Organisation, in 2016 there were more than 3 million deaths related to AUDs (World Health organisation, 2018), which supposes a burden to all health systems.

Unfortunately, the available pharmacological approaches to treat AUDs are inefficient and do not prevent relapse, which continues to be a great challenge. In fact, up to 80% of AUD patients following a treatment relapse within the first year of treatment (Menon and Kandasamy, 2018). This fact has its root in the complexity of alcohol activity and metabolism (Bergmark, 2008; Spanagel 2009).

1.1.1. Mesocorticolimbic system: From reward to drug addiction

Ethanol molecules possess physical characteristics that allow them to act at very different levels within the organism. Concretely, alcohol modifies the permeability of the blood brain barrier (BBB), gaining access to the brain, where it produces neuroadaptations and direct toxic damage (Vore and Deak, 2022). The mesocorticolimbic system (MCLS) is one of the most affected neuronal pathways since it processes reward and aversion. In the absence of a drug of abuse, natural reinforcers activate this system to facilitate behaviours that lead to survival (Fields et al., 2007). In order to do so, it comprises brain areas that play a crucial role in reward management. Concretely, nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala (AMY), ventral tegmental area (VTA), hippocampus and hypothalamus are the main brain structures that shape the neural reward circuitry. All these areas

participate in the three-stage recurring cycle that conceptualises any substance use disorder. Firstly, the learning process is aberrant during the binge/intoxication stage, which leads to the acquisition of non-positive behaviours. Secondly, motivation is highly impacted by the negative affect and stress derived from the withdrawal phase. Finally, brain structures related to executive functions get activated and, under aversive situations or negative emotions, they induce seeking behaviours in the preoccupation/anticipation stage, after which the cycle is repeated (Koob and Volkow, 2016).

Dopamine (DA) is the neurotransmitter of the MCLS par excellence. In fact, in the presence of a natural reinforcer DA neurons start firing and, thereby, DA is released within the structures of the system. Alcohol, among other drugs of abuse, disrupts this mechanism of survival by overstimulating DA neurons. High levels of DA cheat the brain into thinking that the drug is required for the endurance of the organism, which makes it want to repeat the action of taking the drug (Koob and Volkow, 2016). The cell bodies of most of the DA neurons within the MCLS are in the VTA. This brain structure takes part in cognition, reward, and motivation processes (Sanchez-Catalan et al., 2014). VTA DA neurons project to other brain areas of the system, namely NAc and PFC.

On the one hand, researchers have traditionally considered NAc to be the neuralgic centre of reward by raising motivation towards relevant goals and promoting the avoidance of aversive behaviours (Floresco, 2015). It is important to highlight that NAc can be subdivided into two different sections, NAc Shell (NAcS) and Core (NAcC), according to the innervations and cell types in them. When they receive the DA input from VTA, local neurons get activated and filter the information that comes from the reinforcer. NAcS is part of the extended AMY and processes the "liking" part of the reward (Zahm, 1999; Castro and Berridge, 2014), whereas NAcC

is in the inner part of the NAc and processes the reward and reinforcement-related motor function, which facilitates the acquisition of behaviours that lead to new exposures to the reward (Zahm, 1999).

On the other hand, PFC also receives direct DA input from VTA. It makes the connection between the reinforcer and the context in which the reinforcer was acquired. Therefore, PFC determines whether a behaviour needs to be acquired by integrating the information received (Floresco and Ghods-Sharifi, 2007).

1.1.2. Alcohol and neuroinflammation

The immune system, both innate and adapted, is the first line of defence against any pathogen and toxic substance (Zou and Crews, 2010). Several studies demonstrated that alcohol irrupts the normal immune response. In fact, after a long-term alcohol exposure, there is an increase in the incidence of other illnesses such as pneumonia, human immunodeficiency viruses (HIV), and hepatitis C (Moss et al., 2003; Joshi and Guidot, 2007; Szabo and Mandrekar, 2009; Witt, 2010; Szabo and Zakhari, 2011). Although ethanol altering the immune response has been repeatedly proven in the past, there is still a lack of knowledge regarding the thorough mechanisms that underlie this phenomenon.

Microglial cells are the resident macrophages of the brain. Their primary role resides in the protection of the brain tissue by means of inflammatory mechanisms and phagocytosis (Prinz et al., 2019). However, the homeostasis reached by neurons and microglia can be easily disturbed by an overactivation of the latter (Marinelli et al., 2019). Astrocytes also play a role in protective and pathological neuroinflammation, which contributes to neuronal homeostasis (Colombo and Farina, 2016). Both acute and chronic ethanol exposure impact microglia and astrocytes. In fact, they trigger the release of neuroinflammatory mediators such as

cytokines and chemokines and stimulate the production of reactive oxygen species, which, in turn, has a repercussion on the neuronal welfare that might eventually facilitate the development of an AUD (Bachtell et al., 2017; Henriques et al., 2018; Kane and Drew, 2021).

Toll-like receptors (TLRs) seem to play an important role in alcohol-induced neuroinflammation and neurodegeneration in the brain (Vallés et al., 2004; Qin et al., 2008; Fernandez-Lizarbe et al., 2009; Alfonso-Loeches et al., 2010; Zou and Crews, 2010; Cantacorps et al., 2017). TLRs are a type of pattern recognition receptors (PRRs) belonging to the innate immune system, which get activated in the presence of molecules coming from both pathogens and tissue damage (Iwasaki and Medzhitov, 2004). In the brain TLRs are mainly found in glial cells (Okun et al., 2009) and their activation triggers signalling cascades that regulate the activity of transcriptional factors such as nuclear factor (NF)κB and activator protein 1 (AP1), which participate in the transcription of neuroinflammation-involved genes (Kawai and Akira, 2006; 2007). Nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) are intracellular immune receptors that also play a role in acute and chronic alcohol-derived neuroinflammation (Lippai et al., 2013; Alfonso-Loeches et al., 2014; 2015). NLRs participate in the formation of multiprotein complexes known as inflammasomes, the role of which is promoting the proteolytic activity of Caspase1. This process ends with the release of proinflammatory cytokines such as interleukin (IL)1β, and IL18 (Sutterwala et al., 2006; Schroder and Tschopp, 2010; Wen et al., 2013). Finally, there is evidence of a crosstalk between TLRs and NLRs in alcohol pathology in glial cells, which is based on a TLR priming of the NLR function (Alfonso-Loeches et al., 2014; 2015). The mechanism explained in this section is illustrated in Figure 1.

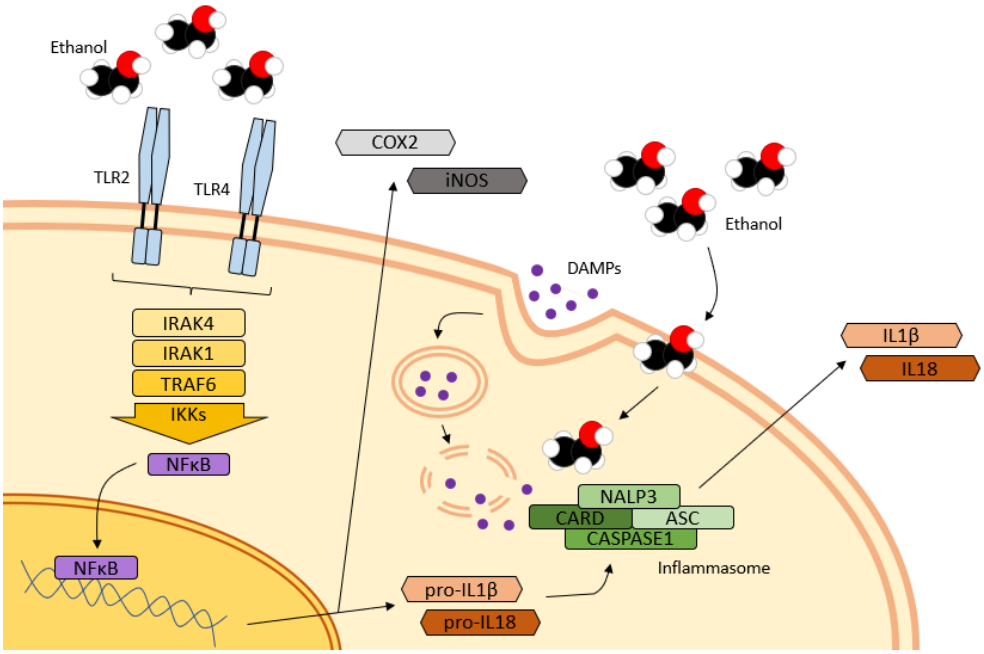

Figure 1. Traditional ethanol exposure-induced neuroinflammatory pathway. *Ethanol exposure might lead to the activation of TLRs, namely TLR2 and TLR4, which translocates the transcriptional factor NFκB to the nucleus. This induces the transcription of neuroinflammatory mediators such as cyclooxygenase 2 (COX2), inducible nitric oxygen synthase (iNOS), and immature cytokines such as IL1β, and IL18. Then cytokines are processed by inflammasomes before their release. The NALP3/Caspase1 inflammasome is ensembled after it detects not only damage-associated molecular patterns (DAMPs) but also ethanol. Figure adapted from Franchi et al. (2012).*

1.2. Pain: General mechanisms

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020). Moreover, pain is a complex pathology that comprises sensorial, emotional, cognitive, and motivational components (Seminowicz and Moayedi, 2017). The most common way of classifying pain is according to its duration. Acute pain refers to a pain condition that exists for less than three months. It usually presents with vegetative nervous system hyperactivity, tachycardia, perspiration, and paleness. However, if the pain condition persists in time and, therefore, beyond normal tissue healing time, it is defined as chronic pain (Raja et al., 2020). Interestingly, chronic pain has a high prevalence in the world, affecting all kinds of people. In fact, epidemiologic data shows that around 18% of adults in developed countries suffer from chronic pain (Reid et al., 2011), which poses a huge burden to healthcare systems. Moreover, chronic pain vastly impacts the MCLS leading to many comorbidities such as anxiety, depression, and AUDs.

1.2.1. Central mechanisms of pain

Pain processing is carried out by an ascending and a descending pathway which comprise several neuronal structures (Figure 2). The ascending nociceptive pathway begins with the nociceptors, which are the receptors in charge of detecting painful stimuli and transmitting the signal to the corresponding nerve fibres that, eventually, will carry the information to the brain areas in charge of processing it (Fenton et al., 2015). These receptors consist of free nerve endings, which are activated when facing mechanical, thermal, and chemical stimuli that could be detrimental for the organism (Pinho-Ribeiro et al., 2017). These free nerve endings

are formed by pseudomonopolar neurons, which are characterised by an axon divided in two branches. One of these branches innervates the peripheral tissues and the other one takes the information to the central nervous system since their cell bodies are in the dorsal root ganglia (DRG) in the spinal cord (Basbaum et al., 2009; Kuner, 2010). These cells transduce noxious stimuli into action potentials and transmit them to the dorsal horn, where they synapse with other neurons that take the information to the brainstem and the thalamus to eventually reach cortical structures that control different aspects of the pain experience, including somatosensory, anterior cingulate and insular cortices (Fields, 2004; Nestler et al., 2009). Interestingly, the thalamus mediates the sensory discriminative and affective-motivational components of pain, which plays a role in the development of pain comorbidities (Lenz et al., 2004).

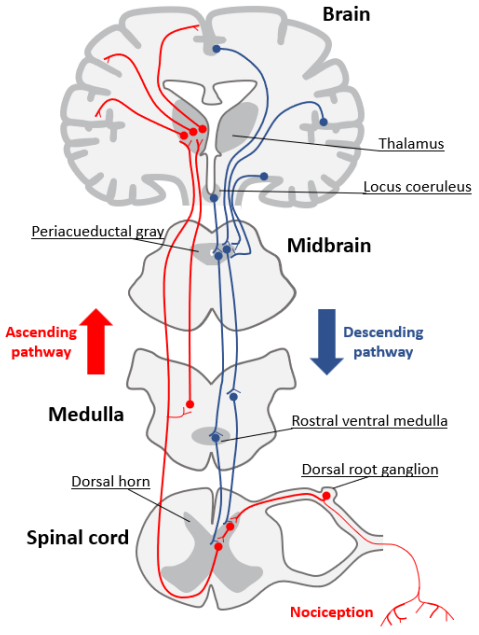

Figure 2. Schematic representation of afferent and efferent pathways underlying the sensation and modulation of pain. *When an organ receives a painful stimulus, it activates the primary afferent nociceptor which takes the information to the dorsal horn. Then, it is transmitted to the thalamus which distributes it to cortical regions, which analyse the danger and exert a response. Moreover, some of these brain areas modulate the pain response by sending axons to the periaqueductal gray, which, with the help of the rostral ventral medulla, interrupts pain signalling. Figure adapted from Fields, (2004).*

The descending nociceptive pathway initiates from pain-associated cortical areas to spinal and trigeminal dorsal horn while synapsing in several brainstem regions. Among those areas, the periaqueductal gray (PAG) stands out for being one of the main actors in endogenous analgesia by facilitating or inhibiting the flow of painful stimuli. PAG merges inputs from the limbic forebrain, the hypothalamus and the neocortex and projecting to the rostral ventromedial medulla (RVM) and the dorsolateral pontine tegmentum (DLPT), the afferents of which get to the spinal cord (Reynolds 1969; Mayer and Liebeskind, 1974; Mayer et al., 1971). RVM can also modulate pain intensity through the activity of its “on cells” and “off cells” (Fields et al., 1995; Urban and Gebhart, 1999; Porreca et al., 2002; Fields 2004).

1.2.2. Pain: A neuroinflammatory disease

Pain processing is tightly related to neuroinflammation. Immune cells participate in enhancing and inhibiting pain conditions at different levels. Nociceptors in the periphery contain receptor for some cytokines, which promote the start of the pain condition (Pinho-Ribeiro et al., 2017). In fact, inhibitors of IL6, IL1β and tumour necrosis factor α (TNFα) attenuate the development of pain (Raoof et al., 2018). In the central nervous system, concretely in the spinal cord, pain induces the chemoattraction of monocytes and macrophages, which contribute to the persistence of the pain condition (von Banchet et al., 2009; Massier et al., 2015). Microglia, on the other hand, play a dual role in pain management. In the presence of a pain condition, they proliferate and change their phenotype in the spinal cord (Gu et al., 2016; Locke et al., 2020). This microgliosis strengthens the synapses in the DRG facilitating long-term potentiation (Ho et al., 2020). This is managed by increasing brain-derived neurotrophic factor (BDNF) release, which contributes to central sensitisation and neuroplasticity (Cappoli et al., 2020). Microglia also release proinflammatory cytokines in the DRG in the presence of a pain condition, modifying

cells excitability and increasing hypersensitivity (Kawasaki et al., 2008; Inoue and Tsuda, 2018). Nonetheless, microglia also play a pivotal role in protecting the organism against pain depending on the environment. If fact, it has been observed that specific microglial phenotypes produce cytokines with analgesic effects such as IL10, IL3, transforming growth factor β (TGFβ) and IL1 receptor antagonist (Vergne-Salle and Bertin, 2021).

1.3. Pain comorbidities: Role of the mesocorticolimbic system

A pain stimulus normally presents with a process of cognitive appraisal, in which pain sufferers try to determine which potential danger induces the feeling of pain and then, react against it. In this sense, organisms perceive pain as a negative stimulus, which evokes equally negative emotions, namely fear, anger and/or sadness and, therefore, activates aversive emotion processing areas belonging to the MCLS (Wiech and Tracey, 2009).

1.3.1. Pain main comorbidities

One of the main disorders that comorbid with pain is depression, with a prevalence of around 30% comorbidity (Chopra and Arora, 2014). Common neurophysiology between both conditions, as shown in human and rodent studies, suggests the association of pain with reduced motivation and depression-like behaviours that relies on hypodopaminergic states in the MCLS (Serafini et al., 2020). Pain has also been shown to act as a stressor. In fact, up to 40% of chronic pain patients suffer from anxiety (Csupak et al., 2018; Lorente et al., 2022). Pain-induced anxiety and depression-like behaviours have their root in the recruitment of the dynorphinergic system within the MCLS, and more specifically within the NAc, leading to decreased DA release (Massaly et al., 2016; Lorente et al., 2022). Indeed, anhedonic state, in which motivation for natural rewards is diminished (Marbach and Lund, 1981), is one of the canonical symptomatologies of depression and anxiety. This DA-dependent behaviour has been observed in animal models of pain using progressive ratio paradigms in self-administration boxes with sucrose as a reward (Schwartz et al., 2014; Massaly et al., 2019).

1.3.2. Pain-induced alcohol use disorders: Motivation, reward, and neuroinflammation

Pain has the evolutionary purpose of eliciting motivations that search for either avoiding or escaping harmful behaviours (Wiech and Tracey, 2013). This is why the relief of pain conditions is rewarding. In fact, pain relief–motivated behaviours can be observed in conditioned place preference (CPP) assays (Navratilova et al., 2012). Chronic pain disrupts reward and motivation processing by altering the MCLS in a molecular, anatomical, and functional way (Seminowicz et al., 2009; Taylor, 2016). This is partially caused by a downregulation in the amount of DA released in NAc (Chang et al., 2014; Taylor et al., 2016), as mentioned in the previous section, and the role of the AMY in decision-making, emotional, and memory processes (Amunts et al., 2005). The NAc is a key brain area in motivation processing and integrates the affective value and saliency of pain stimuli by differentially encoding DA release patterns in each NAc subregion (Schultz et al., 2013). Interestingly, these changes in the NAc are time-dependent and contribute to the shift from acute pain to chronic pain (Apkarian, 2011). In fact, acute pain seems to increase DA in NAc whereas chronic pain leads to a hypodopaminergic state (Porreca and Navratilova, 2017). These hypodopaminergic states modify motivated-behaviours which promote mood disorders that are traditionally presented with chronic pain, among which substance use disorders stand out (Serafini et al., 2020).

Reinforcement has a very strong motivational aspect, which is based on the different phases of the motivated behaviour: the instrumental phase is DA-dependent and involves seeking the reward, whereas the consummatory phase is DA-independent and is based on the obtention of the goal (Salamone and Correa, 2012). In this sense, alcohol irrupts this cycle and promotes changes in DA release in the NAc. Acute ethanol exposure increases DA release, chronic exposure barely

induces changes in DA levels, and withdrawal reduces basal DA levels (Dahchour and Ward, 2022). As previously mentioned, chronic pain patients show hypodopaminergic states in the NAc. Therefore, pain conditions might influence alcohol consumption through dopaminergic mechanisms and, consequently, altering motivation for alcohol. However, there is evidence that inflammatory pain blunts ethanol-evoked DA release in the NAc (Campos-Jurado et al., 2020). This finding might mirror previous published data in the opioid field that showed that pain decreased heroin self-administration when average doses were provided. Interestingly, when heroin concentration was increased, motivation for the reward was rescued in animals in pain (Hipólito et al., 2015). This is further discussed in section 1.5.3.

Interestingly, pain promotes neuroinflammation in brain areas of the MCLS. In fact, neuropathic pain seems to induce microglial activation in the VTA, the NAc, the PFC and the AMY (Taylor et al., 2017). Microglial activation accounts for a disruption in the MCLS and its dopaminergic pathways (Taylor et al., 2015). Therefore, pain-induced neuroinflammation affects emotional and cognitive pain processing areas, linking pain with depression and anxiety disorders (Singhal and Baune, 2017). Thus, based on this ground, it seems plausible that pain and its comorbidities might be related through neuroimmune mechanisms arising in the MCLS.

1.4. Alcohol Use Disorders and pain: Focusing on sex

Men and women are physiologically different, not only at a hormone-related homeostasis, but also in the way their organisms behave when facing an illness, a stressor, or an exposure to exogenous substances. In fact, there is evidence that their immune cells, receptors, and coping mechanisms are different depending on sex (Klein and Flanagan, 2016). Bearing in mind that the immune system is involved in many processes, it is not uncanny that men and women's immune system show different patterns of action under pathological conditions such as Alzheimer's disease, viral and bacterial infections, pain, anxiety, or drug abuse (Fischer et al., 2015; Mapplebeck et al., 2016; Bekhbat and Neigh, 2018; Ferretti et al., 2018; McHugh et al., 2018). This section will revisit sex-derived differences in AUDs and pain found in both preclinical and clinical research.

1.4.1. Sex-derived factors in Alcohol Use Disorders

Epidemiological data indicates that, although men show a higher prevalence of AUD than women (Grant et al., 2015), in the last couple of decades AUDs prevalence in women have increased by 84% whereas in men that increase has been 35% (Grant et al., 2017). This can be explained by the low representation of women in clinical studies until very recently and because traditionally women were excluded from social interactions involving drinking (Sanchis-Segura and Becker, 2016).

AUDs depend on many physiological sex-dependent variables. In fact, women are more susceptible to addictive-like behaviours, alcoholism, and ethylic intoxication (Hommer, 2003; Anker and Carroll, 2010; Sanchis-Segura and Becker, 2016). This is derived from the amount of body water and fat, which is different between men and women, and which play a role in alcohol pharmacokinetics

(Epstein and Menges, 2013; McCaul et al., 2019). Another factor that intervenes is the more prominent vulnerability of women regarding alcohol-induced alterations in stress-related emotional and physiological markers (McCaul et al., 2019). Alcohol exposure also affects hormonal regulation. On the one hand, alcohol alters testosterone levels in men. In fact, low doses of alcohol increase testosterone, whereas high doses in alcohol acute intoxication decrease it (Erol and Karpyak, 2015). On the other hand, in women, oestradiol, progesterone and their metabolites directly correlate with alcohol use and craving (Carroll and Smethells, 2016; McCaul et al., 2019).

Interestingly, not many AUD patients get proper treatment. Concretely, only 23% of men and 15% of women that suffer from an AUD ever get treatment during their lifetimes (Cohen et al., 2007; Alvanzo et al., 2014). These differences between men and women are related to female-specific barriers that distance them from correct therapeutic approaches, namely stigmas and misbeliefs (McCrady et al., 2020). Regardless of the difference in treatment access, both psychological and pharmaceutical therapies seem to have the same outcome for men and women that do receive treatment (Holzhauer et al., 2020). Nonetheless, naltrexone, one of the most used drugs for AUDs, is more efficacious for men than for women (Canidate et al., 2017). In fact, some studies even suggest that naltrexone does not have efficacy whatsoever for women (Garbutt et al., 2005; O'Malley et al., 2007).

Alcohol-induced neuroimmunity is also sex specific. In human adolescence, boys exposed to alcohol have lower plasma levels of chemokines and cytokines than girls of the same age and the same plasma ethanol levels (Pascual et al., 2021). Moreover, TLR4 expression and activation is also greater in young women when visiting an accident and emergency hospital department after a binge drinking session (Pascual et al., 2017). Another study in rodents shows how binge drinking

reduced the number of microglial in the PFC in males but not in females. Additionally, females presented a primed microglial phenotype, which did not occur in males (Barton et al., 2017).

1.4.2. Influence of sex in pain perception and derived inflammation

Pain is another pathological condition that is influenced by the sex of the patient. In fact, chronic pain is 20% more prevalent in women than in men since they are more vulnerable and less tolerant to it (Kurita et al., 2012; Mogil, 2012; Ruau et al., 2012; Landmark et al., 2013; Steingrímsdóttir et al., 2017). Hormones play an important role in pain perception and may be the reason for the great prevalence of chronic pain in women. In this sense, girls show less tolerance to painful stimuli than boys after the age of 12 (Boerner et al., 2014).

Sex also affects pain treatment with opioids. Although women are usually prescribed higher doses than men (Campbell et al., 2010; Cheatle, 2015), it is true that women are more prone to suffer from opioid-derived negative side effects, including respiratory depression, nausea, constipation, dysphoria, vomiting, and headache (Myles et al., 1997; Cepeda et al., 2003; Fillingim et al., 2005; Comer et al., 2010). Nonetheless, male rodents are more likely to report more severe and longer opioid withdrawal symptomatology (Cicero et al., 2002; Diaz et al., 2005). There is controversy regarding sex-dependent opioid analgesia. Some articles suggest that sex does not influence opioid analgesia (Sarton et al., 2000; Fillingim et al., 2005; Bijur et al., 2008), whereas others indicate that opioids are less efficacious in female rodents and women (Berkley, 1997; Cepeda et al., 2003; Miller and Ernst, 2004; Aubrun et al., 2005; Mogil and Bailey, 2010), being this issue still a matter of debate.

The immune system presents sex-dependent differences in how it reacts to pain stimuli. In fact, male and female mice rely on different immune cells in the spinal

cord to process chronic pain-induced behaviour. Females mainly depend on T cells whereas males prefer microglial cells (Sorge et al.,2015). Additionally, immune responses to a pain stimulus differ in male and females, both peripherally and in the central nervous system. This has been widely reviewed by Sorge and Totsch (2017). For example, in an inflammatory pain murine model, researchers observed how males had greater levels of IL1β, IL6, TNFα, and BDNF in the trigeminal ganglia (Kuzawińska et al., 2014). In another study, researchers used lipopolysaccharide (LPS), a TLR4 ligand, which has been shown to cause allodynia. However, allodynic states were only detected in males, although TLR4 mRNA levels in the spinal cord were identical in male and females (Sorge et al., 2011).

1.5. The opioidergic system: Focusing on the mesocorticolimbic system and Mu-Opioid receptors

Both pain-induced comorbidities and AUDs are partially managed by the opioidergic system within the MCLS. This is mainly due to its control over DA release, which plays a pivotal role in reinforcement and mood regulation (Gerrits et al., 2003). This opioidergic system comprises four rhodopsin-like GPCRs and their ligands. These opioid receptors (OR) are called Mu (MOR), Kappa (KOR), Delta (DOR) and Opioid Receptor-Like 1 (ORL1) and their endogenous ligands are endorphins, dynorphins, enkephalins, and nociceptins, respectively. The present thesis studies MORs and how they are involved in pain-induced alcohol relapse while focusing on neuroinflammation within the MCLS. Thereby, Chapter 2 is dedicated to a thorough review of MORs life cycle and Chapter 3 deals with the MOR-neuroinflammation interaction.

1.5.1. Mu-Opioid receptors in Alcohol Use Disorders

Drugs of abuse exert their reinforcing properties through MORs as shown in conditioned place preference, self-administration paradigms, and exploratory locomotion (Shippenberg and Herz 1987; Hand et al., 1989; Devine and Wise 1994; Bontempi and Sharp 1997; Wise, 2004; Le Merrer et al., 2009). This has been suggested to occur through MORs control over dopaminergic transmission. In fact, MORs agonists administered intra-VTA elicit an increase in DA release in the NAc (Gysling and Wang, 1983; Devine et al., 1993; Melis et al., 2000; Jalabert et al., 2011). MORs in the VTA are mainly located on GABAergic inhibitory neurons. Moreover, their activation usually correlates with an inhibition of neuronal activity. Consequently, in the presence of a MOR agonist, MORs stops γ-aminobutyric acid

(GABA)ergic neurons from firing and, therefore, promotes the excitation of dopaminergic neurons (Le Merrer et al., 2009).

Interestingly, ethanol also induces DA release in the NAc when administered not only intra-VTA (Howard et al., 2008), but also intraperitoneally (Imperato and Di Chiara, 1986), intravenously (Howard et al., 2008), and intragastrically (Enrico et al., 2009). Nonetheless, despite the existing evidence regarding ethanol management of DA release in the MCLS, the way ethanol interacts with MOR remains unclear. Ethanol is not an opioid-like molecule, which means that it cannot bind MORs *per se*. In this sense, two major hypotheses have been proposed (Font et al., 2013).

On the one hand, although ethanol is far from being an opioid-like agonist, molecules derived from its metabolism do share a biochemical structure with opioids and therefore are susceptible to binding MORs. When ethanol reaches the brain after crossing the BBB, it can get metabolised to acetaldehyde through pathways that involve catalase, cytochrome CYP2E1 and alcohol dehydrogenase (Hipólito et al., 2007). Acetaldehyde properties make it a highly reactive molecule, which means that it can condensate with a myriad of endogenous molecules creating new ones such as salsolinol (Peana et al., 2017). Salsolinol (1-methyl-6,7-dihidroxy-1,2,3,4-tetrahydroisoquinoline) is formed as result of the reaction of acetaldehyde and DA (Hipólito et al., 2012), has been observed to increase motor activity (Hipólito et al., 2011; Quintanilla et al., 2016), and induces self-administration (Rodd et al., 2008) and conditioned place preference (Hipólito et al., 2011). In most behavioural tests, salsolinol had greater potency than ethanol and acetaldehyde (Peana et al., 2017). Moreover, intra-VTA administration of salsolinol also induces DA release in the NAc (Hipólito et al., 2011; Deehan et al., 2013). Interestingly, salsolinol has been suggested to elicit these behaviours and DA release through the action of MORs due to its similarity in structure with morphine (Davis and Walsh, 1970) and the fact that

it binds ORs (Berríos-Cárcamo et al., 2017). Consequently, salsolinol is considered to be MOR agonist.

On the other hand, some researchers have proposed that the endogenous opioid β-endorphin, a MOR agonist, is the molecule from which ethanol gets its reinforcing properties (Sanchis-Segura et al., 2005; Xiao et al., 2007; Xiao and Ye, 2008). Interestingly, β-endorphinic innervations directly project to the VTA, among other areas (Mansour et al., 1988; Di Chiara and North 1992; Spanagel et al., 1992). There is evidence that ethanol exposure induces the synthesis of β-endorphin (Gianoulakis, 1990) and its liberation in the VTA (Rasmussen et al., 1998; Jarjour et al., 2009; Leriche and Méndez, 2010). Moreover, β-endorphin seems to play a role in ethanol-induced locomotor sensitisation (Dempsey and Grisel, 2012) and conditioned place preference (Pastor et al., 2011). It is important to notice that ethanol-induced β-endorphin release has been reported to be mediated by the presence of ethanol metabolites (Reddy and Sarkar, 1993; Sanchis-Segura et al., 2005). This last evidence suggests that, most probably, both theories work one alongside the other, although further research is required to elucidate this matter.

1.5.2. Mu-Opioid receptors and pain

The opioidergic system plays a pivotal role in pain modulation by promoting analgesia at different points of the pain system. In this sense MORs are key players in analgesia and are highly expressed at all levels of the central pain control network (Benarroch, 2012). Both endogenous and exogenous opioids interact with the opioidergic system to promote analgesia. In the PAG and the RVM, MOR activation leads to the inhibition of GABA release, which in turn activates antinociceptive pathways projecting to the dorsal horn (Mika et al., 2011). MORs are also expressed in the dorsal horn and in DRG primary nociceptive afferents, in fact, their activation

promotes analgesia by both presynaptic and postsynaptic mechanisms (Marvizón et al., 2009; Benarroch, 2012). Finally, MORs are also present in primary nociceptive afferents and, although they are inactive in basal conditions, they participate in systemically administered opioids (Rachinger-Adam et al., 2011).

As previously mentioned, MORs are also present in the MCLS. Interestingly, the existence of a chronic pain condition modifies the availability of ORs within the MCLS. Neuropathic pain patients show a decreased availability of ORs in the PFC and the insula (Maarrawi et al., 2007), whereas arthritic pain patients showed the contrary phenomenon in basal ganglia, insula, caudate, and NAc (Brown et al., 2015). Focusing on MOR, Harris et al. (2007) and DosSantos et al. (2012) observed a decrease in MORs availability in NAc, AMY, and corpus callosum in patients suffering trigeminal neuropathic pain and fibromyalgia. Moreover, the mesocorticolimbical MORs density is also altered in the presence of a pain condition. In fact, chronic neuropathic pain rats had a lower quantity of MORs in the insula and caudate-putamen than controls (Thompson et al., 2018). Finally, MORs function in the MCLS is also compromised under a pain condition. A murine model showed that inflammatory pain blunts or diminished intra-VTA [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO)-induced neuronal activation in basolateral AMY, bed nucleus of the stria terminalis and cingulate cortex (Campos-Jurado et al., 2019).

1.5.3. Pain and Substance Use Disorders: Mu-Opioid receptors as a link

The relationship between opioid use disorders (OUDs) and chronic pain is a well-established one (Sheu et al., 2008). This is mainly due to the high opioid prescription rates to relieve pain sufferers in countries such as the United States of America and Canada (King et al., 2014). In fact, up to 26% of opioid-treated pain patients develop dependence and addictive behaviours (Chou et al., 2015). Pain-

induced OUDs are controlled by the changes in the opioidergic system described in the last section. As seen in many preclinical studies, MOR changes in the MCLS modulate DA release at different levels, which in turn modifies behaviour. In fact, inflammatory pain impairs morphine (8 mg/kg)-induced CPP by a reduction in the levels of morphine-evoked DA release in the NAc (Narita et al., 2005). However, in a model of neuropathic pain, CPP was not induced by a low dose of morphine (3.5 mg/kg) but it was by higher doses (5 or 7 mg/kg; Wu et al., 2014). Something similar occurs in opioid self-administration. Hipólito et al. (2015) showed that inflammatory pain reduces heroin self-administration for low doses, which does not happen for high doses. This effect is not related to the analgesic properties of doses used since the nociception threshold was the same among doses. This suggests that pain-induced changes in the mesocorticolimbical MORs result in a shift in the dose-response curve of opioids.

Opposite to pain-induced opioid-intake alterations, not too much is known about how pain and AUD are related. However, although there is evidence that chronic pain does not predict higher alcohol consumption in patients, it is true that it increases the risk of suffering an AUD (Brown et al., 1996; Brennan et al., 2005; Von Korff et al., 2005; Demyttenaere et al., 2007; Caldeiro et al., 2008; Lawton and Simpson, 2009; Witkiewitz et al., 2015). In this sense, AUDs are 15-20% more probable in pain patients, and if the pain condition interferes with daily activities the probability reaches 50% (Brennan and SooHoo, 2013). In fact, some clinical studies show that the existence of a pain condition is a risk factor leading to alcohol relapse in patients with a previous history of alcohol abuse (Jakubczyk et al., 2015; 2016). Regarding preclinical models, Campos-Jurado et al. (2020) showed for the first time how a low dose of ethanol (52 nmol) directly administered in the VTA failed to induce CPP in the presence of inflammatory pain whereas a higher dose (70 nmol) reverted

this phenomenon. Furthermore, inflammatory pain blunted DA release in the NAc when ethanol was subcutaneously administered at a dose of 1.5 g/kg. These findings are fairly like the ones presented for opioids, which suggests that MORs could be modulating these phenomena.

1.6. References

Alfonso-Loeches, S., Pascual-Lucas, M., Blanco, A. M., Sanchez-Vera, I., & Guerri, C. (2010). Pivotal role of TLR4 receptors in alcohol-induced neuroinflammation and brain damage. *Journal of Neuroscience*, *30*(24), 8285-8295.

Alfonso-Loeches, S., Urena-Peralta, J., Morillo-Bargues, M., Gómez-Pinedo, U., & Guerri, C. (2015). Ethanol-induced TLR4/NLRP3 neuroinflammatory response in microglial cells promotes leukocyte infiltration across the BBB. *Neurochemical Research*, *41*(1), 193-209.

Alfonso-Loeches, S., Ureña-Peralta, J. R., Morillo-Bargues, M. J., Oliver-De La Cruz, J., & Guerri, C. (2014). Role of mitochondria ROS generation in ethanol-induced NLRP3 inflammasome activation and cell death in astroglial cells. *Frontiers in cellular neuroscience*, *8*, 216.

Alvanzo, A. A., Storr, C. L., Mojtabai, R., Green, K. M., Pacek, L. R., La Flair, L. N., ... & Crum, R. M. (2014). Gender and race/ethnicity differences for initiation of alcohol-related service use among persons with alcohol dependence. *Drug and alcohol dependence*, *140*, 48-55.

Amunts, K., Kedo, O., Kindler, M., Pieperhoff, P., Mohlberg, H., Shah, N. J., ... & Zilles, K. (2005). Cytoarchitectonic mapping of the human amygdala, hippocampal region and entorhinal cortex: intersubject variability and probability maps. *Anatomy and embryology*, *210*(5), 343-352.

Anker, J. J., & Carroll, M. E. (2010). Females are more vulnerable to drug abuse than males: evidence from preclinical studies and the role of ovarian hormones. Biological basis of sex differences in psychopharmacology, 73-96.

Apkarian, A. V. (2011). The brain in chronic pain: clinical implications. *Pain management*, *1*(6), 577-586.

Aubrun, F., Salvi, N., Coriat, P., & Riou, B. (2005). Sex-and age-related differences in morphine requirements for postoperative pain relief. *The Journal of the American Society of Anesthesiologists*, *103*(1), 156-160.

Bachtell, R. K., Jones, J. D., Heinzerling, K. G., Beardsley, P. M., & Comer, S. D. (2017). Glial and neuroinflammatory targets for treating substance use disorders. *Drug and alcohol dependence*, *180*, 156-170.Schroder, K., & Tschopp, J. (2010). The inflammasomes. *cell*, *140*(6), 821-832.

Barton, E. A., Baker, C., & Leasure, J. L. (2017). Investigation of sex differences in the microglial response to binge ethanol and exercise. *Brain sciences, 7*(10), 139.

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell*, *139*(2), 267-284.

Bekhbat, M., & Neigh, G. N. (2018). Sex differences in the neuro-immune consequences of stress: Focus on depression and anxiety. Brain, behavior, and immunity, 67, 1-12.

Benarroch, E. E. (2012). Endogenous opioid systems: current concepts and clinical correlations. *Neurology*, *79*(8), 807-814.

Bergmark, A. (2008). On treatment mechanisms—what can we learn from the COMBINE study?. *Addiction*, *103*(5), 703-705.

Berkley, K. J. (1997). Sex differences in pain. *Behavioral and Brain Sciences*, *20*(3), 371-380.

Berríos-Cárcamo, P., Quintanilla, M. E., Herrera-Marschitz, M., Vasiliou, V., Zapata-Torres, G., & Rivera-Meza, M. (2017). Racemic salsolinol and its enantiomers act as agonists of the μ-opioid receptor by activating the Gi protein-adenylate cyclase pathway. *Frontiers in Behavioral Neuroscience*, *10*, 253.

Bijur, P. E., Esses, D., Birnbaum, A., Chang, A. K., Schechter, C., & Gallagher, E. J. (2008). Response to morphine in male and female patients: analgesia and adverse events. *The Clinical journal of pain*, *24*(3), 192-198.

Boerner, K. E., Birnie, K. A., Caes, L., Schinkel, M., & Chambers, C. T. (2014). Sex differences in experimental pain among healthy children: a systematic review and meta-analysis. *PAIN®*, *155*(5), 983-993.

Bontempi, B., & Sharp, F. R. (1997). Systemic morphine-induced Fos protein in the rat striatum and nucleus accumbens is regulated by μ opioid receptors in the substantia nigra and ventral tegmental area. *Journal of Neuroscience*, *17*(21), 8596-8612.

Brennan, P. L., & SooHoo, S. (2013). Pain and use of alcohol in later life: prospective evidence from the health and retirement study. *Journal of aging and health*, *25*(4), 656-677.

Brennan, P. L., Schutte, K. K., & Moos, R. H. (2005). Pain and use of alcohol to manage pain: prevalence and 3-year outcomes among older problem and non-problem drinkers. *Addiction*, *100*(6), 777-786.

Brown, C. A., Matthews, J., Fairclough, M., McMahon, A., Barnett, E., Al-Kaysi, A., ... & Jones, A. K. (2015). Striatal opioid receptor availability is related to acute and chronic pain perception in arthritis: does opioid adaptation increase resilience to chronic pain?. *Pain*, *156*(11), 2267.

Brown, R. L., Patterson, J. J., Rounds, L. A., & Papasouliotis, O. (1996). Substance abuse among patients with chronic back pain. *Journal of family practice*, *43*(2), 152-160.

Caldeiro, R. M., Malte, C. A., Calsyn, D. A., Baer, J. S., Nichol, P., Kivlahan, D. R., & Saxon, A. J. (2008). The association of persistent pain with out-patient addiction treatment outcomes and service utilization. *Addiction*, *103*(12), 1996-2005.

Campbell, C. I., Weisner, C., LeResche, L., Ray, G. T., Saunders, K., Sullivan, M. D., ... & Von Korff, M. (2010). Age and gender trends in long-term opioid analgesic use for noncancer pain. *American journal of public health*, *100*(12), 2541-2547.

Campos-Jurado, Y., Igual-López, M., Padilla, F., Zornoza, T., Granero, L., Polache, A., ... & Hipólito, L. (2019). Activation of MORs in the VTA induces changes on cFos expression in different projecting regions: Effect of inflammatory pain. *Neurochemistry International*, *131*, 104521.

Campos-Jurado, Y., Lorente, J. D., González-Romero, J. L., Granero, L., Polache, A., & Hipólito, L. (2020). Impaired alcohol-induced dopamine release in the nucleus accumbens in an inflammatory pain model: behavioral implications in male rats. *Pain*, *161*(9), 2203-2211.

Canidate, S. S., Carnaby, G. D., Cook, C. L., & Cook, R. L. (2017). A systematic review of naltrexone for attenuating alcohol consumption in women with alcohol use disorders. *Alcoholism: Clinical and Experimental Research*, *41*(3), 466-472.

Cantacorps, L., Alfonso-Loeches, S., Moscoso-Castro, M., Cuitavi, J., Gracia-Rubio, I., López-Arnau, R., ... & Valverde, O. (2017). Maternal alcohol binge drinking induces persistent neuroinflammation associated with myelin damage and behavioural dysfunctions in offspring mice. *Neuropharmacology*, *123*, 368-384.

Cappoli, N., Tabolacci, E., Aceto, P., & Russo, C. D. (2020). The emerging role of the BDNF-TrkB signaling pathway in the modulation of pain perception. *Journal of Neuroimmunology*, *349*, 577406.

Carroll, M. E., & Smethells, J. R. (2016). Sex differences in behavioral dyscontrol: role in drug addiction and novel treatments. Frontiers in Psychiatry, 6, 175.

Castro, D. C., & Berridge, K. C. (2014). Opioid hedonic hotspot in nucleus accumbens shell: mu, delta, and kappa maps for enhancement of sweetness "liking" and "wanting". *Journal of Neuroscience*, *34*(12), 4239-4250.

Cepeda, M. S., Farrar, J. T., Baumgarten, M., Boston, R., Carr, D. B., & Strom, B. L. (2003). Side effects of opioids during short-term administration: Effect of age, gender, and race. *Clinical Pharmacology & Therapeutics*, *74*(2), 102-112.

Chang, P. C., Pollema-Mays, S. L., Centeno, M. V., Procissi, D., Contini, M., Baria, A. T., ... & Apkarian, A. V. (2014). Role of nucleus accumbens in neuropathic pain: linked multi-scale evidence in the rat transitioning to neuropathic pain. *PAIN®*, *155*(6), 1128-1139.

Cheatle, M. D. (2015). Prescription opioid misuse, abuse, morbidity, and mortality: balancing effective pain management and safety. *Pain Medicine*, *16*(suppl_1), S3-S8.

Chopra, K., & Arora, V. (2014). An intricate relationship between pain and depression: clinical correlates, coactivation factors and therapeutic targets. *Expert Opinion on Therapeutic Targets*, *18*(2), 159-176.

Chou, R., Turner, J. A., Devine, E. B., Hansen, R. N., Sullivan, S. D., Blazina, I., ... & Deyo, R. A. (2015). The effectiveness and risks of long-term opioid therapy for chronic pain: a systematic review for a National Institutes of Health Pathways to Prevention Workshop. *Annals of internal medicine*, *162*(4), 276-286.

Cicero, T. J., Nock, B., O'Connor, L., & Meyer, E. R. (2002). Role of steroids in sex differences in morphine-induced analgesia: activational and organizational effects. *Journal of Pharmacology and Experimental Therapeutics*, *300*(2), 695-701.

Cohen, E., Feinn, R., Arias, A., & Kranzler, H. R. (2007). Alcohol treatment utilization: findings from the National Epidemiologic Survey on Alcohol and Related Conditions. *Drug and alcohol dependence*, *86*(2-3), 214-221.

Colombo, E., & Farina, C. (2016). Astrocytes: key regulators of neuroinflammation. *Trends in immunology*, *37*(9), 608-620.

Comer, S. D., Cooper, Z. D., Kowalczyk, W. J., Sullivan, M. A., Evans, S. M., Bisaga, A. M., & Vosburg, S. K. (2010). Evaluation of potential sex differences in the subjective and analgesic effects of morphine in normal, healthy volunteers. *Psychopharmacology*, *208*(1), 45-55.

Csupak, B., Sommer, J. L., Jacobsohn, E., & El-Gabalawy, R. (2018). A population-based examination of the co-occurrence and functional correlates of chronic pain and generalized anxiety disorder. *Journal of anxiety disorders*, *56*, 74-80.

Dahchour, A., & Ward, R. J. (2022). Changes in Brain Dopamine Extracellular Concentration after Ethanol Administration; Rat Microdialysis Studies. *Alcohol and Alcoholism*, *57*(2), 165-175.

Davis, V. E., & Walsh, M. J. (1970). Alcohol, amines, and alkaloids: a possible biochemical basis for alcohol addiction. *Science*, *167*(3920), 1005-1007.

Deehan Jr, G. A., Hauser, S. R., Wilden, J. A., Truitt, W. A., & Rodd, Z. A. (2013). Elucidating the biological basis for the reinforcing actions of alcohol in the mesolimbic dopamine system: the role of active metabolites of alcohol. *Frontiers in behavioral neuroscience*, *7*, 104.

Dempsey, S., & Grisel, J. E. (2012). Locomotor sensitization to EtOH: contribution of β-Endorphin. *Frontiers in Molecular Neuroscience*, *5*, 87.

Demyttenaere, K., Bruffaerts, R., Lee, S., Posada-Villa, J., Kovess, V., Angermeyer, M. C., ... & Von Korff, M. (2007). Mental disorders among persons with chronic back or neck pain: results from the World Mental Health Surveys. *Pain*, *129*(3), 332-342.

Devine, D. P., & Wise, R. A. (1994). Self-administration of morphine, DAMGO, and DPDPE into the ventral tegmental area of rats. *Journal of Neuroscience*, *14*(4), 1978-1984.

Devine, D. P., Leone, P., Pocock, D., & Wise, R. A. (1993). Differential involvement of ventral tegmental mu, delta and kappa opioid receptors in modulation of basal mesolimbic dopamine release: in vivo microdialysis studies. *Journal of Pharmacology and Experimental Therapeutics*, *266*(3), 1236-1246.

Di Chiara, G., & North, R. A. (1992). Neurobiology of opiate abuse. *Trends in pharmacological sciences*, *13*, 185-193.

Diaz, S. L., Kemmling, A. K., Rubio, M. C., & Balerio, G. N. (2005). Morphine withdrawal syndrome: involvement of the dopaminergic system in prepubertal male and female mice. *Pharmacology Biochemistry and Behavior*, *82*(4), 601-607.

DosSantos, M. F., Martikainen, I. K., Nascimento, T. D., Love, T. M., Deboer, M. D., Maslowski, E. C., ... & DaSilva, A. F. (2012). Reduced basal ganglia μ-opioid receptor availability in trigeminal neuropathic pain: a pilot study. *Molecular pain*, *8*, 1744-8069.

Enrico, P., Sirca, D., Mereu, M., Peana, A. T., Lintas, A., Golosio, A., & Diana, M. (2009). Acetaldehyde sequestering prevents ethanol-induced stimulation of mesolimbic dopamine transmission. *Drug and alcohol dependence*, *100*(3), 265-271.

Epstein, E. E., & Menges, D. (2013). Women and addiction.

Erol, A., & Karpyak, V. M. (2015). Sex and gender-related differences in alcohol use and its consequences: Contemporary knowledge and future research considerations. Drug and alcohol dependence, 156, 1-13.

Fenton, B. W., Shih, E., & Zolton, J. (2015). The neurobiology of pain perception in normal and persistent pain. *Pain management*, *5*(4), 297-317.

Fernandez-Lizarbe, S., Pascual, M., & Guerri, C. (2009). Critical role of TLR4 response in the activation of microglia induced by ethanol. *The Journal of Immunology*, *183*(7), 4733-4744.

Ferretti, M. T., Iulita, M. F., Cavedo, E., Chiesa, P. A., Schumacher Dimech, A., Santuccione Chadha, A., ... & Hampel, H. (2018). Sex differences in Alzheimer disease—the gateway to precision medicine. Nature Reviews Neurology, 14(8), 457-469.

Fields, H. (2004). State-dependent opioid control of pain. *Nature Reviews Neuroscience*, *5*(7), 565-575.

Fields, H. L., Hjelmstad, G. O., Margolis, E. B., & Nicola, S. M. (2007). Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. *Annu. Rev. Neurosci.*, *30*, 289-316.

Fields, H. L., Malick, A., & Burstein, R. (1995). Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. *Journal of neurophysiology*, *74*(4), 1742-1759.

Fillingim, R. B., Ness, T. J., Glover, T. L., Campbell, C. M., Hastie, B. A., Price, D. D., & Staud, R. (2005). Morphine responses and experimental pain: sex differences in side

effects and cardiovascular responses but not analgesia. *The Journal of Pain*, *6*(2), 116-124.

Fischer, J., Jung, N., Robinson, N., & Lehmann, C. (2015). Sex differences in immune responses to infectious diseases. Infection, 43(4), 399-403.

Floresco, S. B. (2015). The nucleus accumbens: an interface between cognition, emotion, and action. *Annual review of psychology*, *66*, 25-52.

Floresco, S. B., & Ghods-Sharifi, S. (2007). Amygdala-prefrontal cortical circuitry regulates effort-based decision making. *Cerebral cortex*, *17*(2), 251-260.

Font, L., Luján, M. Á., & Pastor, R. (2013). Involvement of the endogenous opioid system in the psychopharmacological actions of ethanol: the role of acetaldehyde. *Frontiers in behavioral neuroscience*, *7*, 93.

Franchi, L., Muñoz-Planillo, R., & Núñez, G. (2012). Sensing and reacting to microbes through the inflammasomes. *Nature immunology*, *13*(4), 325-332.

Garbutt, J. C., Kranzler, H. R., O'Malley, S. S., Gastfriend, D. R., Pettinati, H. M., Silverman, B. L., ... & Vivitrex Study Group. (2005). Efficacy and tolerability of long-acting injectable naltrexone for alcohol dependence: a randomized controlled trial. *Jama*, *293*(13), 1617-1625.

Gerrits, M. A., Lesscher, H. B., & van Ree, J. M. (2003). Drug dependence and the endogenous opioid system. *European neuropsychopharmacology*, *13*(6), 424-434.

Gianoulakis, C. (1990). Characterization of the effects of acute ethanol administration on the release of β-endorphin peptides by the rat hypothalamus. *European journal of pharmacology*, *180*(1), 21-29.

Grant, B. F., Chou, S. P., Saha, T. D., Pickering, R. P., Kerridge, B. T., Ruan, W. J., ... & Hasin, D. S. (2017). Prevalence of 12-month alcohol use, high-risk drinking, and DSM-IV alcohol use disorder in the United States, 2001-2002 to 2012-2013: results from the National Epidemiologic Survey on Alcohol and Related Conditions. JAMA psychiatry, 74(9), 911-923.

Grant, B. F., Goldstein, R. B., Saha, T. D., Chou, S. P., Jung, J., Zhang, H., ... & Hasin, D. S. (2015). Epidemiology of DSM-5 alcohol use disorder: results from the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA psychiatry, 72(8), 757-766.

Gu, N., Peng, J., Murugan, M., Wang, X., Eyo, U. B., Sun, D., ... & Wu, L. J. (2016). Spinal microgliosis due to resident microglial proliferation is required for pain hypersensitivity after peripheral nerve injury. *Cell reports*, *16*(3), 605-614.

Guha, M. (2014). Diagnostic and Statistical Manual of Mental Disorders: DSM-5 (5th edition), *Reference Reviews, 28*(3), 36-37.

Gysling, K., & Wang, R. Y. (1983). Morphine-induced activation of A10 dopamine neurons in the rat. *Brain research*, *277*(1), 119-127.

Hand, T. H., Stinus, L., & Le Moal, M. (1989). Differential mechanisms in the acquisition and expression of heroin-induced place preference. *Psychopharmacology*, *98*(1), 61-67.

Harris, R. E., Clauw, D. J., Scott, D. J., McLean, S. A., Gracely, R. H., & Zubieta, J. K. (2007). Decreased central μ-opioid receptor availability in fibromyalgia. *Journal of Neuroscience*, *27*(37), 10000-10006.

Henriques, J. F., Portugal, C. C., Canedo, T., Relvas, J. B., Summavielle, T., & Socodato, R. (2018). Microglia and alcohol meet at the crossroads: Microglia as critical modulators of alcohol neurotoxicity. *Toxicology letters*, *283*, 21-31.

Hipólito, L., Martí-Prats, L., Sánchez-Catalán, M. J., Polache, A., & Granero, L. (2011). Induction of conditioned place preference and dopamine release by salsolinol in posterior VTA of rats: involvement of μ-opioid receptors. *Neurochemistry international*, *59*(5), 559-562.

Hipólito, L., Sanchez, M. J., Polache, A., & Granero, L. (2007). Brain metabolism of ethanol and alcoholism: an update. *Current drug metabolism*, *8*(7), 716-727.

Hipólito, L., Sánchez-Catalán, M. J., Martí-Prats, L., Granero, L., & Polache, A. (2012). Revisiting the controversial role of salsolinol in the neurobiological effects of ethanol: old and new vistas. *Neuroscience & Biobehavioral Reviews*, *36*(1), 362-378.

Hipólito, L., Wilson-Poe, A., Campos-Jurado, Y., Zhong, E., Gonzalez-Romero, J., Virag, L., ... & Morón, J. A. (2015). Inflammatory pain promotes increased opioid self-administration: role of dysregulated ventral tegmental area μ opioid receptors. *Journal of Neuroscience*, *35*(35), 12217-12231.

Ho, I. H., Chan, M. T., Wu, W. K., & Liu, X. (2020). Spinal microglia-neuron interactions in chronic pain. *Journal of Leukocyte Biology*, *108*(5), 1575-1592.

Holzhauer, C. G., Cucciare, M., & Epstein, E. E. (2020). Sex and gender effects in recovery from alcohol use disorder. *Alcohol research: current reviews*, *40*(3).

Hommer, D. W. (2003). Male and female sensitivity to alcohol-induced brain damage. Alcohol Research & Health, 27(2), 181.

Howard, E. C., Schier, C. J., Wetzel, J. S., Duvauchelle, C. L., & Gonzales, R. A. (2008). The shell of the nucleus accumbens has a higher dopamine response compared with the core after non-contingent intravenous ethanol administration. *Neuroscience*, *154*(3), 1042-1053.

Imperato, A., & Di Chiara, G. (1986). Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *Journal of Pharmacology and Experimental Therapeutics*, *239*(1), 219-228.

Inoue, K., & Tsuda, M. (2018). Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. *Nature Reviews Neuroscience*, *19*(3), 138-152.

Iwasaki, A., & Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nature immunology*, *5*(10), 987-995.

Jakubczyk, A., Ilgen, M. A., Bohnert, A. S., Kopera, M., Krasowska, A., Klimkiewicz, A., ... & Wojnar, M. (2015). Physical pain in alcohol-dependent patients entering treatment in Poland—prevalence and correlates. *Journal of studies on alcohol and drugs*, *76*(4), 607-614.

Jakubczyk, A., Ilgen, M. A., Kopera, M., Krasowska, A., Klimkiewicz, A., Bohnert, A., ... & Wojnar, M. (2016). Reductions in physical pain predict lower risk of relapse following alcohol treatment. *Drug and Alcohol Dependence*, *158*, 167-171.

Jalabert, M., Bourdy, R., Courtin, J., Veinante, P., Manzoni, O. J., Barrot, M., & Georges, F. (2011). Neuronal circuits underlying acute morphine action on dopamine neurons. *Proceedings of the national academy of sciences*, *108*(39), 16446-16450.

Jarjour, S., Bai, L., & Gianoulakis, C. (2009). Effect of acute ethanol administration on the release of opioid peptides from the midbrain including the ventral tegmental area. *Alcoholism: Clinical and Experimental Research*, *33*(6), 1033-1043.

Joshi, P. C., & Guidot, D. M. (2007). The alcoholic lung: epidemiology, pathophysiology, and potential therapies. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *292*(4), L813-L823.

Kane, C. J., & Drew, P. D. (2021). Neuroinflammatory contribution of microglia and astrocytes in fetal alcohol spectrum disorders. *Journal of neuroscience research*, *99*(8), 1973-1985.

Kawai, T., & Akira, S. (2006). TLR signaling. *Cell Death & Differentiation*, *13*(5), 816-825.

Kawai, T., & Akira, S. (2007). Signaling to NF-κB by Toll-like receptors. *Trends in molecular medicine*, *13*(11), 460-469.

Kawasaki, Y., Zhang, L., Cheng, J. K., & Ji, R. R. (2008). Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1β, interleukin-6, and tumor necrosis factor-α in regulating synaptic and neuronal activity in the superficial spinal cord. *Journal of neuroscience*, *28*(20), 5189-5194.

King, N. B., Fraser, V., Boikos, C., Richardson, R., & Harper, S. (2014). Determinants of increased opioid-related mortality in the United States and Canada, 1990–2013: a systematic review. *American journal of public health*, *104*(8), e32-e42.

Klein, S. L., & Flanagan, K. L. (2016). Sex differences in immune responses. Nature Reviews Immunology, 16(10), 626-638.

Koob, G. F., & Volkow, N. D. (2016). Neurobiology of addiction: a neurocircuitry analysis. *The Lancet Psychiatry*, *3*(8), 760-773.

Kuner, R. (2010). Central mechanisms of pathological pain. *Nature medicine*, *16*(11), 1258-1266.

Kurita, G. P., Sjøgren, P., Juel, K., Højsted, J., & Ekholm, O. (2012). The burden of chronic pain: a cross-sectional survey focussing on diseases, immigration, and opioid use. *PAIN®*, *153*(12), 2332-2338.

Kuzawińska, O., Lis, K., Cudna, A., & Bałkowiec-Iskra, E. (2014). Gender differences in the neurochemical response of trigeminal ganglion neurons to peripheral inflammation in mice. *Acta Neurobiol Exp (Wars)*, *74*(2), 227-232.

Landmark, T., Romundstad, P., Dale, O., Borchgrevink, P. C., Vatten, L., & Kaasa, S. (2013). Chronic pain: one year prevalence and associated characteristics (the HUNT pain study). *Scandinavian journal of pain*, *4*(4), 182-187.

Lawton, J., & Simpson, J. (2009). Predictors of alcohol use among people experiencing chronic pain. *Psychology, health & medicine*, *14*(4), 487-501.

Lenz, F. A., Weiss, N., Ohara, S., Lawson, C., & Greenspan, J. D. (2004). The role of the thalamus in pain. *Supplements to clinical neurophysiology*, *57*, 50-61.

Leriche, M., & Méndez, M. (2010). Ethanol exposure selectively alters β-endorphin content but not [3H]-DAMGO binding in discrete regions of the rat brain. *Neuropeptides*, *44*(1), 9-16.

Lippai, D., Bala, S., Csak, T., Kurt-Jones, E. A., & Szabo, G. (2013). Chronic alcohol-induced microRNA-155 contributes to neuroinflammation in a TLR4-dependent manner in mice. *PloS one*, *8*(8), e70945.

Locke, S., Yousefpour, N., Mannarino, M., Xing, S., Yashmin, F., Bourassa, V., & Ribeiro-da-Silva, A. (2020). Peripheral and central nervous system alterations in a rat model of inflammatory arthritis. *Pain*, *161*(7), 1483-1496.

Lorente, J. D., Cuitavi, J., Campos-Jurado, Y., & Hipólito, L. (2022). Pain-induced alterations in the dynorphinergic system within the mesocorticolimbic pathway: Implication for alcohol addiction. *Journal of Neuroscience Research*, *100*(1), 165-182.

Maarrawi, J., Peyron, R., Mertens, P., Costes, N., Magnin, M., Sindou, M., ... & Garcia-Larrea, L. (2007). Differential brain opioid receptor availability in central and peripheral neuropathic pain. *Pain*, *127*(1-2), 183-194.

Mansour, A., Khachaturian, H., Lewis, M. E., Akil, H., & Watson, S. J. (1988). Anatomy of CNS opioid receptors. *Trends in neurosciences*, *11*(7), 308-314.

Mapplebeck, J. C., Beggs, S., & Salter, M. W. (2016). Sex differences in pain: a tale of two immune cells. Pain, 157, S2-S6.

Marbach, J. J., & Lund, P. (1981). Depression, anhedonia and anxiety in temporomandibular joint and other facial pain syndromes. *Pain*, *11*(1), 73-84.

Marinelli, S., Basilico, B., Marrone, M. C., & Ragozzino, D. (2019). Microglia-neuron crosstalk: Signaling mechanism and control of synaptic transmission. In *Seminars in cell & developmental biology* (Vol. 94, pp. 138-151). Academic Press.

Marvizón, J. C. G., Chen, W., & Murphy, N. (2009). Enkephalins, dynorphins, and β-endorphin in the rat dorsal horn: An immunofluorescence colocalization study. *Journal of Comparative Neurology*, *517*(1), 51-68.

Massaly, N., Copits, B. A., Wilson-Poe, A. R., Hipólito, L., Markovic, T., Yoon, H. J., ... & Morón, J. A. (2019). Pain-induced negative affect is mediated via recruitment of the nucleus accumbens kappa opioid system. *Neuron*, *102*(3), 564-573.

Massaly, N., Morón, J. A., & Al-Hasani, R. (2016). A trigger for opioid misuse: chronic pain and stress dysregulate the mesolimbic pathway and kappa opioid system. *Frontiers in neuroscience*, *10*, 480.

Massier, J., Eitner, A., Segond von Banchet, G., & Schaible, H. G. (2015). Effects of differently activated rodent macrophages on sensory neurons: implications for arthritis pain. *Arthritis & Rheumatology*, *67*(8), 2263-2272.

Mayer, D. J., & Liebeskind, J. C. (1974). Pain reduction by focal electrical stimulation of the brain: an anatomical and behavioral analysis. *Brain research*, *68*(1), 73-93.

Mayer, D. J., Wolfle, T. L., Akil, H., Carder, B., & Liebeskind, J. C. (1971). Analgesia from electrical stimulation in the brainstem of the rat. *Science*, *174*(4016), 1351-1354.

McCaul, M. E., Roach, D., Hasin, D. S., Weisner, C., Chang, G., & Sinha, R. (2019). Alcohol and women: A brief overview. Alcoholism, clinical and experimental research, 43(5), 774.

McCrady, B. S., Epstein, E. E., & Fokas, K. F. (2020). Treatment interventions for women with alcohol use disorder. *Alcohol research: current reviews*.

McHugh, R. K., Votaw, V. R., Sugarman, D. E., & Greenfield, S. F. (2018). Sex and gender differences in substance use disorders. Clinical psychology review, 66, 12-23.

Melis, M., Gessa, G. L., & Diana, M. (2000). Different mechanisms for dopaminergic excitation induced by opiates and cannabinoids in the rat midbrain. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *24*(6), 993-1006.

Menon, J., & Kandasamy, A. (2018). Relapse prevention. *Indian journal of psychiatry*, *60*(Suppl 4), S473.

Mika, J., Obara, I., & Przewlocka, B. (2011). The role of nociceptin and dynorphin in chronic pain: Implications of neuro–glial interaction. *Neuropeptides*, *45*(4), 247-261.

Miller, P. L., & Ernst, A. A. (2004). Sex differences in analgesia: a randomized trial of mu versus kappa opioid agonists. *Southern medical journal*, *97*(1), 35-42.

Ministerio de Sanidad de España. (2023). Encuesta sobre alcohol y drogas en España (EDADES), 1995–2022.

Mogil, J. S. (2012). Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nature Reviews Neuroscience*, *13*(12), 859-866.

Mogil, J. S., & Bailey, A. L. (2010). Sex and gender differences in pain and analgesia. *Progress in brain research*, *186*, 140-157.

Moss, M., Parsons, P. E., Steinberg, K. P., Hudson, L. D., Guidot, D. M., Burnham, E. L., ... & Cotsonis, G. A. (2003). Chronic alcohol abuse is associated with an increased incidence of acute respiratory distress syndrome and severity of multiple organ dysfunction in patients with septic shock. *Critical care medicine*, *31*(3), 869-877.

Myles, P. S., Hunt, J. O., & Moloney, J. T. (1997). Postoperative 'minor'complications: comparison between men and women. *Anaesthesia*, *52*(4), 300-306.

Narita, M., Kishimoto, Y., Ise, Y., Yajima, Y., Misawa, K., & Suzuki, T. (2005). Direct evidence for the involvement of the mesolimbic κ-opioid system in the morphine-induced rewarding effect under an inflammatory pain-like state. *Neuropsychopharmacology*, *30*(1), 111-118.

National Institute on Alcohol Abuse and Alcoholism (2021). Understanding Alcohol Use Disorder

Navratilova, E., Xie, J. Y., Okun, A., Qu, C., Eyde, N., Ci, S., ... & Porreca, F. (2012). Pain relief produces negative reinforcement through activation of mesolimbic reward–valuation circuitry. *Proceedings of the National Academy of Sciences*, *109*(50), 20709-20713.

Nestler, E. J., Hyman, A. E., & Malenka, R. D. (2009). Molecular neuropharmacology: a foundation for clinical neuroscience. *NY: McGraw-Hill Medical*.

Okun, E., Griffioen, K. J., Lathia, J. D., Tang, S. C., Mattson, M. P., & Arumugam, T. V. (2009). Toll-like receptors in neurodegeneration. *Brain research reviews*, *59*(2), 278-292.

O'Malley, S. S., Sinha, R., Grilo, C. M., Capone, C., Farren, C. K., McKee, S. A., ... & Wu, R. (2007). Naltrexone and cognitive behavioral coping skills therapy for the treatment of alcohol drinking and eating disorder features in alcohol-dependent women: a randomized controlled trial. *Alcoholism: Clinical and experimental research*, *31*(4), 625-634.

Pascual, M., López-Hidalgo, R., Montagud-Romero, S., Ureña-Peralta, J. R., Rodríguez-Arias, M., & Guerri, C. (2021). Role of mTOR-regulated autophagy in spine pruning defects and memory impairments induced by binge-like ethanol treatment in adolescent mice. *Brain Pathology, 31*(1), 174-188.

Pascual, M., Montesinos, J., Marcos, M., Torres, J. L., Costa-Alba, P., García-García, F., ... & Guerri, C. (2017). Gender differences in the inflammatory cytokine and chemokine profiles induced by binge ethanol drinking in adolescence. *Addiction biology, 22*(6), 1829-1841.

Pastor, R., Font, L., Miquel, M., Phillips, T. J., & Aragon, C. M. (2011). Involvement of the Beta-Endorphin Neurons of the Hypothalamic Arcuate Nucleus in Ethanol-Induced Place Preference Conditioning in Mice. *Alcoholism: Clinical and Experimental Research*, *35*(11), 2019-2029.

Peana, A. T., Sánchez-Catalán, M. J., Hipólito, L., Rosas, M., Porru, S., Bennardini, F., ... & Acquas, E. (2017). Mystic acetaldehyde: the never-ending story on alcoholism. *Frontiers in Behavioral Neuroscience*, *11*, 81.

Pinho-Ribeiro, F. A., Verri Jr, W. A., & Chiu, I. M. (2017). Nociceptor sensory neuron–immune interactions in pain and inflammation. *Trends in immunology*, *38*(1), 5-19.

Porreca, F., & Navratilova, E. (2017). Reward, motivation and emotion of pain and its relief. *Pain*, *158*(Suppl 1), S43.

Porreca, F., Ossipov, M. H., & Gebhart, G. F. (2002). Chronic pain and medullary descending facilitation. *Trends in neurosciences*, *25*(6), 319-325.

Prinz, M., Jung, S., & Priller, J. (2019). Microglia biology: one century of evolving concepts. *Cell*, *179*(2), 292-311.

Qin, L., He, J., Hanes, R. N., Pluzarev, O., Hong, J. S., & Crews, F. T. (2008). Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. *Journal of neuroinflammation*, *5*(1), 1-17.

Quintanilla, M. E., Rivera-Meza, M., Berríos-Cárcamo, P., Cassels, B. K., Herrera-Marschitz, M., & Israel, Y. (2016). (R)-S alsolinol, a product of ethanol metabolism, stereospecifically induces behavioral sensitization and leads to excessive alcohol intake. *Addiction Biology*, *21*(6), 1063-1071.

Rachinger-Adam, B., Conzen, P., & Azad, S. C. (2011). Pharmacology of peripheral opioid receptors. *Current Opinion in Anesthesiology*, *24*(4), 408-413.

Raja, S. N., Carr, D. B., Cohen, M., Finnerup, N. B., Flor, H., Gibson, S., ... & Vader, K. (2020). The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. Pain. *press. doi*, *10*.

Raoof, R., Willemen, H. L., & Eijkelkamp, N. (2018). Divergent roles of immune cells and their mediators in pain. *Rheumatology*, *57*(3), 429-440.

Rasmussen, D. D., Bryant, C. A., Boldt, B. M., Colasurdo, E. A., Levin, N., & Wilkinson, C. W. (1998). Acute alcohol effects on opiomelanocortinergic regulation. *Alcoholism: Clinical and Experimental Research*, *22*(4), 789-801.

Reddy, B. V., & Sarkar, D. K. (1993). Effect of alcohol, acetaldehyde, and salsolinol on β-endorphin secretion from the hypothalamic neurons in primary cultures. *Alcoholism: Clinical and Experimental Research*, *17*(6), 1261-1267.

Reid, K. J., Harker, J., Bala, M. M., Truyers, C., Kellen, E., Bekkering, G. E., & Kleijnen, J. (2011). Epidemiology of chronic non-cancer pain in Europe: narrative review of prevalence, pain treatments and pain impact. *Current medical research and opinion*, *27*(2), 449-462.

Reynolds, D. V. (1969). Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science*, *164*(3878), 444-445.

Rodd, Z. A., Oster, S. M., Ding, Z. M., Toalston, J. E., Deehan, G., Bell, R. L., ... & McBride, W. J. (2008). The reinforcing properties of salsolinol in the ventral tegmental area: evidence for regional heterogeneity and the involvement of serotonin and dopamine. *Alcoholism: Clinical and Experimental Research*, *32*(2), 230-239.

Ruau, D., Liu, L. Y., Clark, J. D., Angst, M. S., & Butte, A. J. (2012). Sex differences in reported pain across 11,000 patients captured in electronic medical records. *The Journal of Pain*, *13*(3), 228-234.

Salamone, J. D., & Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. *Neuron*, *76*(3), 470-485.

Sanchez-Catalan, M. J., Kaufling, J., Georges, F., Veinante, P., & Barrot, M. (2014). The antero-posterior heterogeneity of the ventral tegmental area. *Neuroscience*, *282*, 198-216.

Sanchis-Segura, C., & Becker, J. B. (2016). Why we should consider sex (and study sex differences) in addiction research. Addiction biology, 21(5), 995-1006.

Sanchis-Segura, C., Correa, M., Miquel, M., & Aragon, C. M. (2005). Catalase inhibition in the Arcuate nucleus blocks ethanol effects on the locomotor activity of rats. *Neuroscience letters*, *376*(1), 66-70.

Sarton, E., Olofsen, E., Romberg, R., den Hartigh, J., Kest, B., Nieuwenhuijs, D., ... & Dahan, A. (2000). Sex differences in morphine analgesia: an experimental study in healthy volunteers. *The Journal of the American Society of Anesthesiologists*, *93*(5), 1245-1254.

Schultz, W. (2013). Updating dopamine reward signals. *Current opinion in neurobiology*, *23*(2), 229-238.

Schwartz, N., Temkin, P., Jurado, S., Lim, B. K., Heifets, B. D., Polepalli, J. S., & Malenka, R. C. (2014). Decreased motivation during chronic pain requires long-term depression in the nucleus accumbens. *Science*, *345*(6196), 535-542.

Seminowicz, D. A., & Moayedi, M. (2017). The dorsolateral prefrontal cortex in acute and chronic pain. *The Journal of Pain*, *18*(9), 1027-1035.

Seminowicz, D. A., Laferriere, A. L., Millecamps, M., Jon, S. C., Coderre, T. J., & Bushnell, M. C. (2009). MRI structural brain changes associated with sensory and emotional function in a rat model of long-term neuropathic pain. *Neuroimage*, *47*(3), 1007-1014.

Serafini, R. A., Pryce, K. D., & Zachariou, V. (2020). The mesolimbic dopamine system in chronic pain and associated affective comorbidities. *Biological psychiatry*, *87*(1), 64-73.

Sheu, R., Lussier, D., Rosenblum, A., Fong, C., Portenoy, J., Joseph, H., & Portenoy, R. K. (2008). Prevalence and characteristics of chronic pain in patients admitted to an outpatient drug and alcohol treatment program. *Pain Medicine*, *9*(7), 911-917.

Shippenberg, T. S., & Herz, A. (1987). Place preference conditioning reveals the involvement of D1-dopamine receptors in the motivational properties of μ-and κ-opioid agonists. *Brain research*, *436*(1), 169-172.

Singhal, G., & Baune, B. T. (2017). Microglia: an interface between the loss of neuroplasticity and depression. *Frontiers in cellular neuroscience*, *11*, 270.

Sorge, R. E., & Totsch, S. K. (2017). Sex differences in pain. *Journal of neuroscience research*, *95*(6), 1271-1281.

Sorge, R. E., LaCroix-Fralish, M. L., Tuttle, A. H., Sotocinal, S. G., Austin, J. S., Ritchie, J., ... & Mogil, J. S. (2011). Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. *Journal of Neuroscience*, *31*(43), 15450-15454.

Sorge, R. E., Mapplebeck, J., Rosen, S., Beggs, S., Taves, S., Alexander, J. K., ... & Mogil, J. S. (2015). Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nature neuroscience*, *18*(8), 1081-1083.

Spanagel, R. (2009). Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiological reviews*, *89*(2), 649-705.

Spanagel, R., Herz, A., & Shippenberg, T. S. (1992). Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proceedings of the National Academy of Sciences*, *89*(6), 2046-2050.

Steingrímsdóttir, Ó. A., Landmark, T., Macfarlane, G. J., & Nielsen, C. S. (2017). Defining chronic pain in epidemiological studies: a systematic review and meta-analysis. *Pain*, *158*(11), 2092-2107.

Sutterwala, F. S., Ogura, Y., Szczepanik, M., Lara-Tejero, M., Lichtenberger, G. S., Grant, E. P., ... & Flavell, R. A. (2006). Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity*, *24*(3), 317-327.

Szabo, G., & Mandrekar, P. (2009). A recent perspective on alcohol, immunity, and host defense. *Alcoholism: Clinical and Experimental Research*, *33*(2), 220-232.

Szabo, G., & Zakhari, S. (2011). Mechanisms of alcohol-mediated hepatotoxicity in human-immunodeficiency-virus-infected patients. *World journal of gastroenterology: WJG*, *17*(20), 2500.

Taylor, A. M., Becker, S., Schweinhardt, P., & Cahill, C. (2016). Mesolimbic dopamine signaling in acute and chronic pain: implications for motivation, analgesia, and addiction. *Pain*, *157*(6), 1194.

Taylor, A. M., Castonguay, A., Taylor, A. J., Murphy, N. P., Ghogha, A., Cook, C., ... & Cahill, C. M. (2015). Microglia disrupt mesolimbic reward circuitry in chronic pain. *Journal of Neuroscience*, *35*(22), 8442-8450.

Taylor, A. M., Mehrabani, S., Liu, S., Taylor, A. J., & Cahill, C. M. (2017). Topography of microglial activation in sensory-and affect-related brain regions in chronic pain. *Journal of neuroscience research*, *95*(6), 1330-1335.

Thompson, S. J., Pitcher, M. H., Stone, L. S., Tarum, F., Niu, G., Chen, X., ... & Bushnell, M. C. (2018). Chronic neuropathic pain reduces opioid receptor availability with associated anhedonia in rat. *Pain*, *159*(9), 1856.

Urban, M. O., & Gebhart, G. F. (1999). Supraspinal contributions to hyperalgesia. *Proceedings of the National Academy of Sciences*, *96*(14), 7687-7692.

Vallés, S. L., Blanco, A. M., Pascual, M., & Guerri, C. (2004). Chronic ethanol treatment enhances inflammatory mediators and cell death in the brain and in astrocytes. *Brain pathology*, *14*(4), 365-371.

Vergne-Salle, P., & Bertin, P. (2021). Chronic pain and neuroinflammation. *Joint Bone Spine*, *88*(6), 105222.

Von Banchet, G. S., Boettger, M. K., Fischer, N., Gajda, M., Bräuer, R., & Schaible, H. G. (2009). Experimental arthritis causes tumor necrosis factor-α-dependent infiltration of macrophages into rat dorsal root ganglia which correlates with pain-related behavior. *PAIN®*, *145*(1-2), 151-159.

Von Korff, M., Crane, P., Lane, M., Miglioretti, D. L., Simon, G., Saunders, K., ... & Kessler, R. (2005). Chronic spinal pain and physical–mental comorbidity in the United States: results from the national comorbidity survey replication. *Pain*, *113*(3), 331-339.

Vore, A. S., & Deak, T. (2022). Alcohol, inflammation, and blood-brain barrier function in health and disease across development. In International Review of Neurobiology (Vol. 161, pp. 209-249). Academic Press.

Wen, H., Miao, E. A., & Ting, J. P. Y. (2013). Mechanisms of NOD-like receptor-associated inflammasome activation. *Immunity*, *39*(3), 432-441.

Wiech, K., & Tracey, I. (2009). The influence of negative emotions on pain: behavioral effects and neural mechanisms. *Neuroimage*, *47*(3), 987-994.

Wiech, K., & Tracey, I. (2013). Pain, decisions, and actions: a motivational perspective. *Frontiers in neuroscience*, *7*, 46.

Wise, R. A. (2004). Dopamine, learning and motivation. *Nature reviews neuroscience*, *5*(6), 483-494.

Witkiewitz, K., Vowles, K. E., McCallion, E., Frohe, T., Kirouac, M., & Maisto, S. A. (2015). Pain as a predictor of heavy drinking and any drinking lapses in the COMBINE study and the UK Alcohol Treatment Trial. *Addiction*, *110*(8), 1262-1271.

Witt, E. D. (2010). Research on alcohol and adolescent brain development: opportunities and future directions. *Alcohol*, *44*(1), 119-124.

World Health Organization (2008). G*lobal report on falls prevention in older age*.

World Health Organization. (2018). *Global status report on alcohol and health 2018*.

Wu, Y., Na, X., Zang, Y., Cui, Y., Xin, W., Pang, R., ... & Liu, X. (2014). Upregulation of tumor necrosis factor-alpha in nucleus accumbens attenuates morphine-induced rewarding in a neuropathic pain model. *Biochemical and biophysical research communications*, *449*(4), 502-507.

Xiao, C., & Ye, J. H. (2008). Ethanol dually modulates GABAergic synaptic transmission onto dopaminergic neurons in ventral tegmental area: role of μ-opioid receptors. *Neuroscience*, *153*(1), 240-248.

Xiao, C., Zhang, J., Krnjević, K., & Ye, J. H. (2007). Effects of ethanol on midbrain neurons: role of opioid receptors. *Alcoholism: Clinical and Experimental Research*, *31*(7), 1106-1113.

Zahm, D. S. (1999). Functional-anatomical implications of the nucleus accumbens core and shell subterritories. *Annals of the New York Academy of Sciences*, *877*(1), 113-128.

Zou, J., & Crews, F. (2010). Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF-κB and proinflammatory cytokines. *Alcoholism: Clinical and Experimental Research*, *34*(5), 777-789.

CHAPTER 2

State of the Art: Mu-Opioid Receptors

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Review

The Life Cycle of the Mu-Opioid Receptor

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Opioid receptors (ORs) are undisputed targets for the treatment of pain. Unfortunately, targeting these receptors therapeutically poses significant challenges including addiction, dependence, tolerance, and the appearance of side effects, such as respiratory depression and constipation. Moreover, misuse of prescription and illicit narcotics has resulted in the current opioid crisis. The mu-opioid receptor (MOR) is the cellular mediator of the effects of most commonly used opioids, and is a prototypical G protein-coupled receptor (GPCR) where new pharmacological, signalling and cell biology concepts have been coined. This review summarises the knowledge of the life cycle of this therapeutic target, including its biogenesis, trafficking to and from the plasma membrane, and how the regulation of these processes impacts its function and is related to pathophysiological conditions.

Highlights

SNPs of *OPRM1*, resulting in amino acid changes in the protein sequence (e.g., N40D), may have effects in pain and reward processing.

Epigenetic changes, as well as positive and negative transcription factors, are key controllers of receptor expression.

Alternative splicing generates over 20 different isoforms of mu-opioid receptor (MOR) with distinct pharmacological characteristics; these include isoforms with 7-TM, 6-TM, and 1-TM.

MicroRNAs affect *OPRM1* mRNA stability upon treatment with opioid drugs.

Ligand-dependent regulation of MOR has been the subject of intense research focussed on the development of improved analgesics.

Pain might be a risk factor towards drug addiction. The role of MORs in pain and reward processes highlights the role of this receptor in such comorbidity.

The Dark Side of Opioid Pain Relief

In the past couple of decades, the USA has experienced what is now known as 'the opioid epidemic'. This devastating situation started due to the indiscriminate prescription of therapeutic opioids to ease painful conditions, suffered by approximatively 30% of American citizens. It is estimated that 3–26% of chronic pain sufferers treated with opioids become addicts, and 61% of drug overdoses in 2014 in the USA involved pharmaceutical opioids. Many countries in the world have followed the path of the USA, with Canada having a similar prevalence of opioid misuse and other countries, such as the UK and The Netherlands, reporting an increase in opioid misuse and overdose.

In light of these events, research on the biology of ORs, the cellular mediators of opioid-induced effects, has gained significant momentum. Since their discovery in the second half of the 20th century, many techniques have been used to study ORs; from radioligand binding in the 1970s, and *in situ* hybridisation and molecular cloning in the 1990s [1], to the most recent visualisation of these receptors and their signalling *in vitro* and *in vivo* [2]. Of the four ORs subtypes [Mu (MOR), Delta (DOR), Kappa (KOR), and Opioid Receptor-Like 1 (ORL-1)], the MOR stands out for its role in opioid-induced analgesia and reward processing [3]. MORs play key roles in pain management, euphoria, sedation, miosis, addiction, truncation rigidity, nausea, and respiratory control in the central nervous system. Therefore, changes in their structure or function have significant consequences on those behaviours. Herein, the life cycle of the MOR is reviewed, and the journey of this receptor, from its birth (biogenesis) to its death (degradation) is captured, highlighting the most relevant variations and disruptions along the way.

A New Beginning: A Messenger Is Born

The four OR subtypes are part of the rhodopsin-like GPCRs superfamily, and are 60% identical to each other. The greatest identity is found in the regions that encode for the 7-transmembrane domain (TM) and intracellular loops (70–90%), while the areas encoding for their N and C termini, as well as the extracellular loops, are more divergent. The MOR is encoded by the *OPRM1* gene in humans and the *Oprm1* gene in mice, and it is conserved across species. In humans the *OPRM1* gene is in the sixth chromosome (6q25.2) and contains at least 19 exons [4]. The rodent

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and human MOR share 94% sequence identity, with the N terminus of the receptor being the area of highest divergence (with 65% sequence identity) [5]. This overall high sequence similarity explains why, to date, no significant differences in the structure, pharmacology, and function have been reported across these species.

SNPs in the coding regions of the MOR have been widely studied, linked to alterations in signalling, and suggested to underlie altered responses to opioids [6]. The rs1799971 SNP, located at position 118 in the exon 1 of the *OPRM1* gene, is the most studied, and it encompasses the change of an adenine (A) to a guanine (G). Interestingly, the G-containing allele is present in 15–30% of Europeans, 40–50% of Asians, and 1–3% of Latinos and African Americans [7]. At a protein level, this SNP results in a change of amino acid at position 40, located in the N terminus of the receptor; from an asparagine (Asn, N) to an aspartate (Asp, D). This N40D change removes a potential site for asparagine-linked glycosylation, which has been suggested to alter MOR affinity for different ligands, its transduction cascade [8], as well as the half-life of the receptor at the membrane [9]. Moreover, G118 adds a methylation site, which has been reported to result in a reduction of the levels of MOR messenger RNA (mRNA) [10]. Both alteration on glycosylation profiles as well as changes in the methylation patterns can disrupt the normal activity of the receptor. Some reports suggest that the rs1799971 SNP interferes with the analgesic power of some opioids [11]. However, the effect of this SNP depends on the type of pain and/or opioid analgesic under evaluation [12]. Numerous groups have also investigated the relationship between this polymorphism, and postoperative reactions to anaesthesia such as vomiting, nausea, dizziness, and pruritus, albeit with some discrepancies [13,14]. As in the case of opioid-induced side effects, there is contradicting data with regards to the effects of this SNP in alcoholism [15], nicotine addiction [16], heroin misuse and relapse [17], and gambling [18]. Interestingly, pain and reward processing are not the only behaviours reported to be affected by the rs1799971 SNP. Indeed, this SNP has been assessed for the role that MORs play in the **Hypothalamic–Pituitary–Adrenal (HPA) axis** (see Glossary), including stress, separation anxiety [7], aggressive behaviours [19], and suicidal ideation [20]. Finally, other SNPs have also been associated with pathological conditions related to MOR signalling (Figure 1).

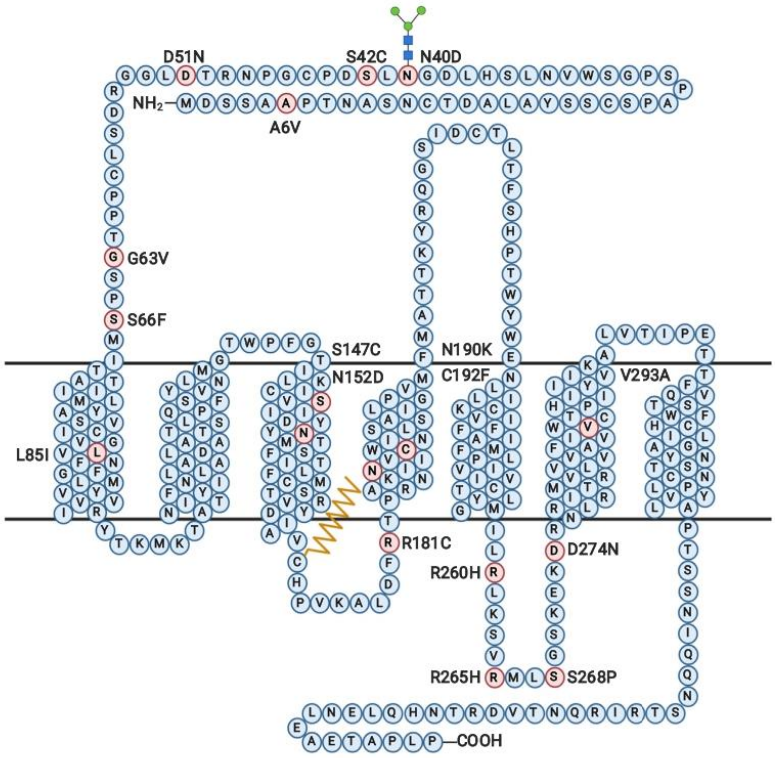
OPRM1 and *Oprm1* transcriptional regulation has been previously reviewed by Wei and Loh [21]. Under normal conditions, MORs are expressed mainly in the central nervous system, the HPA axis, and the testis. Both the human and the mouse genes are regulated by two promoters; the distal and the proximal promoters, with the latter accounting for approximatively 95% of the transcriptional activity [21]. These regions contain many GpC sites that can be epigenetically modified [22] (Figure 2A). It has been shown that when these promoters are highly methylated, the expression of the *OPRM1/Oprm1* gene is supressed [23], and in the organs where the receptor is expressed, these GpC sites are usually unmethylated or hypomethylated.

Methylation of the CpG sites of the *OPRM1* promoter has been associated with several pathological conditions. *OPRM1* promoter hypermethylation is related to the risk for alcohol dependence [24]. Interestingly, a specific CpG cluster from the *OPRM1* promoter has been shown to be altered by naltrexone treatment of alcohol dependence in an age and ethnicity-dependent way [22]. Increased DNA methylation in the *OPRM1* gene is also associated with opioid dependence. The *OPRM1* promoter is hypermethylated in blood cells of opioid addicts. This methylation pattern is conserved in the sperm, suggesting an epigenetic heritability of opioid abuse or dependence phenotypes [25]. Increased *OPRM1* promoter methylation has also been reported in blood cells of males with opioid use disorder [26], in lymphocytes of former heroin addicts treated with methadone [27], and has been associated with worse neonatal abstinence syndrome outcomes [28].

Glossary

Dimer: protein complex formed by two proteins. Homodimers contain two identical protomers while heterodimers contain two different protomers.
Histone acetylation: addition of an acetyl residue to N-terminal tail lysine's of the histone core, mediated by the histone acetyltransferase enzyme. This modification eases the DNA-histones interaction, facilitating transcription. Histone acetylation can be reversed by the action of the histone deacetylase enzyme, which impedes DNA transcription.
Histone methylation: some amino acids from the histone core N-terminal tails can be methylated by methyltransferases. Depending on the methylated residue, this epigenetic modification can be repressive or activating.
Hypothalamic–Pituitary–Adrenal (HPA) axis: this axis that comprises the hypothalamus, the pituitary gland, and the adrenal glands, is part of the neuroendocrine system and its connection with the central nervous system. It plays a major role in stress management and steroid hormones production.
Let-7: Let-7 was the first miRNA family discovered in humans, although it was first found in *C. elegans*, and is conserved across different species.
Mesocorticolimbic system (MCLS): this system is a dopamine-dependent pathway that facilitates behaviours that lead to survival. It has been associated with the behaviour modulation for reward and motivation but also aversion.
MicroRNAs (miRNAs): single strand RNA that can regulate gene expression. miRNAs need to be transcripted and processed. Drosha and Dicer are two proteins with catalytic power and carry out miRNAs processing, the former within the nucleus and the latter in the cytoplasm. In between the action of each of them, exportin 5 takes the non-mature miRNA out of the nucleus. After being processed, miRNAs are incorporated to the RNA-induced silencing complex (RISC), which in turn, by complementary binding of the 3′-UTR, sequester the mRNA, which is then degraded by exonucleases.
Re-initiation: event that happens when ribosomes resume scanning, and re-initiate at downstream sites when the first AUG is followed shortly by a frame terminator codon.

A6V	rs1799972	R181C	rs79910351
N40D	rs1799971	N190K	rs34074916
S42C	rs76546679	C192F	rs62638690
D51N	rs1042753	R260H	rs1799974
G63V	rs9282817	R265H	rs376950705
S66F	rs9282819	S268P	rs200811844
L85I	rs76773039	D274N	rs17174829
S147C	rs17174794	V293A	rs11575856
N152D	rs17174801		



Ubiquitination: this post-translational modification requires three enzymes (E). E1-activating enzyme is the first of them and activates the ubiquitin molecule at its C-terminal by using ATP. Afterwards, an E2-conjugating enzyme binds the activated ubiquitin through a cysteine residue. Finally, an E3-ubiquitin ligase ends the process by transferring the ubiquitin to the substrate.

Untranslated region (UTR): regions flanking the mRNAs coding region. Both the 3′-UTR and the 5′-UTR regions play an important role in mRNA regulation and translation.

β-Arrestin: cytosolic proteins with two crescent-shaped beta-sandwiches and a central crest that bind to phosphorylated GPCRs, to promote receptor internalisation. For some GPCRs β-arrestins have been proposed to scaffold the formation of signalling complexes.

Figure 1. Human MOR Structural SNPs and Post-translational Modifications. Glycosylation occurs on the asparagine at position 40 whereas palmitoylation occurs on the cysteine at position 172 (C3.55). Figure adapted from Knapman and Connor [6]. Abbreviation: MOR, mu-opioid receptor.

The decrease in MOR expression caused by an increased *OPRM1* promoter methylation, may account for dampened responses to endogenous and exogenous opioids. High methylation levels have been used as a biomarker to predict acute and chronic postsurgical pain [29], and *Oprm1* silencing in primary sensory neurons of the dorsal root ganglia has been observed under neuropathic pain conditions. Finally, *OPRM1* promoter methylation has been investigated in the context of Alzheimer's disease (AD) with increased methylation detected

in AD patients [30]. Intriguingly, MOR activation has recently been shown to attenuate Aβ oligomer-induced neurotoxicity, suggesting another biomarker for AD diagnosis.

The *OPRM1/Oprm1* promoter can be epigenetically regulated by other modifications apart from DNA methylation. For example, **histone acetylation** and **methylation**, have also been described to regulate *OPRM1* expression in global ischemia patients [31]. Nonetheless, epigenetics is not the only mechanism that participates in *OPRM1/Oprm1* transcription. Transcriptional factors that enhance (positive) or repress (negative) gene expression, also play important roles in MOR expression (Figure 2B). In their extensive review, Wei and Loh list all of the positive and negative transcriptional factors for mouse and human *MOR* gene [21].

While epigenetics and transcriptional factors control *OPRM1/Oprm1* transcription, once the mRNA is transcribed, it must reach its maturity by undergoing further regulatory mechanisms that will further influence the levels of the final protein product.

Mature but Not Enough

Alternative splicing is commonly seen across GPCRs. However, the extensive alternative splicing of the *OPRM1* gene (generating more than 30 splice variants) is unusual and conserved in rodents and human [4,32]. There are three main groups of *OPRM1* isoforms according to the number of TM domains of the resulting protein (Figure 2C). The first group is the one with the traditional 7-TM domains structure. Proteins in this group preserve exons 1, 2, and 3, and differ on the exon with the STOP codon (C-terminal tail of the protein). The second group predominantly swaps exon 1 for exon 11, which results in 6-TM domains. However, some studies suggest that there are other exons that might also form 6-TM variants [33]. Finally, the last group only has 1-TM domain, and the isoforms within it are non-functional. Expression of these isoforms is region specific in the brain [34], and their distribution differs between sexes [35].

The pharmacology of the MOR splice variants has been thoroughly investigated, most prominently by the groups of Pasternak and Pan [36,37]. Generally, opioids produce analgesia through 7-TM variants. However, it has been suggested that morphine uses both 7-TM and 6-TM variants, whereby 7-TM activation would trigger analgesia, reward, and respiratory depression, while chronic 6-TM activation participates in opioid-induced hyperalgesia (OIH), tolerance, and dependence. In this context, heteromerisation of 6-TM variants with the β2-adrenoreceptors (β2-ARs), has been suggested to contribute to OIH, and in rodent models, it has been proposed that β2-ARs antagonists can efficiently block the hyperalgesic effects induced by 6-TM activation [38]. Endomorphin analogues and buprenorphine [39] have also been shown to act through 7-TM and 6-TM isoforms to produce analgesia. Drugs that only activate 6-TM, such as 3-iodobenzoyl naltrexamine, have been shown to have reduced side-effects while keeping their therapeutic potential [40], suggesting that bias towards a specific MOR isoform is a promising avenue for potential therapeutic interventions, that may avoid the classical opioid adverse effects.

MicroRNAs (miRNAs) provide an additional regulatory mechanism for the *OPRM1*. Interestingly, morphine upregulates **let-7** miRNA expression in neuroblastoma-like cells, and in a mouse model of opioid tolerance. Let-7 supressed MOR translation without altering *OPRM1* transcription [41], a let-7 miRNA inhibitor partially reduced morphine-induced antinociceptive tolerance, and miRNAs members of the let-7 family were upregulated in plasma after oral hydromorphone or oxycodone administration in humans [42], supporting the relationship between let-7 miRNAs and tolerance. miR-132 and miR-212 are miRNAs expressed in tandem [43], and regulated by the cAMP-response element binding protein (CREB). In zebrafish, morphine has been shown to regulate the miR-212/132 cluster, which in turn represses *OPRM1* mRNA translation [44].

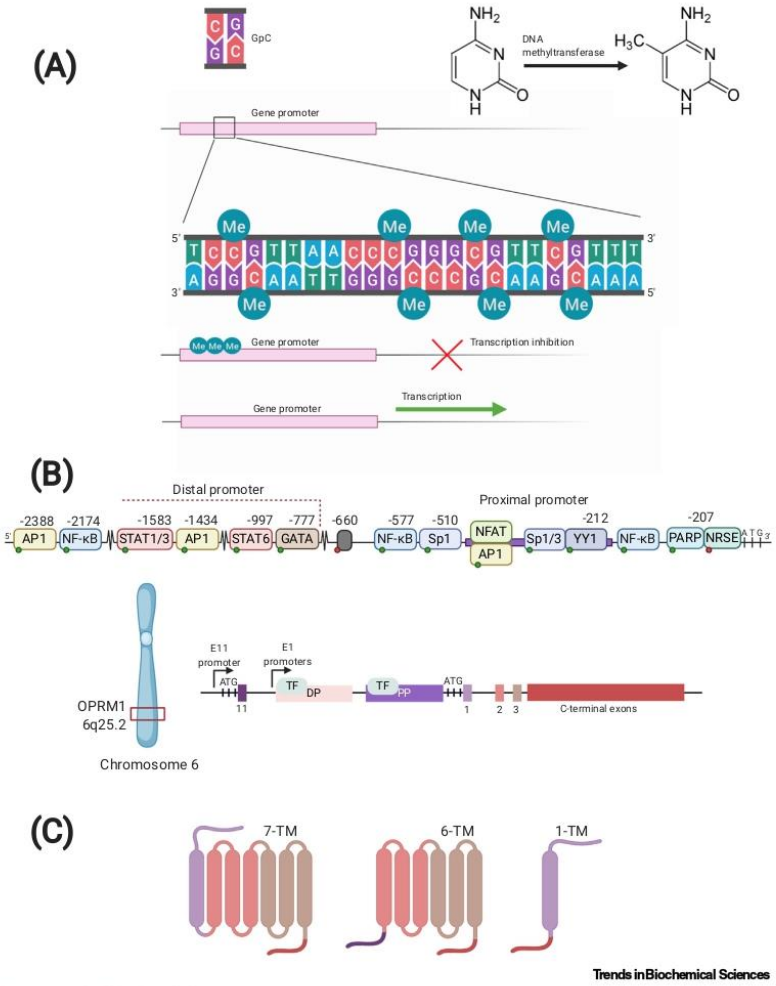


Figure 2. *OPRM1* Regulation. (A) GpC islands methylation by DNA methyltransferase. Addition of a methyl group to cysteines impedes transcriptional factors (TFs) promoter recognition and transcription. (B) Main exons of the *OPRM1* gene, with the three groups of splicing variants, and exon 1 proximal promoters (PP) and distal promoters (DP) regulation by TFs. Green spot TFs promote *OPRM1* transcription, whereas red spot TFs inhibit it. Figure adapted from Wei and Loh [21] and Puig and Gutstein [106]. (C) MOR isoforms. The colours of the fractions within each isoform match the colours of the exons in Figure 2B. Abbreviation: MOR, mu-opioid receptor.

Other miRNAs that can alter MOR mRNA stability upon opioid treatment, or in different pain conditions have also been reported, including miR-16 in lymphocytes [45], miR-134 in SH-SY5Y cells and dorsal root ganglia [46], and miR-339-3 in the hippocampus [47]. Some miRNAs have been shown to regulate the mature mRNA of a specific MOR isoform. For example, chronic morphine treatment upregulates miR-103 and miR-107 in the striatum of morphine-tolerant mice, which in turn, specifically upregulates the isoform MOR-1A [48]. Chronic morphine treatment also

increases miR-378a-3p expression in the brainstem of morphine tolerant mice, which in turn, decreases the expression of the MOR-1B3 and the MOR-1B4 isoforms [49].

After alternative splicing and miRNA regulation, the mRNA reaches its maturity. Since GPCRs are plasma membrane proteins, the *OPRM1* messenger is translated by ribosomes located on the rough endoplasmic reticulum (ER) at the surface of the plasma membrane. The *OPRM1* mRNA 5′-**untranslated region (UTR)** contains four AUG codons. The first and the third AUG codons can function efficiently to initiate translation, however, translation from the third AUG negatively affects *OPRM1* expression at the level of translation [50]. It has also been suggested that the weak expression of MOR under normal conditions is due to **re-initiation** mechanisms.

Upon translation, MOR must be correctly folded. It has been suggested that the 1-TM isoforms act as chaperones for the 7-TM isoforms, facilitating their correct folding in the ER [51]. Several reports suggest that the 6-TM variants of MOR have altered subcellular expression, and may require coexpression with other proteins (such as ORL-1 or β2-ARs) to reach the plasma membrane [33]. Moreover, hydrophobic ligands such as naloxone or etorphine, can act as pharmacological chaperones, helping the newly synthesised receptors to adopt their correct conformation and reach the plasma membrane [52] (Box 1).

MORs can undergo four different post-translational modifications (PTMs): glycosylation, palmitoylation, phosphorylation, and **ubiquitination**, which have recently been reviewed by Duarte and Devi [53]. This section provides a description of the two PTMs that occur before and facilitate the anchorage of the receptor into the plasma membrane (Figure 1). Glycosylation consists of the attachment of sugar molecules to a protein that will be located in the plasma membrane or secreted. In the ER, monosaccharides covalently attach to what will be the extracellular part of the protein and formation of more complex glycans occurs in the Golgi apparatus [54]. There are five asparagine residues located at the N-terminal of the MOR that can potentially be glycosylated (N-glycosylation). Interestingly, the MOR has been suggested to have brain-region specific glycosylation levels [55]. As mentioned above, the A118G polymorphism of the *OPRM1* gene eliminates Asn40 and, therefore, a key site of glycosylation. This has been suggested to reduce the half-life of the receptor at the membrane [9], and alter MOR binding and signalling properties [8]. The other PTM that occurs just after MORs translation is palmitoylation, namely the covalent union between a palmitate group and a cysteine through a thiol bond. It occurs in the ER and contributes to GPCR normal functioning by facilitating their incorporation to the plasma membrane. At the mMOR, Cys170 (Cys172 in hMOR) in TM3, has been reported to be a palmitoylation site, contributing to the interaction with cholesterol at the membrane, and facilitating MOR homodimerisation [56].

MOR at Work: A Freshman at the Cell Membrane

The plasma membrane is the classical site of action of GPCRs like MORs. MORs bind a great variety of ligands (Box 2) and the consequences of their activation depend on the ligand itself as well as the site of action within the cell and the organism. Once at the membrane, the minimal functional unit of the MOR has been demonstrated to be a monomer [57]. However, MORs can form **dimers** namely homodimers [58], as well as heterodimers with other OR subtypes, or with other GPCRs. There is evidence that MOR heterodimerisation with ORL-1, KORs, and DORs, can alter MOR-induced responses *in vitro* and *in vivo* [59]. Moreover, other GPCRs such as β2-ARs, and dopamine and cannabinoid receptors, have also been shown to heterodimerise with MORs [60]. Nonetheless, further research is required to support the physiological functions of MORs heterodimers, and whether they represent potential therapeutic targets.

Trends in Biochemical Sciences

CellPress

Box 1. Protein Structures of the Mu-Opioid Receptor

The first crystal structure of the MOR (Figure I) was first described in 2012 [98]. It belonged to a construct of the mouse MOR (where the third intracellular loop was replaced with a T4 lysozyme) bound to a morphinian-like antagonist. This structure showed the typical 7-TM organisation of the receptor, and has been used for significant *in silico* drug discovery efforts [99]. In 2015, the crystal structure of an agonist-bound mouse MOR and a G protein mimetic nanobody (Nb39) was solved, representing the first active state structure of the OR family [100]. This structure and related NMR studies [101] revealed how agonist and G protein binding at the MOR induce a conformational change with conserved shifts in domain positioning, characteristic of GPCR activation mechanisms. This change in receptor conformation, then facilitates the conformational changes in the Gαi G protein that permits nucleotide exchange. Finally, in 2018, Koehl *et al.* [102] presented the cryo-electron microscopy structure of a DAMGO-bound MOR in complex with the Gαiβγ heterotrimeric protein in the nucleotide free state. This was one of the first structures of a GPCR-Gαi complex. While this active structure was almost identical to the one previously described by Huang *et al.* [100] in 2015, it showed the receptor-induced changes in the Gαi protein conformation, that permit signalling via GDP/GTP exchange.

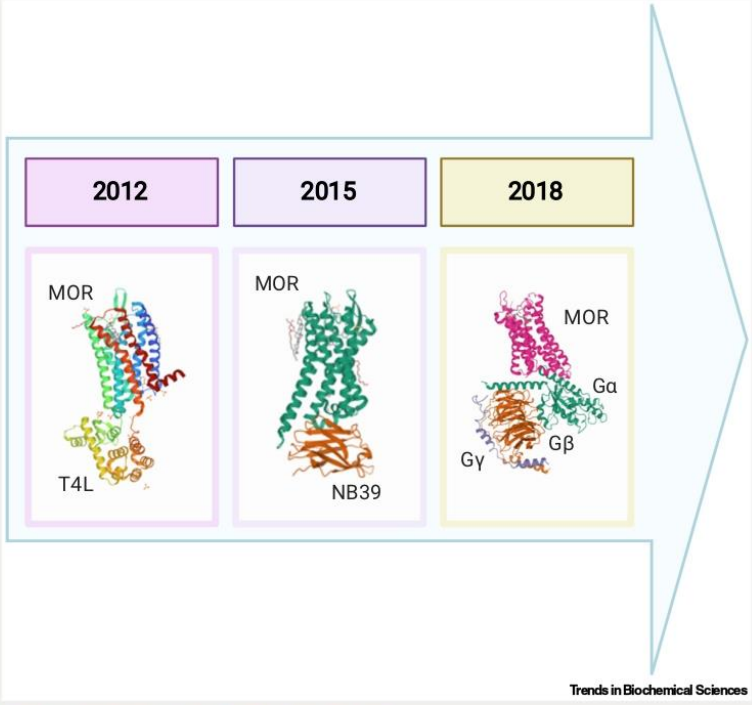


Figure I. Chronology of MOR Structure Determinations.

The classical signalling cascade triggered by the MOR is through the activation of inhibitory G proteins (Figure 3A). MOR activation results in neuron hyperpolarisation and an inhibition of neuronal firing which is crucial in the modulation of pain sensation. MORs are widely expressed at different levels in the pain pathway, from somatosensory neurons of the dorsal root ganglia [61], dorsal horn nociceptive neurons (including excitatory interneurons and neurons from lamina I from the anterolateral tract), to brain areas of the descending modulatory pathway [62]. In the dorsal horn, when MORs are activated, there is an inhibition of neuropeptide release (Substance P and CGRP), which promotes analgesia. However, the major sites for the analgesic properties

of MOR agonists are the periaqueductal grey and the rostral ventromedial medulla. These two areas of the descending modulatory pain pathway are characterised by two different populations of neurons that enhance (on-cells) or attenuate (off-cells) pain sensation by modulating dorsal horn nociceptive neurons activity. Because of the different locations of the MOR, in the on-cells or in inhibitory neurons controlling off-neurons activity, in both cases, the activation of MOR leads to analgesia [63].

MOR regulatory function of the **mesocorticolimbic system** (MCLS) is prominent in motivation, reward, and aversion. This system is composed of many interconnected brain regions, including canonical dopaminergic pathways. MOR inhibition of GABAergic neurons from the ventral tegmental area (VTA), is key for the role of this receptor in processing the reward, not only from stimuli of natural reinforcers such as food, drink, social interaction, and sex, but also from opioid and alcohol addiction [64]. Those neurons inhibit VTA dopaminergic neurons that fire to nucleus accumbens (NAc). Therefore, when MORs are activated in these neurons, they cannot fire and dopamine is released in NAc, an event that is essential to process reinforcement [65–67]. Furthermore, MORs can also be found in NAc, and its specific location in this area encodes for reward or aversion [68]. In addition, the activation of MORs located in NAc increases social interaction and might also be crucial to understanding psychiatric disorders that involve social impairment [69]. MOR is also expressed in the prefrontal cortex (PFC). However, while it is still not known exactly where MORs are located within the complex neural network in this brain area, research suggests that their activation leads to the excitation of glutamatergic pyramidal neurons through a disinhibition mechanism. It is also interesting to notice that MOR upregulation and signalling in the PFC might increase the responsiveness towards opiate-like drugs and natural rewards. In fact, MOR activation in PFC may also account for food-seeking behaviour and increased alcohol drinking in rats. Moreover, MORs in PFC also seem modulate inhibitory

Box 2. Endogenous and Exogenous Ligands of the Mu-Opioid Receptor

Endogenous opioid peptides derive from the proteolytic cleavage of larger prepropeptides, and they all can bind the mu-opioid receptor (MOR) albeit with different affinities (Figure I). β-endorphins have high affinity for MOR and are potent analgesics with long lasting effects. Furthermore, β-endorphins also participate in reward processing and drug addiction. Enkephalins (Met-enkephalin and Leu-enkephalin) display higher affinity for DORs, although they also bind and activate MORs. Similarly, dynorphins, prototypical KOR ligands, can also bind MOR with low affinity. Finally, endomorphins are potent and selective MORs agonists, even though the gene encoding for these tetrapeptides or their protein precursors have not been identified.

MORs are also activated by multiple exogenous ligands. These include not only opioids but also metabolites of other substances such as alcohol. Morphine is one of the most used analgesic drugs in clinic, and its actions have been widely proven to be mediated by the MOR [103]. Morphine derivatives, such as codeine and oxycodone, are also used for the treatment of mild pain and cough or severe pain, respectively, although they still display common opioid side effects. Another family of drugs that can activate MORs are piperidine derivatives, among which the most common is fentanyl. Fentanyl and its derivatives are over a hundred times more potent than morphine and thus, they are used to ease moderate to severe pain in clinic. However, they still present opioid-induced side effects and their recreational use mixed with other drugs of abuse has recently skyrocketed. Finally, methadone (R enantiomer) and buprenorphine are MOR agonists with different efficacies that are widely used as maintenance treatments for opioid addiction.

Naloxone is the prototypical OR antagonist. It is one of the most widely used opioids, the primary treatment for overdose, and its use has been instrumental in understanding MOR actions in preclinical studies. Other antagonists such as β-funaltrexamine are highly selective for MOR, however, their pharmacokinetic properties (e.g., irreversible antagonist) represent significant caveats for their clinical use, and it has mainly been used to study OR pharmacology.

Drugs that undergo metabolic reactions within the organism can generate molecules that bind and activate MORs. For example, (R/S)-salsolinol is a tetrahydroisoquinoline that has been proposed as the ethanol fraction that binds MORs [104]. Studies suggest that (R/S)-salsolinol binding pose at the MORs is like that of morphine [105]. *In vivo*, salsolinol triggers a response in the MCLS, like the one triggered by opioids, further supporting the action of this molecule as an MOR ligand.

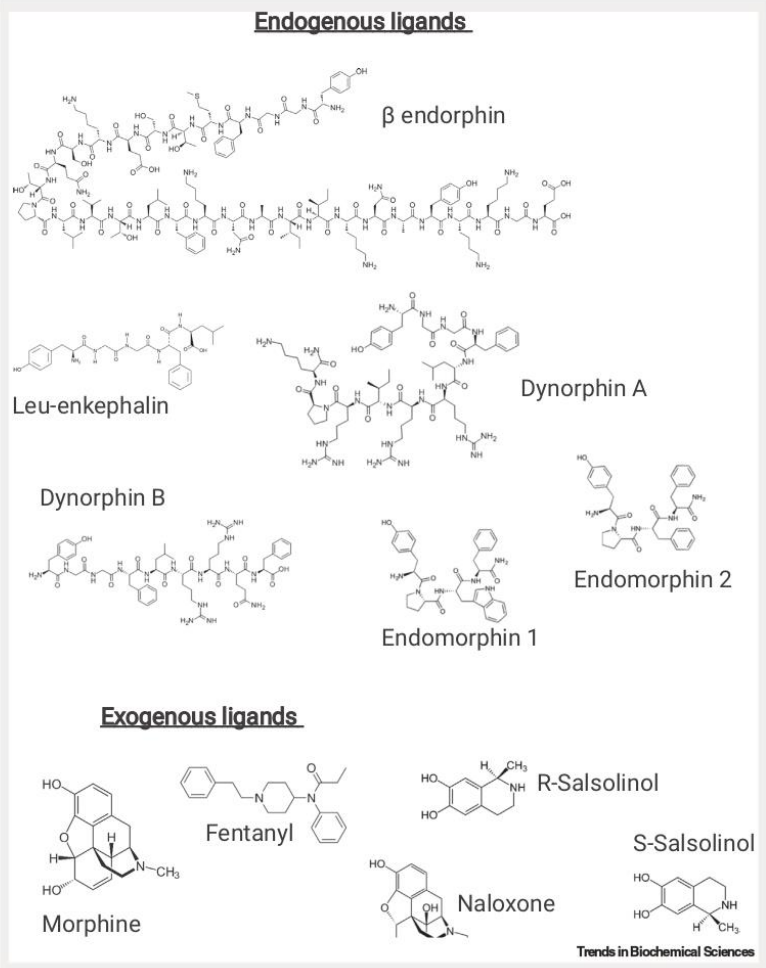


Figure I.

control and impulsivity, since MOR knockout mice, and rats treated with a MOR antagonist, show decreased impulsivity while carrying out various tasks [70].

There is accumulating evidence that relates painful conditions to drug abuse and misuse. Interestingly, MORs play a role in this comorbidity since, even though pain and reward are opposed processes [71], they both need MORs [3,61]. In fact, pain negatively impacts on motivation by altering the MCLS normal functioning [72–74]. Inflammatory pain blunts neuronal activation induced by intra-VTA DAMGO administration in some VTA projecting areas. Therefore, inflammatory pain induces MOR desensitisation in VTA, which is relevant for addictive behaviours

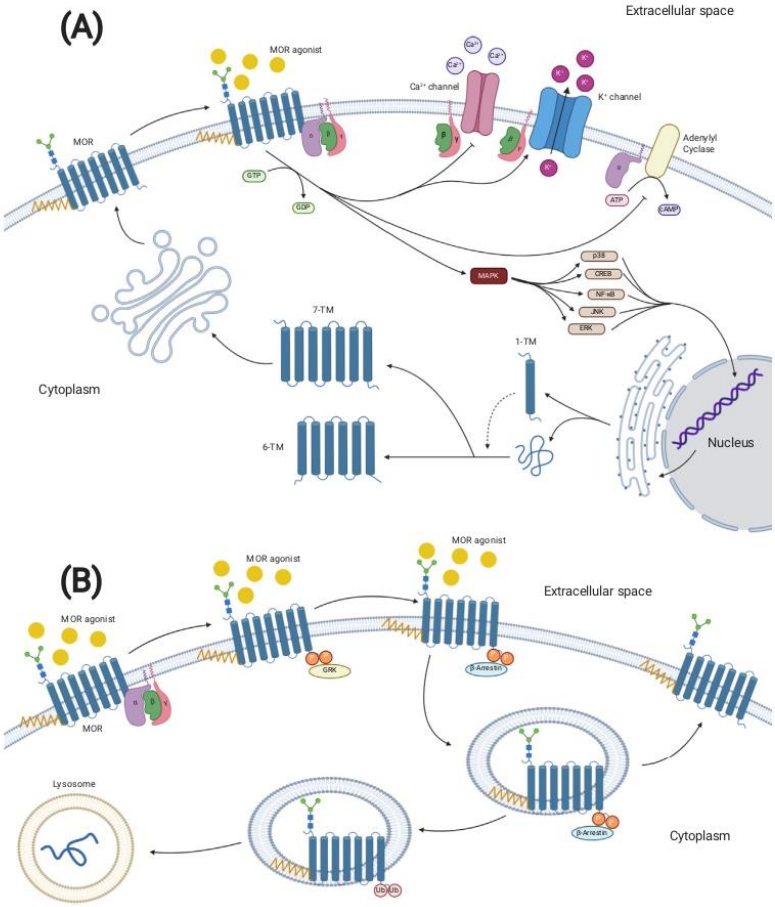


Figure 3. MOR Life Cycle. (A) From DNA to its activation. MOR mRNA is transcribed and translocated to the rough endoplasmic reticulum in the ribosomes where translation occurs. 1-TM splicing variant chaperons the 6-TM and 7-TM splicing variants. The latter undergoes post-translational modifications in the Golgi apparatus, and gets into the cell membrane. Upon the binding of an agonist, activation of MOR promotes dissociation of inhibitory Gαi and Gβγ protein subunits. Gαi subunits typically suppress adenylate cyclase (AC), resulting in decreases in intracellular cAMP. Presynaptically, Gβγ subunits inhibit voltage-gated calcium channel (VGCC) opening. Postsynaptically, Gβγ subunits activate G-protein inwardly rectifying potassium (GIRK) channels. Altogether, this results in reduced neurotransmitter release and membrane hyperpolarisation. MORs activation also triggers kinase cascades that end with the translocation to the nucleus of some transcriptional factors. (B) MOR internalisation, recycling, and degradation. After MORs activation triggers the G protein-induced cascade, GRK recruitment and MOR phosphorylation occur. These events are followed by β-arrestin recruitment and receptor internalisation. Upon ligand and arrestin dissociation, MORs are either recycled to the cell membrane or ubiquitinated and degraded in lysosomal vesicles. Abbreviation: MOR, mu-opioid receptor.

[73]. Interestingly, Hipólito and collaborators discovered that MORs from the MCLS were desensitised in animals that suffered inflammatory pain and had a previous history of heroin consumption. This alteration promoted relapse into heroin consumption [72].

Finally, prolonged use of opioids can also lead to OIH, a significant clinical problem that defines a state of nociceptive sensitisation, and is characterised by a paradoxical response, whereby a patient receiving opioids to treat pain becomes more sensitive to certain painful stimuli. The precise molecular mechanisms underlying OIH are not yet understood, and are the subject of intense study [75].

The Decline After a Life of Service

Following activation, MORs undergo rapid phosphorylation, which triggers a decline in their G protein signalling as well as the recruitment of proteins that will result in receptor internalisation (Figure 3B). Phosphorylation of the MOR and its impact on receptor desensitisation and internalisation has been widely studied [76,77]. While this phosphorylation is mostly mediated by G protein receptor kinases (GRKs) [78], there is also evidence that other intracellular kinases such as Ca^{2+}/calmodulin-dependent protein kinase II, proto-oncogene tyrosine-protein kinase, and protein kinase C can phosphorylate the receptor [53]. Importantly, the phosphorylation barcode, as well as its signalling consequences, are highly dependent on the ligand bound to the MOR. Sequential and hierarchical phosphorylation of MORs results in the recruitment of the cytosolic protein **β-arrestin**. MOR phosphorylation and β-arrestin recruitment result in MORs desensitisation, namely the uncoupling of the G protein signalling cascades, which has been proposed to be the initial step leading to opioid tolerance [79].

MOR can recruit β-arrestin1 and 2 isoforms, although this seems to be agonist dependent. While β-arrestins are essential to initiate MORs endocytosis via clathrin-coated pits, not all ligands that recruit arrestins induce robust receptor internalisation. This differential ability of agonists to induce receptor internalisation, has been linked to the phosphorylation barcodes mentioned earlier [76,77]. As with other GPCRs, β-arrestins have also been suggested to participate in MOR signalling [80], although the physiological relevance from this signalling is still not fully understood [81]. Moreover, β-arrestin1 can promote MOR ubiquitination by acting as E3-ubiquitin ligase adaptors [53]. This ubiquitination is also ligand dependent (occurs with high efficacy ligands such as DAMGO, but not with morphine) and results in degradation of the MOR within the lysosomes. However, MOR is not always degraded after endocytosis. Instead, and as opposed to the DOR, internalised MORs are usually recycled back to the plasma membrane, in a process known as resensitisation [82]. Each MOR isoform has been suggested to have different recycling rates and the mechanisms underlying receptor recycling are starting to be elucidated [83]. More recently, elegant microscopy studies have provided unprecedented insight into the mechanisms of MOR internalisation and recycling in presynaptic terminals, and how it differs from the classical internalisation and resensitisation paradigms that operate post-synaptically [84]. It is clear that MOR internalisation, recycling, and degradation are processes related to the development of tolerance. However, the molecular and cellular mechanisms, as well as the neural circuits changes which lead to reduced opioid responsiveness, are just starting to be unravelled [85].

Interestingly, the regulatory mechanisms of MOR have been the focus of intense research in an attempt to generate opioid analgesics devoid of, or with limited, side effects. In addition to tolerance, opioid treatment also results in significant respiratory depression, constipation, dependence, and addiction. Thus, efforts to separate the therapeutic and adverse effects of MOR agonists have dominated drug discovery at this receptor. These efforts were spearheaded by the observation of enhanced and prolonged morphine-induced analgesia and decreased tolerance in β-arrestin2 knockout mice, presumably due to decreased receptor desensitisation [86,87]. These β-arrestin2 knockout mice were later shown to display decreased morphine-induced respiratory depression and constipation [88]. This finding then led to investigations

focused on the discovery of MOR agonists that would avoid the 'β-arrestin pathway' while still promoting G protein signalling; namely G protein-biased agonists [89].

There are now numerous descriptions of MOR G protein-biased agonists. While some of these descriptions are limited to observations in recombinant cell lines, others have been tested *in vivo*, showing promising results. TRV130 (oliceridine, Olinvyk) has been the only new opioid agonist that has reached clinical trials and FDA applications [90], and was recently approved for the management of moderate to severe acute pain. SR17018 and mitragynine pseudoindoxyl are other compounds that have shown decreased opioid-induced side effects in pre-clinical models [91,92]. However, there is now mounting evidence that suggests that while these compounds may still provide improved therapeutic profiles, the mechanism underlying these profiles is unlikely to be linked to an arrestin-dependent signal. Perhaps the most significant evidence is that derived from studies revisiting the initial hypothesis in β-arrestin2 knock-out mice which shows that morphine-induced respiratory depression is independent of β-arrestin2 signalling [93]. Additionally, in knock-in mice expressing a MOR unable to recruit β-arrestin (by deletion of the phosphorylation sites that facilitate this recruitment), the opioid side effects of respiratory depression, constipation, and withdrawal are exacerbated while only tolerance seems to be diminished [94]. In light of these data, the classical pharmacological concepts of partial agonism and low intrinsic efficacy have been proposed as potential explanations for the different therapeutic windows of the so-called G protein biased opioids [81,95,96]. Finally, it is important to highlight that while this research has focused on the development of tolerance, respiratory depression, and constipation, most of the novel opioids described as biased still result in significant abuse liability [97].

Concluding Remarks

From birth as an mRNA to death as a heavily modified protein, MORs are essential for many vital activities. However, these events can be thwarted by genetic variations, alterations in expression patterns, and changes in MOR signalling and regulation. While research has provided invaluable information that sheds light on MORs function, in many aspects we are still partially blind and further research is needed to untangle the ins and outs of the MOR life cycle and how it changes in diseased conditions. MOR hetero- and homodimerisation, pain comorbidities, biased/partial agonism, MOR isoforms and their role in neuroinflammation, chronic pain, and other pathologies represent some of the areas in which the field is moving at a vertiginous pace (see Outstanding Questions).

Outstanding Questions

Can epigenetics, mRNA processing, or PTMs of the MOR gene or protein, be targeted therapeutically for the generation of novel, safer analgesics?

Can epigenetic modifications in the *OPRM1*, triggered by painful conditions or a drug use disorder, be reversed?

Can the methylation state of the *OPRM1* promoter be used as a biomarker for the assessment of risk associated with substance use disorders?

What are the cellular mechanisms that explain the improved side effect profiles of novel opioids?

Do miRNAs represent a viable strategy for the treatment of opioid tolerance?

What are the cellular and neuronal mechanisms for controlling alterations of MORs in the MCLS system alongside pain that have consequences for addiction and reward processes? Are they ligand-dependent?

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References

1. Snyder, S.H. and Pasternak, G.W. (2003) Historical review: opioid receptors. *Trends Pharmacol. Sci.* 24, 198–205
2. Jullié, D. *et al.* (2020) Opioid pharmacology under the microscope. *Mol. Pharmacol.* 98, 425–432
3. Darcq, E. and Kieffer, B.L. (2018) Opioid receptors: drivers to addiction? *Nat. Rev. Neurosci.* 19, 499–514
4. Piltonen, M. *et al.* (2018) Alternative splicing of the delta-opioid receptor gene suggests existence of new functional isoforms. *Mol. Neurobiol.* 56, 2855–2869
5. Pándy-Szekeres, G. *et al.* (2018) GPCRdb in 2018: adding GPCR structure models and ligands. *Nucleic Acids Res.* 46, D440–D446
6. Knapman, A. and Connor, M. (2015) Cellular signalling of non-synonymous single-nucleotide polymorphisms of the human mu-opioid receptor (*OPRM1*). *Br. J. Pharmacol.* 172, 349–363
7. Boparai, S. *et al.* (2018) Interaction between the opioid receptor *OPRM1* gene and mother-child language style matching prospectively predicts children's separation anxiety disorder symptoms. *Res. Dev. Disabil.* 82, 120–131
8. Mura, E. *et al.* (2013) Consequences of the 118A>G polymorphism in the OPRM1 gene: translation from bench to bedside? *J. Pain Res.* 6, 331–353
9. Huang, P. *et al.* (2012) A common single nucleotide polymorphism A118G of the mu opioid receptor alters its N-glycosylation and protein stability. *Biochem. J.* 441, 379–386
10. Oertel, B.G. *et al.* (2012) Genetic–epigenetic interaction modulates mu-opioid receptor regulation. *Hum. Mol. Genet.* 21, 4751–4760
11. Bartošová, O. *et al.* (2019) Epidural analgesia with sufentanil in relation to *OPRM1* and ABCB1 polymorphisms. *Physiol. Res.* 68, S59–S64

12. Matic, M. *et al.* (2020) OPRM1 and COMT polymorphisms: implications on postoperative acute, chronic and experimental pain after cardiac surgery. *Pharmacogenomics* 21, 181–193
13. Sia, A.T. *et al.* (2013) Influence of mu-opioid receptor variant on morphine use and self-rated pain following abdominal hysterectomy. *J. Pain* 14, 1045–1052
14. Kong, Y. *et al.* (2018) Opioid receptor mu 1 (*OPRM1*) A118G polymorphism (rs1799971) and postoperative nausea and vomiting. *Am. J. Transl. Res.* 10, 2764–2780
15. Samochowiec, A. *et al.* (2019) The role of *OPRM1* polymorphism in the etiology of alcoholism. *Adv. Clin. Exp. Med. Off. Organ Wroclaw Med. Univ.* 28, 199–202
16. Kong, X. *et al.* (2017) Association of opioid receptor mu 1 (*OPRM1*) A118G polymorphism (rs1799971) with nicotine dependence. *Oncotarget* 8, 84329–84337
17. Levran, O. *et al.* (2017) The mu-opioid receptor nonsynonymous variant 118A>G is associated with prolonged abstinence from heroin without agonist treatment. *Pharmacogenomics* 18, 1387–1391
18. Kim, K.M. *et al.* (2019) Associations among the opioid receptor gene (*OPRM1*) A118G polymorphism, psychiatric symptoms, and quantitative EEG in Korean males with gambling disorder: a pilot study. *J. Behav. Addict.* 8, 463–470
19. Tchalova, K. *et al.* (2019) Variation in the mu-opioid receptor gene (*OPRM1*) and experiences of felt security in response to a romantic partner's quarrelsome behavior. *Mol. Psychiatry* 1–11
20. Nobile, B. *et al.* (2019) Polymorphism A118G of opioid receptor mu 1 (*OPRM1*) is associated with emergence of suicidal ideation at antidepressant onset in a large naturalistic cohort of depressed outpatients. *Sci. Rep.* 9, 2569
21. Wei, L. and Loh, H.H. (2011) Transcriptional and epigenetic regulation of opioid receptor genes: present and future. *Annu. Rev. Pharmacol. Toxicol.* 51, 75–97
22. Lin, Y. *et al.* (2020) An analysis of the effect of mu-opioid receptor gene (*OPRM1*) promoter region DNA methylation on the response of naltrexone treatment of alcohol dependence. *Pharmacogenomics J.* 20, 672–680
23. Knothe, C. *et al.* (2016) Pharmacoepigenetics of the role of DNA methylation in mu-opioid receptor expression in different human brain regions. *Epigenomics* 8, 1583–1599
24. Zhang, H. *et al.* (2012) Hypermethylation of *OPRM1* promoter region in European Americans with alcohol dependence. *J. Hum. Genet.* 57, 670–675
25. Chorbov, V.M. *et al.* (2011) Elevated levels of DNA methylation at the *OPRM1* promoter in blood and sperm from male opioid addicts. *J. Opioid Manag.* 7, 258–264
26. Ebrahimi, G. *et al.* (2018) Elevated levels of DNA methylation at the OPRM1 promoter region in men with opioid use disorder. *Am. J. Drug Alcohol Abuse* 44, 193–199
27. Nielsen, D.A. *et al.* (2009) Increased *OPRM1* DNA methylation in lymphocytes of methadone-maintained former heroin addicts. *Neuropsychopharmacol.* 34, 867–873
28. Wachman, E.M. *et al.* (2014) Epigenetic variation in the mu-opioid receptor gene in infants with neonatal abstinence syndrome. *J. Pediatr.* 165, 472–478
29. Chidambaran, V. *et al.* (2017) DNA methylation at the mu-1 opioid receptor gene (*OPRM1*) promoter predicts preoperative, acute, and chronic postsurgical pain after spine fusion. *Pharmacogenomics Personalized Med.* 10, 157–168
30. Xu, C. *et al.* (2018) Elevated methylation of *OPRM1* and *OPRL1* genes in Alzheimer's disease. *Mol. Med. Rep.* 18, 4297–4302
31. Formisano, L. *et al.* (2007) Ischemic insults promote epigenetic reprogramming of mu opioid receptor expression in hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A.* 104, 4170–4175
32. Pasternak, G.W. and Pan, Y. (2013) Mu opioids and their receptors: evolution of a concept. *Pharmacol. Rev.* 65, 1257–1317
33. Convertino, M. *et al.* (2015) mu-Opioid receptor 6-transmembrane isoform: a potential therapeutic target for new effective opioids. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 62, 61–67
34. Pan, Y. *et al.* (2001) Generation of the mu Opioid Receptor (MOR-1) protein by three new splice variants of the Oprm gene. *Proc. Natl. Acad. Sci. U. S. A.* 98, 14084–14089
35. Liu, A. *et al.* (2018) Sex associated differential expressions of the alternatively spliced variants mRNA of OPRM1 in brain regions of C57BL/6 Mouse. *Cell. Physiol. Biochem.* 50, 1441–1459
36. Pasternak, G.W. (2018) Mu opioid pharmacology: 40 years to the promised land. *Adv. Pharm. (San Diego, Calif.)* 82, 261–291
37. Pasternak, G.W. *et al.* (2020) Emerging insights into mu opioid pharmacology. *Handb. Exp. Pharmacol.* 258, 89–125
38. Samoshkin, A. *et al.* (2015) Structural and functional interactions between six-transmembrane mu-opioid receptors and β2-adrenoreceptors modulate opioid signaling. *Sci. Rep.* 5, 18198
39. Grinnell, S.G. *et al.* (2016) Mediation of buprenorphine analgesia by a combination of traditional and truncated mu opioid receptor splice variants. *Synapse* 70, 395–407
40. Lu, Z. *et al.* (2018) Truncated mu opioid receptors with six transmembrane domains are essential for opioid analgesia. *Anesth. Analg.* 126, 1050–1057
41. He *et al.* (2010) Regulation of opioid tolerance by let-7 family microRNA targeting the μ opioid receptor. *J. Neurosci.* 30, 10251–10258
42. Toyama, K. *et al.* (2017) Identification of circulating miRNAs differentially regulated by opioid treatment. *Int. J. Mol. Sci.* 18, 1991
43. Remenyi, J. *et al.* (2010) Regulation of the miR-212/132 locus by MSK1 and CREB in response to neurotrophins. *Biochem. J.* 428, 281–291
44. Garcia-Concejo, A. *et al.* (2018) Opioid and Notch signaling pathways are reciprocally regulated through miR- 29a and miR-212 expression. *BBA Gen. Sub.* 1862, 2605–2612
45. Hou, W. *et al.* (2016) Simian immunodeficiency virus impacts MicroRNA-16 mediated post-transcriptional regulation of mu opioid receptor in CEM x174 Cells. *J. Cell. Biochem.* 117, 84–93
46. Ni, J. *et al.* (2013) Regulation of mu-opioid type 1 receptors by microRNA134 in dorsal root ganglion neurons following peripheral inflammation. *Eur. J. Pain* 17, 313–323
47. Wu, Q. *et al.* (2013) MicroRNA 339 down-regulates mu-opioid receptor at the post-transcriptional level in response to opioid treatment. *FASEB J.* 27, 522–535
48. Lu, Z. *et al.* (2014) Morphine regulates expression of μ-opioid receptor MOR-1A, an intron-retention carboxyl terminal splice variant of the mu-opioid receptor (*OPRM1*) gene via miR-103/miR-107. *Mol. Pharmacol.* 85, 368–380
49. Lu, Z. *et al.* (2020) Morphine modulates the expression of mu-opioid receptor exon 5-associated full-length C-terminal splice variants by upregulating miR-378a-3p. *FASEB J.* 34, 4540–4556
50. Song, K.Y. *et al.* (2010) uAUG-mediated translational initiations are responsible for human mu opioid receptor gene expression. *J. Cell. Mol. Med.* 14, 1113–1124
51. Xu, J. *et al.* (2014) Isolating and characterizing three alternatively spliced mu opioid receptor variants: mMOR-1A, mMOR-1O, and mMOR-1P. *Synapse* 68, 144–152
52. Chaipatikul, V. *et al.* (2003) Rescuing the traffic-deficient mutants of rat mu-opioid receptors with hydrophobic ligands. *Mol. Pharmacol.* 64, 32–41
53. Duarte, M.L. and Devi, L.A. (2020) Post-translational modifications of opioid receptors. *Trends Neurosci.* 43, 417–432
54. Wei, L. (2004) Post-transcriptional regulation of opioid receptors in the nervous system. *Front. Biosci.* 9, 1665–1679
55. Huang, P. *et al.* (2008) Brain region-specific N-glycosylation and lipid rafts association of the rat mu opioid receptor. *Biochem. Biophys. Res. Commun.* 365, 82–88
56. Zheng, H. *et al.* (2012) Palmitoylation and membrane cholesterol stabilize mu-opioid receptor homodimerization and G protein coupling. *BMC Cell Biol.* 13, 6
57. Kuszak, A.J. *et al.* (2009) Purification and functional reconstitution of monomeric mu-opioid receptors. *J. Biol. Chem.* 284, 26732–26741
58. Möller, J. *et al.* (2020) Single-molecule analysis reveals agonist-specific dimer formation of mu-opioid receptors. *Nat. Chem. Biol.* 16, 946–954
59. Gomes, I. *et al.* (2016) G Protein-Coupled Receptor heteromers. *Annu. Rev. Pharmacol. Toxicol.* 56, 403–425

60. Ugur, M. *et al.* (2018) Heteromerization modulates mu opioid receptor functional properties in vivo. *Front. Pharmacol.* 9, 1240
61. Wang, D. *et al.* (2018) Functional divergence of delta and mu opioid receptor organization in CNS pain circuits. *Neuron* 98, 90–108 e5
62. Aicher, S.A. *et al.* (2000) mu-Opioid receptors often colocalize with the substance p receptor (NK1) in the trigeminal dorsal horn. *J. Neurosci.* 20, 4345–4354
63. Fields, H. (2004) State-dependent opioid control of pain. *Nat. Rev. Neurosci.* 5, 565–575
64. Le Merrer, J. *et al.* (2009) Reward processing by the opioid system in the brain. *Physiol. Rev.* 89, 1379–1412
65. Kim, K.M. *et al.* (2012) Optogenetic mimicry of the transient activation of dopamine neurons by natural reward is sufficient for operant reinforcement. *PLoS One* 7, e33612
66. Kawahara, Y. *et al.* (2013) Food reward-sensitive interaction of ghrelin and opioid receptor pathways in mesolimbic dopamine system. *Neuropharmacology* 67, 395–402
67. Ikemoto, S. and Bonci, A. (2014) Neurocircuitry of drug reward. *Neuropharmacology* 76, 329–341
68. Castro, D.C. and Berridge, K.C. (2014) Advances in the neurobiological bases for food 'liking' versus 'wanting'. *Physiol. Behav.* 136, 22–30
69. Trezza, V. *et al.* (2011) Nucleus accumbens mu-opioid receptors mediate social reward. *J. Neurosci.* 31, 6362–6370
70. Baldo, B.A. (2016) Prefrontal cortical opioids and dysregulated motivation: a network hypothesis. *Trends Neurosci.* 39, 366–377
71. Apkarian, A.V. *et al.* (2013) Neural mechanisms of pain and alcohol dependence. *Pharmacol. Biochem. Behav.* 112, 34–41
72. Hipólito, L. *et al.* (2015) Inflammatory pain promotes increased opioid self-administration: role of dysregulated ventral tegmental area μ opioid receptors. *J. Neurosci.* 35, 12217–12231
73. Campos-Jurado, Y. *et al.* (2019) Activation of MORs in the VTA induces changes on cFos expression in different projecting regions: effect of inflammatory pain. *Neurochem. Int.* 131, 104521
74. Campos-Jurado, Y. *et al.* (2020) Impaired alcohol-induced dopamine release in the nucleus accumbens in an inflammatory pain model: behavioural implications in male rats. *Pain* 161, 2203–2211
75. Roeckel, L. *et al.* (2017) Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide. *Sci. Rep.* 7, 10406–10415
76. Yousuf, A. *et al.* (2015) Role of phosphorylation sites in desensitization of mu-opioid receptor. *Mol. Pharmacol.* 88, 825–835
77. Miess, E. *et al.* (2018) Multisite phosphorylation is required for sustained interaction with GRKs and arrestins during rapid mu-opioid receptor desensitization. *Sci. Signal.* 11, eaas9609
78. Just, S. *et al.* (2013) Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. *Mol. Pharmacol.* 83, 633–639
79. Williams, J.T. *et al.* (2013) Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol. Rev.* 65, 223–254
80. Halls, M.L. *et al.* (2016) Plasma membrane localization of the mu-opioid receptor controls spatiotemporal signaling. *Sci. Signal.* 9, ra16
81. Gillis, A. *et al.* (2020) Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. *Sci. Signal.* 13, eaaz3140
82. Tanowitz, M. and Zastrow, M.V. (2003) A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J. Biol. Chem.* 278, 45978–45986
83. Kunselman, J.M. *et al.* (2019) Homologous regulation of mu opioid receptor recycling by G βγ , Protein Kinase C, and receptor phosphorylation. *Mol. Pharmacol.* 96, 702–710
84. Jullié, D. *et al.* (2020) A discrete presynaptic vesicle cycle for neuromodulator receptors. *Neuron (Cambridge, Mass.)* 105, 663–677 e8
85. Arttamangkul, S. *et al.* (2019) Separation of acute desensitization and long-term tolerance of mu-opioid receptors is determined by the degree of C-Terminal phosphorylation. *Mol. Pharmacol.* 96, 505–514
86. Bohn, L.M. *et al.* (2000) mu-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408, 720–723
87. Bohn, L.M. *et al.* (2002) Differential mechanisms of morphine antinociceptive tolerance revealed in beta arrestin-2 knockout mice. *J. Neurosci.* 22, 10494–10500
88. Raehal, K.M. *et al.* (2005) Morphine side effects in beta-arrestin 2 knockout mice. *J. Pharmacol. Exp. Ther.* 314, 1195–1201
89. Siuda, E.R. *et al.* (2016) Biased mu-opioid receptor ligands: a promising new generation of pain therapeutics. *Curr. Opin. Pharmacol.* 32, 77–84
90. Viscusi, E.R. *et al.* (2019) APOLLO-1: a randomized placebo and active-controlled phase III study investigating oliceridine (TRV130), a G protein-biased ligand at the mu-opioid receptor, for management of moderate-to-severe acute pain following bunionectomy. *J. Pain Res.* 12, 927–943
91. Váradi, A. *et al.* (2016) Mitragynine/corynantheidine pseudoindoxyls as opioid analgesics with mu agonism and delta antagonism, which do not recruit β-arrestin-2. *J. Med. Chem.* 59, 8381–8397
92. Schmid, C.L. *et al.* (2017) Bias factor and therapeutic window correlate to predict safer opioid analgesics. *Cell* 171, 1165–1175 e13
93. Kliewer, A. *et al.* (2020) Morphine-induced respiratory depression is independent of β-arrestin2 signalling. *Br. J. Pharmacol.* 177, 2923–2931
94. Kliewer, A. *et al.* (2019) Phosphorylation-deficient G-protein-biased mu-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat. Commun.* 10, 367
95. Yudin, Y. and Rohacs, T. (2019) The G-protein-biased agents PZM21 and TRV130 are partial agonists of mu-opioid receptor-mediated signalling to ion channels. *Br. J. Pharmacol.* 176, 3110–3125
96. Conibear, A.E. and Kelly, E. (2019) A biased view of mu-opioid receptors? *Mol. Pharmacol.* 96, 542–549
97. Altarifi, A.A. *et al.* (2017) Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. *J. Psychopharmacol.* 31, 730–739
98. Manglik, A. *et al.* (2012) Crystal structure of the mu-opioid receptor bound to a morphinan antagonist. *Nature* 485, 321–326
99. Manglik, A. *et al.* (2016) Structure-based discovery of opioid analgesics with reduced side effects. *Nature* 537, 185–190
100. Huang, W. *et al.* (2015) Structural insights into mu-opioid receptor activation. *Nature* 524, 315–321
101. Sounier, R. *et al.* (2015) Propagation of conformational changes during mu-opioid receptor activation. *Nature (London)* 524, 375–378
102. Koehl, A. *et al.* (2018) Structure of the mu-opioid receptor–G, protein complex. *Nature* 558, 547–552
103. Matthes, H.W. *et al.* (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* 383, 819–823
104. Hipólito, L. *et al.* (2011) Induction of conditioned place preference and dopamine release by salsolinol in posterior VTA of rats: involvement of mu-opioid receptors. *Neurochem. Int.* 59, 559–562
105. Berríos-Cárcamo, P. *et al.* (2019) Molecular modeling of salsolinol, a full G, protein agonist of the mu-opioid receptor, within the receptor binding site. *Chem. Biol. Drug Design* 94, 1467
106. Puig, S. and Gutstein, H.B. (2017) A "tail" of opioid receptor variants. *J. Clin. Invest.* 127, 1221–1224

CHAPTER 3

State of the Art: Mu-Opioid Receptors and Neuroinflammation

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Review article

Crosstalk between Mu-Opioid receptors and neuroinflammation: Consequences for drug addiction and pain

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ABSTRACT

Mu-Opioid Receptors (MORs) are well-known for participating in analgesia, sedation, drug addiction, and other physiological functions. Although MORs have been related to neuroinflammation their biological mechanism remains unclear. It is suggested that MORs work alongside Toll-Like Receptors to enhance the release of pro-inflammatory mediators and cytokines during pathological conditions. Some cytokines, including TNF-α, IL-1β and IL-6, have been postulated to regulate MORs levels by both avoiding MOR recycling and enhancing its production. In addition, Neurokinin-1 Receptor, also affected during neuroinflammation, could be regulating MOR trafficking. Therefore, inflammation in the central nervous system seems to be associated with altered/increased MORs expression, which might regulate harmful processes, such as drug addiction and pain. Here, we provide a critical evaluation on MORs' role during neuroinflammation and its implication for these conditions. Understanding MORs' functioning, their regulation and implications on drug addiction and pain may help elucidate their potential therapeutic use against these pathological conditions and associated disorders.

1. Mu-Opioid receptors

Mu-Opioid Receptors (MORs) belong to the family of Opioid Receptors (ORs), which are metabotropic receptors well-known for binding opioidergic drugs such as morphine and heroin. Other members of the ORs family include Delta (DOR), Kappa (KOR) and opioid receptor like-1 (ORL1). All ORs can bind with differential affinity distinct endogenous (own produced by the organisms) opioids, including endorphins (recognized by MORs), enkephalins (DORs), dynorphins (KORs) and nociceptins (ORL1) (Bodnar, 2022). ORs also differ in their distribution within the nervous system. MORs, DORs and ORL1 are widespread in the central nervous system (CNS), whereas KORs are more restricted to the midline and ventral structures (Allen Institute for Brain Science, 2011). ORs participate in many processes including analgesia, neuroprotection, respiratory control, ionic homeostasis, peristalsis, mood regulation, cardioprotection and sedation, among others (reviewed elsewhere: Shenoy and Lui, 2018).

The effects of endogenous opioids also depend on where in the nervous system they bind their receptors, the cell type they have their effects on, and the specific receptor(s) to which they bind. In particular, they can produce analgesia, euphoria, reinforcement, sedation, dysphoria, miosis, addiction, truncal rigidity, hedonia, aversion, nausea, and reduce the rate of respiration and cough reflex in the CNS (McNicol et al., 2003; Hyman et al., 2006; Le Merrer et al., 2009; Al-Hasani and Bruchas, 2011; Castro and Berridge, 2014a). Interestingly, activation of the different ORs might result in opposite outcomes. Indeed, MORs activation within the mesocorticolimbic system (MCLS) induces euphoria, whereas KORs and DORs activation leads to dysphoric

Abbreviations: Akt, Protein Kinase B; CFA, Complete Freund's Adjuvant; CNS, Central Nervous System; DAMGO, (D-Ala2, N-MePhe4, Gly-ol)-enkephalin; DOR, Delta Opioid Receptor; ERK, Extracellular signal-regulated Kinase; GRK2, G Protein-coupled Receptor Kinase 2; i.p., Intraperitoneal; IKKs, IKappaB Kinases; IL, Interleukin; JNK, c-Jun NH2-Terminal Kinase; KOR, Kappa Opioid Receptor; LPS, Lipopolysaccharide; MAPK, Mitogen-Activated Protein Kinase; MCLS, Mesocorticolimbic System; MEK, Mitogen-Activated Protein Kinase Kinase; MORs, Mu-Opioid Receptors; mRNA, Messenger Ribonucleic Acid; NAc, Nucleus Accumbens; NF-kB, Nuclear Factor Kappa B; NKR1, Neurokinin Receptor 1; OIH, Opioid Induced Hyperalgesia; ORL1, Opioid Receptor like-1; ORs, Opioid Receptors; PFC, Prefrontal Cortex; PKC, Protein Kinase C; ROS, Reactive Oxygen Species; siRNA, Small interfering RNA; SNPs, Single Nucleotide Polymorphisms; TLRs, Toll-like Receptors; TNF-α, Tumour Necrosis Factor alpha; VTA, Ventral Tegmental Area.

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behaviors (Anand et al., 2010). ORL1 participates in pain modulation, anxiety, mood, and memory formation (Thompson et al., 2012). Although these are the main effects of the ORs activation, readers should be aware that ORs might behave differently even when activated with the same agonist and within the same brain area, creating what it is known as 'hotspots' and 'coldspots' (Hipólito et al., 2008; Castro and Berridge, 2014b, 2017; Al-Hasani et al., 2015; Campos-Jurado et al., 2017).

MORs are widely distributed throughout the brain (see Fig. 1) and participate in many mechanisms regulating CNS functions, including pain processing, reinforcement, and related processes. Due to MORs pivotal role in the reward and pain systems (Cuitavi et al., 2021a), changes in their regulation at either gene, mRNA or protein level might result in disruption of these physiological processes and thus, predispose to pathological state or disorders such as addiction, opioid-induced hyperalgesia, and pain comorbidities. Interestingly, these affections are related to dysregulation of the intracellular signaling cascades triggered by MORs after their activation through G proteins (see Fig. 2). Among the different G protein types, MORs are known to couple with G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_{o2}, G_z and G_{16}, although with different frequency or potency (Connor and Christie, 1999).

In the last decade, an increasing amount of studies have demonstrated that MORs are implicated in neuroinflammatory regulation (Merighi et al., 2013; Gessi et al., 2016; Cahill and Taylor, 2017; Shrivastava et al., 2017; Reiss et al., 2022). A critical evaluation of the crosstalk between MORs and neuroinflammation, and its implication for drug addiction and pain processing, might help clinical and pre-clinical research to uncover novel therapeutic opportunities for neurological disorders where the endogenous opioid system plays a key role.

2. Neuroimmunity: A new way of understanding MORs

Up until very recently, the brain was conceived as an immune privileged organ. The blood-brain barrier, composed by endothelial cells, astrocytes and pericytes, protects the CNS from pathogens and other chemical substances (Kadry et al., 2020). Nonetheless, the discovery of immune cells and processes within the brain changed this misconception. On the one hand, microglia, resident macrophages in the CNS, stand as the main protectors of the brain. Under non-pathological conditions, microglial cells are responsible for a myriad of functions such as neuronal survival and death, synaptogenesis, and protection against infection (Li and Barres, 2018). On the other hand, astrocytes also play a pivotal role in neuroinflammation. Interestingly, based on the stimuli that activates astrocytes, their response can be protective or detrimental (Colombo and Farina, 2016). MORs seem to be active players in inducing immune responses through glial cells in the CNS. However, in a systemic level, MOR agonists have traditionally shown immunosuppression and hypothesis about the effect of opioid therapies increasing vulnerability to infection or uncontrolled tumor growth are nowadays under careful evaluation (Plein and Rittner, 2018; Sekandarzad et al., 2017). Thereby, although we focus on the neuroinflammatory processes induced by MOR activation in the CNS in this section we also give a brief update on MOR-mediated systemic immunosuppression.

2.1. MOR and systemic immunosuppression

Opioids have traditionally linked to immunosuppression in a systemic level (Zhang et al., 2020). In fact, there is evidence that they, directly or indirectly, can reduce the activity of a myriad of immune cells such as natural killers, T and B cells, neutrophils, mast cells and dendritic cells, which can be reversed by treating with MORs antagonists. In fact, MOR mRNA and protein has been identified in immune cells at

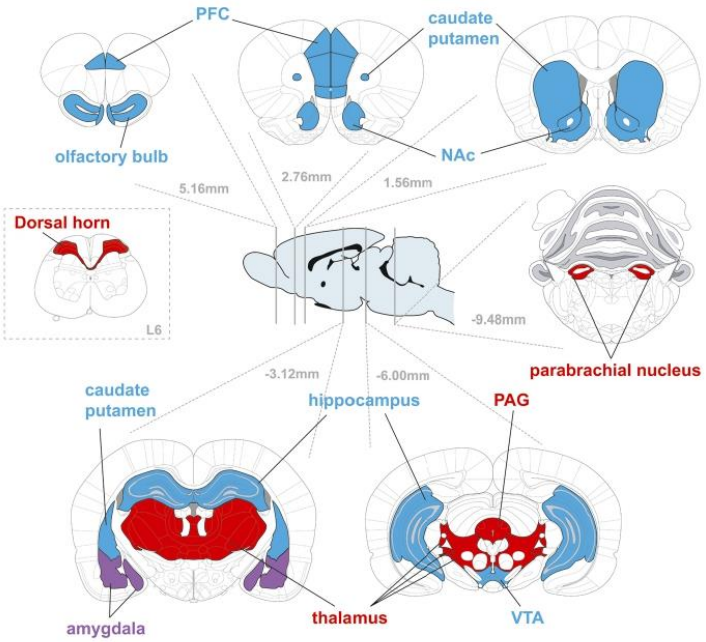

Fig. 1. Distribution of MORs within the Central Nervous system. Regions in blue represent areas involved in addiction. Regions in red represent areas related to pain regulation. Regions in purple account for areas related to both, addiction, and pain. NAc: Nucleus Accumbens; PAG: Periaqueductal Grey area; PFC: Prefrontal Cortex; VTA: Ventral-Tegmental Area.
Schematics are adapted from Paxinos and Watson (Paxinos and Watson, 2006).

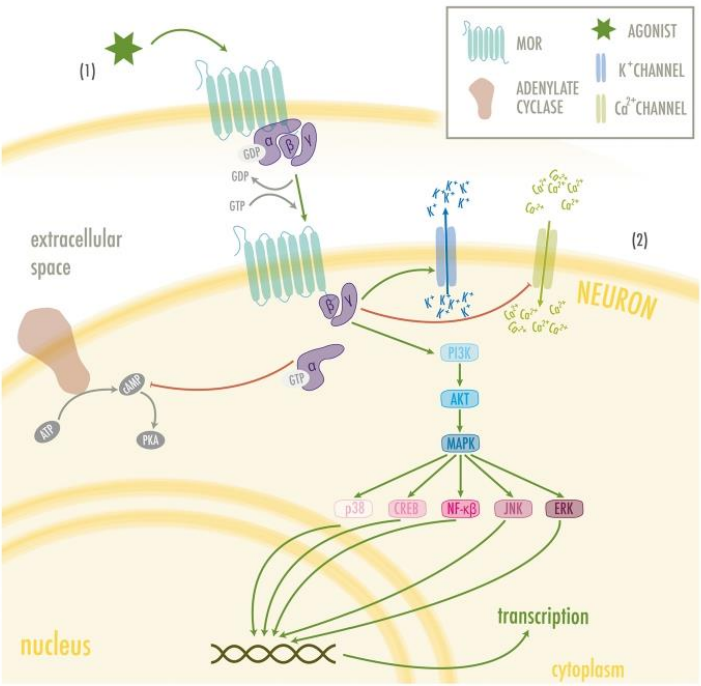

Fig. 2. MOR activation pathway. After binding an agonist, MORs get activated and promote the dissociation of the associated G protein. On the one hand, Gαi subunits suppresses adenylate cyclase, which results in a decrease of cAMP inside de cell. On the other hand, Gβγ subunits promote the exit of potassium ions (K^+) to the extracellular space and inhibit the entrance of calcium ions (Ca^{2+}), which leads to hyperpolarization of the membrane. Upon activation, MORs also induce a kinase cascade leading to the activation of transcriptional factors.

peripheral but also at central level (Machelska and Celik, 2020). This information has been recently reviewed by Plein and Rittner (2018) and Eisenstein (2019), however we present a summary in this section highlighting the most recent data.

Natural killer cells are lymphocytes belonging to the innate immune system, which play a role in limiting the spread of microbial infections and tumors (Vivier et al., 2008). Several reports reviewed at Einsenstein 2019 have shown the suppressive effect of MOR agonists on NK cells activity at peripheral but also when administered directly in the periaqueductal grey nucleus (weber and Pert Science 1989). Interestingly, recent data of Maher et al., (2019, 2020) has increased the knowledge in this specific matter. In the first experiment Maher et al. (2019) showed that opioids such as morphine, methadone and buprenorphine reduced natural killer cells cytotoxicity on K562 tumor cell line in vitro through MORs. Here, authors extracted cells from human plasma and treated them with opioids for 2 h. It is important to notice that when cells were pretreated with naloxone the decrease in the activity was not observed. This very same phenomenon was observed again in a later experiment in which they treated cells with morphine as their opioid of choice (Maher et al., 2020). Paradoxically, authors also observed a MORs-mediated increase in the levels of granzymes A and B, secreted serine proteases involved in apoptotic processes, which were significantly reduced when naloxone was used as pretreatment. Interestingly, IL6, a proinflammatory cytokine, show a tendency to increase in the medium after treating NK cells with morphine in the presence of K562 cells that was completely impaired by the pretreatment with naloxone. Altogether these data may indicate a dynamic response to morphine of the NK cells from initial activation to final inhibition of its cytotoxic properties that deserved to be further investigated. It is also important to remark that Maher et al., (2019, 2020) only used NK isolated from male humans' plasma and it would be interesting to study is the same changes can be found in women. Moreover, some opioids seem to be more immunosuppressive than others. In fact, oxycodone, hydromorphone, and buprenorphine do no alter natural killer cells activity (Meserve et al., 2014) and tramadol even increases it (Santamaria et al., 2010).

B lymphocytes belong to the adaptative immune system and oversee antibody production and immune memory through their different phenotypes (Sharonov et al., 2020). D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), a synthetic MOR agonist, reduces antibody production, which is reversed by the addition of naloxone (Reviewed at Eisenstein, 2019). Moreover, morphine decreases the expression of major histocompatibility complex class II (MHC-II), essential to recognize pathogens, in B cells (Plein and Rittner, 2018). However, a gap in the literature aiming the specific pathways by opioids alter lymphocytes B function makes it difficult to understand the effect of opioids, chronic or acutely given in antibody production. Together with this reported action in B lymphocytes, opioids have also shown to alter T lymphocytes function. T lymphocytes, which are adaptive immune system pathogen presenter cells, are also compromised when an opioid is present. In fact, morphine inhibits T lymphocyte-mediated apoptosis and alters T cells differentiation and function (Roy et al., 2011). Other opioids such as methadone decreased the expression of the CD8 + T markers of activation CD69 and CD25 in human patients (Mazahery et al., 2020). Finally, sufentanil, an opioid used in postoperative analgesia, has been shown to reduce the levels of different phenotypes of T cells in rats, including T helpers and regulatory T cells (Peng et al., 2020).

Regarding the data presented in this section, it is important to pinpoint that, although there is evidence of MOR-induced systemic immunosuppression, this subject is very complex and holds very variables. On top of the aforementioned differences found in natural killer cells activity when different opioids are used, there are further data suggesting that opioid could act systemically without promoting immunosuppression. Some studies using breast cancer mouse models observed that morphine did not promote tumor growth or angiogenesis (Doornebal et al., 2013, 2015). In fact, there is a lack of evidence regarding opioid-induced metastasis (Hooijmans et al., 2015;

Sekandarzad et al., 2017) or opioid increased incidence of infection (Häuser et al., 2015). Taking all of this into account, and as Plein and Rittner (2018) pointed out in their review "there is not strong enough data to establish a clinical relevance of opioid-induced immunosuppression". Further research is required to shed light on this specific topic by investigating which opioids, which doses and the temporal dynamics that may lead to immunosuppressive effect of these medications. This information can help to design the best therapeutical approach.

2.2. MORs and neuroinflammation

Many of the functions carried out by microglia and astrocytes involve neuroinflammatory-mediated signaling cascades which promote oxidative stress and regulate inflammatory cytokines, chemokines, and mediators through the action of the toll-like receptors (TLRs) (Kumar, 2019). Although opioids have been classically posed as immunosuppressors at systemic but also in the nervous system (central and peripheral), data available are contradictory. Curiously, recent research places microglial MORs as neuroinflammation promoters in the CNS as summarized in Table 1 (Merighi et al., 2013; Gessi et al., 2016; Cahill and Taylor, 2017; Shrivastava et al., 2017; Zhang et al., 2020; Reiss et al., 2022). However, the mechanisms by which they function remain unclear, since the relation that connects the TLR and the MOR pathway, although hinted, is yet to be scientifically proven (see Fig. 3) (Zhang et al., 2020). Nonetheless, the PKCe–Akt–ERK1/2 pathway stands out as the best candidate to connect both receptors. In an in vitro experiment where microglia cell cultures were treated with both lipopolysaccharides (LPS, a model of inflammation) and morphine, it was reported that this pathway, which leads to the release of inflammatory mediators, reached its maximal activation 10 min after the treatment (Limiroli et al., 2002). Furthermore, this morphine-induced activation was higher than that of LPS alone (Merighi et al., 2013). Similarly, Gessi et al. (2016) found that morphine increases NF-κB translocation to the nucleus in microglia cell cultures activated with LPS. Moreover, IKappaB Kinases (IKKs) activation was higher when morphine or DAMGO were added alongside LPS. It is important to notice that, in all these cases, morphine and DAMGO seem to increase an existing neuroinflammatory response and fail to induce one on their own. In this sense, both Merighi et al. (2013) and Gessi et al. (2016) suggest that MORs activation might be enhancing TLR-promoted response by an upregulation of proinflammatory cytokines. Nonetheless, there is evidence that these responses were pharmacologically blocked with Naloxone, a MOR antagonist, and by silencing MOR by using siRNAμ.

Further research points at pro- and anti-inflammatory cytokines as markers confirming the relationship between MORs and neuroinflammatory events. Shrivastava et al. (2017) found that some proinflammatory cytokines such as TNF-α, IL-1β and IL-6, known to promote inflammation, were upregulated, whereas the anti-inflammatory cytokines IL-4 and IL-13 were downregulated in primary microglia cell cultures from rats treated with DAMGO. These findings were further validated in vivo in the hypothalamus of rat pups. This very same study also used ethanol, which stimulates immune responses (Cantacorps et al., 2017) and promotes MOR activation through several mechanisms (Reviewed by Peana et al., 2017), obtaining a similar outcome. Other studies carried out in different immune cell types show the downregulation of anti-inflammatory interleukins when MORs are activated. Concretely, Duncker et al. (2012) described how mesenteric lymphocytes obtained from mice subcutaneously treated with DAMGO showed lower levels of IL-4 and IL-10. Another study observed how acute dosage/administration of morphine in vivo in a mice model inhibited the production of IL-10 in thioglycolate-elicited peritoneal macrophages when treated with LPS (Limiroli et al., 2002), although this study only shows how morphine is acting again as a potentiator of LPS. MOR activation also seems to be related to astrocytosis. Sil et al. (2018) demonstrated both in vitro and in vivo that morphine induces astrocyte activation and neuroinflammation, which are reverted after a treatment with naltrexone. Moreover, this mechanism has been proposed to promote morphine tolerance via extracellular vesicles signaling (Ma et al., 2021).

Notwithstanding this information, there are different studies that suggests that an acute or chronic treatment with morphine induces glial immunosuppression by different mechanisms. *In vitro* studies showed that morphine acute treatment impaired chemotaxis and release of proinflammatory cytokines or chemokines in microglia and astrocytes cultured cells (Chao et al., 1997; Hu et al., 2000; Mahajan et al., 2002, 2005). Very recently Peng and colleagues showed that morphine chronic treatment impaired lysosomal activity and reducing mitophagy both in vitro and in vivo (Peng et al., 2022).

2.3. Role of neuroinflammation as MORs expression and trafficking controller

The aforementioned neuroimmune mechanisms seem to correlate with MORs expression. Although cytokines' role regulating the gene expression of MORs was previously described in different cell types (Ruzicka et al., 1996; Vidal et al., 1998; Kraus et al., 2001, 2003; Börner et al., 2004), it was not until the last decade that this was further confirmed. Langsdorf et al. (2011) stimulated cultured macrophages (line TPA-HL-60) with LPS and, as expected, cytokines and Reactive Oxygen Species (ROS) were upregulated in the cell medium. Afterwards, they treated SH-SY5Y cells, which are a neuroblastoma cell line, with that medium and found an increase in the MOR messenger levels. Finally, Byrne et al. (2012) treated U97 MG human astrocytoma cells with IL-1β and observed an upregulation of both mRNA and protein levels for MOR. This study also showed that morphine downregulates the levels of MOR in the membrane of the astrocytoma cells. However, those levels were restored when IL-1β was administered. Although no mechanism has been proposed for this event, it is known that the presence of Il-1β reduces the amount of the G Protein-Coupled Receptor Kinase 2 (GRK2) when it is released, producing allodynia (Kleibeuker et al., 2008; Willemen et al., 2010). Therefore, since GRK2 is a kinase phosphorylating MORs and that is needed for MORs' internalization (Møller et al., 2020), this suggests that the release of IL-1β prevents MOR from being endocytosed.

Another possible mechanism for neuroinflammation-induced MOR alterations involves Neurokinin Receptors (NKRs). These receptors, which are G protein coupled receptors, spread all along the nervous system and are involved in the regulation of stress, pain, muscle contraction and inflammation responses (Almeida et al., 2004). NK1Rs, a type of Neurokinin receptor, are closely related to MORs. In fact, MOR activation has been suggested to inhibit the release of Substance P, an agonist of NK1R (Xiao et al., 2016). Furthermore, in some instances, the activation of NK1R seems to result in the release of pro-inflammatory mediators (Zieglgänsberger, 2019). For example, treating RAW 264.7 cells (murine macrophages) with Substance P at nanomolar concentrations activates intracellular pathways (ERK1/2 and p38 MAPK) leading to a release in pro-inflammatory mediators (Sun et al., 2008). Additionally, NK1Rs can regulate MORs' availability stage by endosomal encapsulation when activated (Schmidlin et al., 2002; Bowman et al., 2015). Similarly, NK1Rs can be upregulated by the action of IL-1β (Guo et al., 2004). Nonetheless, the stated MOR-NK1R relationship remains a hypothesis that needs further confirmation.

3. Neuroimmunity as a driver of drug addiction

Drug addiction is a neuropsychiatric disorder characterized by a recurring desire to continue taking the drug despite harmful consequences (Liu and Li, 2018). There are many substances that can lead to addiction including both legal and illegal drugs, such as cannabis, opioids, alcohol, cocaine, tobacco, caffeine, etc. (American Psychiatric Association, 2013). All of them activate a common neurochemical pathway in the mesocorticolimbic system (MCLS) due to their properties

Table 1
Levels of neuroinflammatory mediators in in vivo and in vitro experiments after the administration of a MOR agonist.

Neuroinflammatory Mediator	Increases (+) Decreases (-)	Animal	Treatment	Administration	Tissue/cell culture	Reference
iNOS	+	Mouse	LPS (1 μg/ml) + morphine (100 nM)	In cell culture medium	Microglia cell culture	Gessi et al. (2016)
	+	Mouse	LPS (1 μg/ml) + morphine (100 nM) or LPS (1 μg/ml) + DAMGO (100 nM)	In cell culture medium	Microglia cell culture	Merighi et al. (2013)
COX-2	+	Mouse	LPS (1 μg/ml) + morphine (100 nM)	In cell culture medium	Microglia cell culture	Merighi et al. (2013)
IL-1β	+	Mouse	LPS (1 μg/ml) + morphine (100 nM)	In cell culture medium	Microglia cell culture	Merighi et al. (2013)
	+	Mouse	LPS (1 μg/ml) + morphine (100 nM) or LPS (1 μg/ml) + DAMGO (100 nM)	In cell culture medium	Microglia cell culture	Gessi et al. (2016)
	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
	+	Rat	Morphine (10 mg/kg)	Subcutaneus injections twice a day for 5 days into the spinal space	Spinal cord	Tian et al. (2015)
	+	Rat	Morphine (10 μM)	20 h incubation	Spinal neuron culture	Tian et al. (2015)
	+	Rat	Morphine (10 μM)	Injection twice a day for 7 days through a catheter	Spinal cord	Bai et al. (2014)
	+	Rat	Morphine (10 μg)	Intrathecal infusion (10 μg in 2 μl of saline) for 5 days	Spinal cord	Johnston et al. (2004)
	+	Rat	Morphine (15 μg/h)	Intrathecal infusion for 5 days	Spinal cord	Lin et al. (2010)
	+	Rat	Complete Freund's Adjuvant (CFA) suspension (0,1 mg/0,1 ml)	Injection into the plantar surface in the hind-paw	Paw skin	Bianchi et al. (2008)
IL-6	+	Mouse	LPS (1 μg/ml) + morphine (100 nM)	In cell culture medium	Microglia cell culture	Merighi et al. (2013)
	+	Mouse	LPS (1 μg/ml) + morphine (100 nM) or LPS (1 μg/ml) + DAMGO (100 nM)	In cell culture medium	Microglia cell culture	Gessi et al. (2016)
	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
	+	Rat	Morphine (10 μg)	Intrathecal infusion (10 μg in 2 μl of saline) for 5 days	Spinal cord	Johnston et al. (2004)
	+	Rat	Morphine (15 μg/h)	Intrathecal infusion for 5 days	Spinal cord	Lin et al. (2010)
	+	Rat	Complete Freund's Adjuvant (CFA) suspension (0,1 ml)	Injection into the plantar surface in the hind-paw	Serum	Tekieh et al. (2011)
	+	Rat	Complete Freund's Adjuvant (CFA) suspension (0,1 ml)	Injection into the plantar surface in the hind-paw	Serum	Zaringhalam et al. (2013)
TNF-α	+	Mouse	LPS (1 μg/ml) + morphine (100 nM)	In cell culture medium	Microglia cell culture	Merighi et al. (2013)
	+	Mouse	Morphine (100 nM)	In cell culture medium	Microglia cell culture	Gessi et al. (2016)
	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
	+	Rat	Ethanol (2,5 g/kg/day)	Solution fo 11,34% ethanol in milk to pups for 5 days, twice a day	Hypothalamus	Shrivastava et al. (2017)
	+	Rat	Morphine (10 μM)	Injection twice a day for 7 days through a catheter	Spinal cord	Bai et al. (2014)
	+	Rat	Morphine (10 μg)	Intrathecal infusion (10 μg in 2 μl of saline) for 5 days	Spinal cord	Johnston et al. (2004)
	+	Rat	Morphine (15 μg/h)	Intrathecal infusion for 120 h	Spinal cord	Tsai et al. (2016)
	+	Rat	Morphine (15 μg/h)	Intrathecal infusion for 5 days	Spinal cord	Lin et al. (2010)
	+	Rat	Complete Freund's Adjuvant (CFA) suspension (0,1 mg/0,1 ml)	Injection into the plantar surface in the hind-paw	Paw skin	Bianchi et al. (2008)
	+	Rat	Complete Freund's Adjuvant (CFA) suspension (0,1 ml)	Injection into the plantar surface in the hind-paw	Serum	Akhtari et al. (2012)
NF-κB	+	Mouse	LPS (1 μg/ml) + morphine (0,1 μM or 10 μM)	In cell culture medium for 15 min	Microglia cell culture	Gessi et al. (2016)
	+	Rat	Morphine (10 μM)	Injection twice a day for 7 days through a catheter	Spinal cord	Bai et al. (2014)
p-AKT	+	Mouse	Morphine (100 nM)	In cell culture medium	Microglia cell culture	Gessi et al. (2016)
	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
IL-1α	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
IL-1	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
MCP-1	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
CXCL1	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)

(continued on next page)

Table 1 (*continued*)

Neuroinflammatory Mediator	Increases (+) Decreases (-)	Animal	Treatment	Administration	Tissue/cell culture	Reference
IBA-1	+	Rat	Ethanol (2,5 g/kg/day)	Solution fo 11,34% ethanol in milk to pups for 5 days, twice a day	Hypothalamus	Shrivastava et al. (2017)
CSFR1	+	Rat	Ethanol (2,5 g/kg/day)	Solution fo 11,34% ethanol in milk to pups for 5 days, twice a day	Hypothalamus	Shrivastava et al. (2017)
MCP1	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
	+	Rat	Ethanol (2,5 g/kg/day)	Solution fo 11,34% ethanol in milk to pups for 5 days, twice a day	Hypothalamus	Shrivastava et al. (2017)
IL-4	–	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
	–	Mouse	DAMGO (5 mg/kg)	Subcutaneus injection	Mesenteric lymphocytes	Duncker et al. (2012)
IL-13	–	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
MIP-3α	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
OX-6	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
IFN-γ	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
p38 MAPK	+	Rat	Ethanol (50 mM) + DAMGO (50 μM)	In cell culture medium	Microglia cell culture	Shrivastava et al. (2017)
IL-10	–	Mouse	DAMGO (5 mg/kg)	DAMGO (5 mg/kg)	Mesenteric lymphocytes	Duncker et al. (2012)

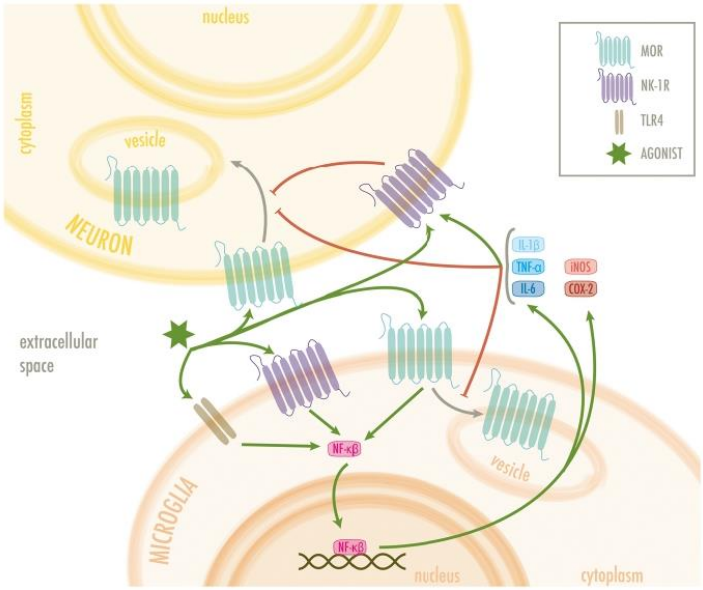

Fig. 3. Hypothesis of MOR-neuroinflammation feedback. Opioid agonists activate several receptors on microglial cells (MOR, NK1R, and TLR4) which promotes MOR internalization and release of proinflammatory mediators. These mediators, at the same time, regulate MOR trafficking in both neurons and microglia possibly through the action of NK1Rs.

as reinforcers (Liu and Li, 2018). This system, which processes reward and aversion, is one of the most affected neuronal circuits in disorders associated with use of drugs. Natural reinforcers activate this system to facilitate behaviors that increase survival (Fields et al., 2007). In order to do so, the MCLS comprises brain areas that play a crucial role in reward management. Concretely, Nucleus Accumbens (NAc), Prefrontal Cortex (PFC), extended amygdala, Ventral Tegmental Area (VTA), hippocampus and hypothalamus are the main brain structures that shape the neural reward circuitry (Koob and Volkow, 2016).

Opioid receptors in the MCLS play an important role in modulating reward responses when activated by both endogenous and exogenous opioids (Darcq and Kieffer, 2018). Interestingly, exogenous opioids can disrupt MCLS's normal activity. Opioids such as morphine or heroin can bind to MORs at GABAergic neurons of the VTA (Fields and Margolis, 2015). MORs' activation inhibits GABAergic neurons, which in turn disinhibits VTA dopaminergic neurons. Thus, opioid receptors activation increases the release of dopamine within the NAc, which is the main target of VTA dopaminergic neurons (Le Merrer et al., 2009). In

addition, MORs agonists have also shown to modulate DA extracellular levels within the NAc, thus regulating DA release at terminal level (Hipólito et al., 2008). Depending on the subarea studied within the NAc, the administration of DAMGO differentially regulated DA extracellular levels, suggesting that MORs might be located in a different neuron type or location in the shell and in the core of the NAc. The increase in the dopaminergic activity accounts for the reinforcing effect, which is essential in opioid and other drugs reinforcing events that ultimately are crucial for the development of addiction (Becker and Chartoff, 2019).

3.1. The role of cytokines in the MCLS for drug addiction

Multiple drugs can promote the release of neuroinflammatory mediators within different areas from the MCLS (Cahill and Taylor, 2017; Cantacorps et al., 2017). Similarly, the expression of different cytokines can also interfere with the process of drug addiction. IL-2 participates through modulating the sensitivity of the dopaminergic circuitry within this system (Zalcman, 2001). IL-6 is essential in promoting methamphetamine-induced dopamine neurotoxicity (Ladenheim et al., 2000). Another cytokine that interferes in the MCLS is TNF-α (Lewitus et al., 2016; Norlin et al., 2021). Pre-treatment with an intraperitoneal (i.p.) injection of TNF-α in mice was able to reduce the extracellular striatal dopamine release evoked by methamphetamine (Nakajima et al., 2004). Likewise, markers of neuroinflammation are related to stress and depression (Maydych, 2019). Krasnova et al. (2016) demonstrated that methamphetamine self-administration in rats triggered neuroinflammation in the striatum. They also suggest that this event could be related to the classical neurocognitive dysfunctions found in human methamphetamine users including depression. Moreover, Cahill and Taylor (2017) suggested that events associated with neuroinflammation within the MCLS may trigger depression.

Multiple studies have demonstrated that predisposition to addiction by drugs of abuse has a significant genetic component (Marcos et al., 2008; Bevilacqua and Goldman, 2009; Liu et al., 2009). Accordingly, some studies have found genetic associations between neuroinflammation and drug addiction. On the one hand, a study comparing the *IL-1β* gene from 60 opioid addicts versus 60 healthy people showed a significant difference in two different single nucleotide polymorphisms (SNPs), both associated with an increased production of IL-1β. Those SNPs were found at a higher frequency in opioid addicts, thus suggesting neuroinflammation plays a role in addiction (Liu et al., 2009). On the other hand, another SNP at the *IL-10* gene promoter seems associated with a decrease in this anti-inflammatory interleukin. One study quantified the amount of people with this polymorphism in both alcoholic patients and healthy controls. Interestingly, this SNP was more present in alcoholics than in healthy people (Marcos et al., 2008).

3.2. Neuroinflammation: The key to understanding drug relapse and tolerance?

Recent studies have provided a direct connection between neuroinflammation and relapse (Brown et al., 2018; Berríos-Cárcamo et al., 2020; Cuitavi et al., 2021b; Fernández-Rodríguez et al., 2022). Glial cells are involved in synaptic plasticity; they modulate the effects of drugs of abuse and vice versa (Jiménez-González et al., 2021). Moreover, microglial activation has been related to addiction and to an increased expression of cytokines (Schwarz and Bilbo, 2013; Loftis and Janowsky, 2014; Taylor et al., 2016; Cantacorps et al., 2017). Therefore, 3-isobutyryl-2-isopropyl pyrazolo-[1,5-a] pyridine (ibudilast), which is an anti-inflammatory drug that decreases glial activation, has been used in the past to prevent relapse into drug use disorders (Linker et al., 2019). Hutchinson et al. (2009) also used ibudilast in Sprague–Dawley rats and discovered that it attenuated the pro-inflammatory cytokine release induced by morphine and, therefore, it reduced opioid withdrawal. Additionally, studies in humans showed that heroin craving was significantly reduced when patients were treated with ibudilast (Metz et al., 2017). In another investigation, a traumatic brain injury in the frontal cortex in Long-Evans rats led to less IL-10 when compared to controls. Interestingly, animals were more prone to relapse into cocaine consumption as assessed by the operant conditioning chambers paradigm (Vonder Haar et al., 2018).

Curiously, there is an increase of pro-inflammatory cytokines in morphine tolerance rats (Hutchinson et al., 2008), and the inhibition of the expression of pro-inflammatory mediators restores the effect of opioids (Raghavendra et al., 2004) thus suggesting they participate in the development of opioid tolerance. Furthermore, the PKCε pathway alongside Akt and ERK phosphorylation are involved in an increase in the release of pro-inflammatory mediators in microglia due to opioid administration (Zheng et al., 2011; Merighi et al., 2013), since PKCε inhibition can reduce opioid tolerance (Smith et al., 2007). Moreover, a chronic morphine treatment also induces the activation of the PI3k/Akt signaling pathway by interacting with MORs, which produces an increase in IL-1β levels and the development of morphine tolerance (Xu et al., 2014; Tian et al., 2015).

All things considered, morphine tolerance is enhanced when combined with glial activation since the levels of pro-inflammatory cytokines are higher than with just glial activation. These pro-inflammatory cytokines are produced via NF-kβ through different pathways that are activated by morphine and nociceptive stimulation, and alter MOR transcriptional levels (Zhou et al., 2021).

4. Pain-derived neuroinflammation: role of MORs

Pain, defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage" by the International Association for the Study of Pain (Raja et al., 2020), is a very complex function of the nervous system. Physiological pain is needed to promote survival, and it must be well regulated since both down- and up-regulation can be detrimental (Breivik, 2008). When pain persists beyond the healing process (3 months or more) and is no longer physiologically relevant, it can be considered chronic (Nicholas et al., 2019).

Chronic pain is one of the most prevalent illnesses in developed countries, with an increasing number of patients over the years (Reid et al., 2011; Breivik et al., 2013; Souza et al., 2017; Steingrímsdóttir et al., 2017). Moreover, chronic pain shares comorbidities with other illnesses, such as depression, anxiety, sleep disturbances, drug use disorders, and lack of energy, all of which interfere with people's quality of life (Banks and Kerns, 1996; Anisman and Hayley, 2012; Tajerian et al., 2014; Hipólito et al., 2015; Lorente et al., 2022a). Interestingly, the number of sufferers increases with age, and it is more prevalent in women. Thus, chronic pain represents a disruption in the normal life for many individuals and a significant economic burden for our societies (Kurita et al., 2012; Landmark et al., 2013; Sá et al., 2019).

Despite the recent number of reviews on pain, its mechanism, its comorbidities, and potential therapeutics (Garcia-Larrea and Quesada, 2022; Haanpää and Treede, 2022; Hu et al., 2022; Lorente et al., 2022b; Song et al., 2022), the crosstalk between neuroinflammation, opioids and pain remains poorly understood. Nonetheless, numerous in vivo experiments in rats have shown that morphine administration provokes the release of inflammatory mediators in the dorsal horn of the spinal cord, which is a key area in pain management (Johnston, 2004; Bai et al., 2014; Tsai et al., 2016; Eidson and Murphy, 2019). Specifically, Bai et al. (2014) injected 10 μg of morphine twice a day for 7 days to Sprague Dawley rats through a catheter located into the dorsal horn of the spinal cord. After that period rats were sacrificed, and the levels of some proteins were measured by western blotting. Results showed that morphine administration triggered the inflammatory pathway; there was an increased translocation of NF-κB to the nucleus and higher levels of TNF-α and IL-1β. The authors suggested that this effect is not only promoted by MOR, but also by TLRs, since its blockage diminished the effect of morphine administration.

4.1. Neuroinflammation to understand MOR-based Opioid-induced hyperalgesia and tolerance

Nowadays, opioids are the best analgesic treatment for chronic pain. However, prolonged opioid administration can induce different adverse effects including tolerance, opioid-induced hyperalgesia (OIH) and addiction (Furlan and Murphy, 2022). It has been reported that the development of opioid tolerance leads to higher levels of opioid consumption, which in turn enhances OIH in a process that seems to be modulated by neuroinflammatory mechanisms. Raghavendra et al. (2002) demonstrated a positive synergistic effect by which the neuroinflammation induced by neuropathic pain and morphine treatment was higher than that produced just by neuropathic pain. Therefore, this suggests that opioid treatment can aggravate the neuroinflammation produced by certain pathologies.

As previously mentioned, opioid treatments can produce neuroinflammation without the presence of pain. Furthermore, spinal glial activation in neuropathic pain and opioid treatment accounts for the release of pro-inflammatory cytokines, which leads to a reduction in the antinociceptive effect of morphine or morphine tolerance (Song and Zhao, 2001; Watkins et al., 2005; Tsai et al., 2008). In fact, IL-1β inhibition allows morphine to have its antinociceptive effect, showing the role of pro-inflammatory cytokines in opioid tolerance and in neuropathic pain (Johnston et al., 2004; Shavit et al., 2005; Sung et al., 2005; Watkins et al., 2005). Moreover, naloxone, a MOR antagonist, reduced morphine tolerance and the levels of spinal TNF-α, IL-1β and IL-6 in animals that followed an opioid treatment (Lin et al., 2010). Additionally, IL-10 in spinal cord induces nociception since it inhibits spinal glial activation, decreases the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), and attenuates morphine tolerance (Milligan et al., 2006; Watkins et al., 2007; Lin et al., 2010). Altogether, these results suggest that MORs activation produces an increase in pro-inflammatory mediators, which leads to a major sensitivity towards a noxious stimulus.

One of the main causes of OIH is cytokine signaling to neurons of the CNS (Vanderwall and Milligan, 2019). Cytokines can cross the blood brain barrier, attributable to an increased permeability produced by neuroinflammation (Matsuda et al., 2019), and then emit signals through brain endothelial cells or through glossopharyngeal and vagus sensory nerves (Watkins and Maier, 2005). In an animal model of arthritis produced by a Complete Freund's Adjuvant (CFA) injection in the knee of the animal, three cytokines, TNF-α, IL-1β and IL-6, were upregulated, and this increase was associated with hyperalgesia via MORs regulation (Bianchi et al., 2008; Tekieh et al., 2011; Akhtari et al., 2012; Zaringhalam et al., 2013). Moreover, blocking these cytokines reduced hyperalgesia (Delery and Edwards, 2020). Additionally, at least one part of its anti-hyperalgesic effects is mediated by an increased MORs spinal expression (Tekieh et al., 2011; Akhtari et al., 2012; Zaringhalam et al., 2013). By blocking IL-6 the anti-hyperalgesic effects are only present in the seven first days of treatment. In the second and third weeks of treatment the anti-IL-6 has a hyperalgesic effect which indicates that IL-6 regulates hyperalgesia in a time dependent manner (Tekieh et al., 2011). In agreement, it has previously been shown that certain analgesic drugs (i.e. Bisphosphonate ibandronate) can lead to a reduction in hyperalgesia and release of pro-inflammatory cytokines produced by CFA (Bianchi et al., 2008).

The release of pro-inflammatory cytokines seems to result in reduced MOR expression. Conversely, the activation of MORs produces an upregulation of pro-inflammatory cytokines. An in vitro study has demonstrated that a c-Jun NH2-terminal kinase (JNK) inhibition increases MORs gene expression, and it can be reversed by inhibitors of P38 MAPK and NF-kβ (Wagley et al., 2013). An in vivo study has shown that JNK activation regulates G proteins coupled to opioid receptors, which can interfere with the tolerance produced by DAMGO (Melief et al., 2010). Furthermore, it has been observed in spinal astrocytes that the activation of the JNK pathway by an acute ultra-low dose of morphine leads to thermal hyperalgesia (Sanna et al., 2015). Additionally, inhibition of either NF-kβ or MEK1/2, when in combination with morphine administration, attenuates neuropathic pain development and thermal and mechanical hyperalgesia in another murine model (Popiolek-Barczyk et al., 2014). Therefore, this suggests that the relationship between hyperalgesia and neuroinflammation is bidirectional.

5. Conclusion, limitations and way forward

This review presents an updated summary of current discoveries on MORs and their role in neuroinflammatory processes. In just the last 10 years, this field of research has seen an exponential growth, which indicates that elucidating the relationship between MORs and neuroinflammation poses a significant interest to basic and translational researchers as well as clinicians. Elucidating MOR-neuroinflammation relationship will serve as a promising venue for better personalized and accurate treatments for pain, drug addiction, and other MOR-related conditions. Importantly, neuroinflammatory mediators seem to regulate MORs presence on the cell membrane through positive feedback. This relationship is of special relevance to better assess and treat associated phenomena such as drug addiction, OIH, and opioid tolerance.

New treatments targeting the MOR-neuroinflammation relationship to address addiction and tolerance find a great limitation. In this reviewer, we have emphasized the dualistic nature of MOR activation, which can result in immunostimulation and/or immunosuppression. The outcome of how an opioid has an effect in the immune system might be different in and out of the CNS and have different consequences for patients treated with opioids, which is a huge limitation in the existing literature. In particular, this issue is a matter of debate among anesthesiologist and surgeons since the use of perisurgical opioids for tumor removal, could result in immunodepression. The same applies when alleviating a cancer-induced pain condition or a non-malignant pain treated with opioids. In this sense, finding new ways of safely administering opioids including the idea of targeting specific regions will be of key interest. Be that as it may, further research is warranted to verify the clinical relevance of the crosstalk between opioids and immune system observed in vitro and in vivo using animal models.

Moreover, in order to further understand this relationship, the first and most important aspect that must be elucidated is the specific link(s) connecting MORs and the TLR pathways. As suggested by Zhang et al. (2020), the PKCe–Akt–ERK1/2 is the main candidate, although further research is required to find out which molecule, or composition of several molecules are playing a key linker role. Additionally, it is of crucial importance to ascertain possible sex-dependent factors which could have an impact on the above-mentioned aspects. Traditionally, there has been a huge gap in many scientific fields regarding potential sex differences and this one is not an exception, with only a few recent research papers accounting for this factor (Shrivastava et al., 2017; Doyle and Murphy, 2018; Cuitavi et al., 2021b). Moreover, as shown in this review, nearly all the articles presented work with either cell cultures or animal models (see Table 1). Nonetheless, results seem promising and establish the basis of the crosstalk between MORs and neuroinflammation, which positively impacts its translational value. Therefore, the next step should be confirming this crosstalk in humans and assess its impact on addiction and pain conditions.

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CRediT authorship contribution statement

Javier Cuitavi: Conceptualization, Writing – original draft, Writing – review & editing. **Jose Vicente Torres-Pérez:** Supervision, Writing – review & editing. **Jesús David Lorente:** Writing – original draft, Writing – review & editing. **Yolanda Campos-Jurado:** Writing – review & editing, Table and figures. **Paula Andrés-Herrera:** Writing – review & editing, Table and figures. **Ana Polache:** Supervision. **Carmen Agustín-Pavón:** Supervision, Writing – review & editing. **Lucía Hipólito:** Resources, Supervision, Writing – review & editing. All authors contributed to the article and approved the submitted version.

Data Availability

No data was used for the research described in the article.

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References

Akhtari, Z., Zaringhalam, J., Eidi, A., Manaheji, H., Tekieh, E., 2012. Bidirectional effects of serum TNF alpha level and spinal P38MAPK phosphorylation on hyperalgesia variation during CFA-induced arthritis. EXCLI J. 11, 373, 10.17877/DE290R-10360.

Al-Hasani, R., Bruchas, M.R., 2011. Molecular mechanisms of opioid receptor-dependent signaling and behavior. J. Am. Soc. Anesthesiol. 115 (6), 1363–1381. https://doi.org/10.1097/ALN.0b013e318238bba6.

Al-Hasani, R., McCall, J.G., Shin, G., Gomez, A.M., Schmitz, G.P., Bernardi, J.M., Bruchas, M.R., 2015. Distinct subpopulations of nucleus accumbens dynorphin neurons drive aversion and reward. Neuron 87 (5), 1063–1077. https://doi.org/10.1016/j.neuron.2015.08.019.

Almeida, T.A., Rojo, J., Nieto, P.M., Pinto, F.M., Hernandez, M., Martin, J.D., Candenas, M.L., 2004. Tachykinins and tachykinin receptors: structure and activity relationships. Curr. Med. Chem. 11 (15), 2045–2081. https://doi.org/10.2174/0929867043364748.

Anand, K.J., Willson, D.F., Berger, J., Harrison, R., Meert, K.L., Zimmerman, J., Carcillo, J., Newth, C.J.L., Prodhan, P., Dean, J.M., Nicholson, C., 2010. Tolerance and withdrawal from prolonged opioid use in critically ill children. Pediatrics 125 (5), e1208–e1225. https://doi.org/10.1542/peds.2009-0489.

Anisman, H., Hayley, S., 2012. Inflammatory factors contribute to depression and its comorbid conditions. pe45-pe45 Sci. Signal. 5 (244). https://doi.org/10.1126/scisignal.2003579.

AnonAllen Institute for Brain Science (2011). Allen Reference Atlas. Available from atlas.brain-map.org.

Bai, L., Zhai, C., Han, K., Li, Z., Qian, J., Jing, Y., Zhang, W., Xu, J.T., 2014. Toll-like receptor 4- mediated nuclear factor-κB activation in spinal cord contributes to chronic morphine-induced analgesic tolerance and hyperalgesia in rats. Neurosci. Bull. 30 (6), 936–948. https://doi.org/10.1007/s12264-014-1483-7.

Banks, S.M., Kerns, R.D., 1996. Explaining high rates of depression in chronic pain: A diathesis-stress framework. Psychol. Bull. 119 (1), 95. https://doi.org/10.1037/0033-2909.119.1.95.

Becker, J.B., Chartoff, E., 2019. Sex differences in neural mechanisms mediating reward and addiction. Neuropsychopharmacology 44 (1), 166–183. https://doi.org/10.1038/s41386-018-0125-6.

Berríos-Cárcamo, P., Quezada, M., Quintanilla, M.E., Morales, P., Ezquer, M., Herrera-Marschitz, M., Ezquer, F., 2020. Oxidative stress and neuroinflammation as a pivot in drug abuse. A focus on the therapeutic potential of antioxidant and anti-inflammatory agents and biomolecules. Antioxidants 9 (9), 830. https://doi.org/10.3390/antiox9090830.

Bevilacqua, L., Goldman, D., 2009. Genes and addictions. Clin. Pharmacol. Ther. 85 (4), 359. https://doi.org/10.1038/clpt.2009.6.

Bianchi, M., Franchi, S., Ferrario, P., Sotgiu, M.L., Sacerdote, P., 2008. Effects of the bisphosphonate ibandronate on hyperalgesia, substance P, and cytokine levels in a rat model of persistent inflammatory pain. Eur. J. Pain. 12 (3), 284–292. https://doi.org/10.1016/j.ejpain.2007.06.005.

Bodnar, R.J., 2022. Endogenous opiates and behavior: 2020. Peptides, 170752. https://doi.org/10.1016/j.peptides.2022.170752.

Börner, C., Kraus, J., Schröder, H., Ammer, H., Höllt, V., 2004. Transcriptional regulation of the human μ-opioid receptor gene by interleukin-6. Mol. Pharmacol. 66 (6), 1719–1726. https://doi.org/10.1124/mol.104.003806.

Bowman, S.L., Soohoo, A.L., Shiwarski, D.J., Schulz, S., Pradhan, A.A., Puthenveedu, M. A., 2015. Cell-autonomous regulation of Mu-opioid receptor recycling by substance P. Cell Rep. 10 (11), 1925–1936. https://doi.org/10.1016/j.celrep.2015.02.045.

Breivik, H., Borchgrevink, P.C., Allen, S.M., Rosseland, L.A., Romundstad, L., Breivik Hals, E.K., Stubhaug, A., 2008. Assessment of pain. BJA Br. J. Anaesth. 101 (1), 17–24. https://doi.org/10.1093/bja/aen103.

Breivik, H., Eisenberg, E., O'Brien, T., 2013. The individual and societal burden of chronic pain in Europe: the case for strategic prioritisation and action to improve knowledge and availability of appropriate care. BMC Public Health 13 (1), 1229. https://doi.org/10.1186/1471-2458-13-1229.

Brown, K.T., Levis, S.C., O'Neill, C.E., Northcutt, A.L., Fabisiak, T.J., Watkins, L.R., Bachtell, R.K., 2018. Innate immune signaling in the ventral tegmental area contributes to drug-primed reinstatement of cocaine seeking. Brain Behav., Immun. 67, 130–138. https://doi.org/10.1016/j.bbi.2017.08.012.

Byrne, L.S., Peng, J., Sarkar, S., Chang, S.L., 2012. Interleukin-1 beta-induced up-regulation of opioid receptors in the untreated and morphine-desensitized U87 MG human astrocytoma cells. J. Neuroinflamm. 9 (1), 252. https://doi.org/10.1186/1742-2094-9-252.

Cahill, C.M., Taylor, A.M., 2017. Neuroinflammation—a co-occurring phenomenon linking chronic pain and opioid dependence. Curr. Opin. Behav. Sci. 13, 171–177. https://doi.org/10.1016/j.cobeha.2016.12.003.

Campos-Jurado, Y., Martí-Prats, L., Zornoza, T., Polache, A., Granero, L., Cano-Cebrián, M.J., 2017. Regional differences in mu-opioid receptor-dependent modulation of basal dopamine transmission in rat striatum. Neurosci. Lett. 638, 102–108. https://doi.org/10.1016/j.neulet.2016.12.024.

Cantacorps, L., Alfonso-Loeches, S., Moscoso-Castro, M., Cuitavi, J., Gracia-Rubio, I., López- Arnaud, R., Escubedo, E., Guerri, C., Valverde, O., 2017. Maternal alcohol binge drinking induces persistent neuroinflammation associated with myelin damage and behavioural dysfunctions in offspring mice. Neuropharmacology 123, 368–384. https://doi.org/10.1016/j.neuropharm.2017.05.034.

Castro, D.C., Berridge, K.C., 2014a. Advances in the neurobiological bases for food 'liking'versus 'wanting'. Physiol. Behav. 136, 22–30. https://doi.org/10.1016/j.physbeh.2014.05.022.

Castro, D.C., Berridge, K.C., 2014b. Opioid hedonic hotspot in nucleus accumbens shell: mu, delta, and kappa maps for enhancement of sweetness "liking" and "wanting". J. Neurosci. 34 (12), 4239–4250. https://doi.org/10.1523/JNEUROSCI.4458-13.2014.

Castro, D.C.and Berridge, K.C. (2017). Opioid and orexin hedonic hotspots in rat orbitofrontal cortex and insula. Proceedings of the National Academy of Sciences, *114*(43), E9125-E9134. DOI: 〈10.1073/pnas.1705753114〉.

Chao, C.C., Hu, S., Shark, K.B., Sheng, W.S., Gekker, G., Peterson, P.K., 1997. Activation of mu opioid receptors inhibits microglial cell chemotaxis. J. Pharmacol. Exp. Ther. 281 (2), 998–1004 (DOI: n/a).

Colombo, E., Farina, C., 2016. Astrocytes: key regulators of neuroinflammation. Trends Immunol. 37 (9), 608–620. https://doi.org/10.1016/j.it.2016.06.006.

Connor, M., Christie, M.J., 1999. Opioid receptor signalling mechanisms. Clin. Exp. Pharmacol. Physiol. 26 (7), 493–499. https://doi.org/10.1046/j.1440-1681.1999.03049.x.

Cuitavi, J., Hipólito, L., Canals, M., 2021a. The life cycle of the mu-opioid receptor. Trends Biochem. Sci. 46 (4), 315–328. https://doi.org/10.1016/j.tibs.2020.10.002.

Cuitavi, J., Lorente, J.D., Campos-Jurado, Y., Polache, A., Hipólito, L., 2021b. Neuroimmune and Mu-Opioid receptor alterations in the mesocorticolimbic system in a sex-dependent inflammatory pain-induced alcohol relapse-like rat model. Front. Immunol. 3722. https://doi.org/10.3389/fimmu.2021.689453.

Darcq, E., Kieffer, B.L., 2018. Opioid receptors: drivers to addiction? Nat. Rev. Neurosci. 19 (8), 499–514. https://doi.org/10.1038/s41583-018-0028-x.

Delery, E.C., Edwards, S., 2020. Neuropeptide and cytokine regulation of pain in the context of substance use disorders. Neuropharmacology 174, 108153. https://doi.org/10.1016/j.neuropharm.2020.108153.

Doornebal, C.W., Klarenbeek, S., Braumuller, T.M., Klijn, C.N., Ciampricotti, M., Hau, C. S., de Visser, K.E., 2013. A preclinical mouse model of invasive lobular breast cancer metastasisA preclinical mouse model of breast cancer metastasis. Cancer Res. 73 (1), 353–363. https://doi.org/10.1158/0008-5472.CAN-11-4208.

Doornebal, C.W., Vrijland, K., Hau, C.S., Coffelt, S.B., Ciampricotti, M., Jonkers, J., Hollmann, M.W., 2015. Morphine does not facilitate breast cancer progression in two preclinical mouse models for human invasive lobular and HER2+ breast cancer. Pain 156 (8), 1424–1432. https://doi.org/10.1097/j.pain.0000000000000136.

Doyle, H.H., Murphy, A.Z., 2018. Sex-dependent influences of morphine and its metabolites on pain sensitivity in the rat. Physiol. Behav. 187, 32–41. https://doi.org/10.1016/j.physbeh.2017.11.030.

Duncker, S.C., Philippe, D., Martin-Paschoud, C., Moser, M., Mercenier, A., Nutten, S., 2012. Nigella sativa (Black Cumin) seed extract alleviates symptoms of allergic diarrhea in mice, involving opioid receptors. PloS One 7 (6), e39841. https://doi.org/10.1371/journal.pone.0039841.

Eidson, L.N., Murphy, A.Z., 2019. Inflammatory mediators of opioid tolerance: implications for dependency and addiction. Peptides 115, 51–58. https://doi.org/10.1016/j.peptides.2019.01.003.

Eisenstein, T.K., 2019. The role of opioid receptors in immune system function. Front. Immunol. 10, 2904. https://doi.org/10.3389/fimmu.2019.02904.

Fernández-Rodríguez, S., Cano-Cebrián, M.J., Rius-Pérez, S., Pérez, S., Guerri, C., Granero, L., Polache, A., 2022. Different brain oxidative and neuroinflammation status in rats during prolonged abstinence depending on their ethanol relapse-like drinking behavior: Effects of ethanol reintroduction. Drug Alcohol Depend. 232, 109284, 10.1016/j.drugalcdep.2022.109284.

Fields, H.L., Margolis, E.B., 2015. Understanding opioid reward. Trends Neurosci. 38 (4), 217–225. https://doi.org/10.1016/j.tins.2015.01.002.

Fields, H.L., Hjelmstad, G.O., Margolis, E.B., Nicola, S.M., 2007. Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. Annu. Rev. Neurosci. 30, 289–316. https://doi.org/10.1146/annurev.neuro.30.051606.094341.

Furlan, A.D., Murphy, L., 2022. Opioids. Clin. Pain. Manag. A Pract. Guide 188–197. https://doi.org/10.1002/9781119701170.ch18.

Garcia-Larrea, L., Quesada, C., 2022. Cortical stimulation for chronic pain: from anecdote to evidence. Eur. J. Phys. Rehabil. Med. https://doi.org/10.23736/S1973-9087.22.07411-1.

Gessi, S., Borea, P.A., Bencivenni, S., Fazzi, D., Varani, K., Merighi, S., 2016. The activation of μ-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia. FEBS Lett. 590 (17), 2813–2826. https://doi.org/10.1002/1873-3468.12313.

Guo, C.J., Douglas, S.D., Gao, Z., Wolf, B.A., Grinspan, J., Lai, J.P., Riedel, E., Ho, W.Z., 2004. Interleukin-1β upregulates functional expression of neurokinin-1 receptor (NK-1R) via NF-κB in astrocytes. Glia 48 (3), 259–266. https://doi.org/10.1002/glia.20079.

Haanpää, M., Treede, R.D., 2022. Neuropathic pain. Clin. Pain. Manag. A Pract. Guide 373–380. https://doi.org/10.1002/9781119701170.ch36.

Häuser, W., Bock, F., Engeser, P., Hege-Scheuing, G., Hüppe, M., Lindena, G., Petzke, F., 2015. Recommendations of the updated LONTS guidelines. Long. Term. Opioid Ther. Chronic Noncancer Pain. Schmerz 29 (1), 109–130. https://doi.org/10.1007/s00482-014-1463-x.

Hipólito, L., Sánchez-Catalán, M.J., Zanolini, I., Polache, A., Granero, L., 2008. Shell/core differences in mu-and delta-opioid receptor modulation of dopamine efflux in nucleus accumbens. Neuropharmacology 55 (2), 183–189. https://doi.org/10.1016/j.neuropharm.2008.05.012.

Hipólito, L., Wilson-Poe, A., Campos-Jurado, Y., Zhong, E., Gonzalez-Romero, J., Virag, L., Morón, J.A., 2015. Inflammatory pain promotes increased opioid self-administration: role of dysregulated ventral tegmental area μ opioid receptors. J. Neurosci. 35 (35), 12217–12231. https://doi.org/10.1523/JNEUROSCI.1053-15.2015.

Hooijmans, C.R., Geessink, F.J., Ritskes-Hoitinga, M., Scheffer, G.J., 2015. A systematic review and meta-analysis of the ability of analgesic drugs to reduce metastasis in experimental cancer models. Pain 156 (10), 1835.

Hu, S., Chao, C.C., Hegg, C.C., Thayer, S., Peterson, P.K., 2000. Morphine inhibits human microglial cell production of, and migration towards, RANTES. J. Psychopharmacol. 14 (3), 238–243. https://doi.org/10.1177/026988110001400307.

Hu, W., Bian, Q., Zhou, Y., Gao, J., 2022. Pain management with transdermal drug administration: a review. Int. J. Pharm., 121696 https://doi.org/10.1016/j.ijpharm.2022.121696.

Hutchinson, M.R., Coats, B.D., Lewis, S.S., Zhang, Y., Sprunger, D.B., Rezvani, N., Baker, E.M., Jekich, B.M., Wieseler, J.L., Somogyi, A.A., Martin, D., Poole, S., Judd, C.M., Maier, S.F., Watkins, L.R., 2008. Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. Brain Behav., Immun. 22 (8), 1178–1189. https://doi.org/10.1016/j.bbi.2008.05.004.

Hutchinson, M.R., Lewis, S.S., Coats, B.D., Skyba, D.A., Crysdale, N.Y., Berkelhammer, D. L., Brzeski, A., Northcutt, A., Vietz, C.M., Judd, C.M., Maier, S.F., Watkins, L.R., Johnson, K.W., 2009. Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). Brain Behav., Immun. 23 (2), 240–250. https://doi.org/10.1016/j.bbi.2008.09.012.

Hyman, S.E., Malenka, R.C., Nestler, E.J., 2006. Neural mechanisms of addiction: the role of reward-related learning and memory. Annu. Rev. Neurosci. 29, 565–598. https://doi.org/10.1146/annurev.neuro.29.051605.113009.

Jiménez-González, A., Gómez-Acevedo, C., Ochoa-Aguilar, A., Chavarría, A., 2021. The role of Glia in addiction: dopamine as a modulator of glial responses in addiction. Cell. Mol. Neurobiol. 1–12. https://doi.org/10.1007/s10571-021-01105-3.

Johnston, I.N., Milligan, E.D., Wieseler-Frank, J., Frank, M.G., Zapata, V., Campisi, J., Langer, S., Martin, D., Green, P., Fleshner, M.F., Leinwand, L., Maier, S.F., Watkings, L.R., 2004. A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. J. Neurosci. 24 (33), 7353–7365. https://doi.org/10.1523/JNEUROSCI.1850-04.2004.

Kadry, H., Noorani, B., Cucullo, L., 2020. A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity. Fluids Barriers CNS 17 (1), 1–24. https://doi.org/10.1186/s12987-020-00230-3.

Kleibeuker, W., Gabay, E., Kavelaars, A., Zijlstra, J., Wolf, G., Ziv, N., Yirmiya, R., Shavit, Y., Tal, M., Heijnen, C.J., 2008. IL-1β signaling is required for mechanical allodynia induced by nerve injury and for the ensuing reduction in spinal cord neuronal GRK2. Brain Behav. Immun. 22 (2), 200–208. https://doi.org/10.1016/j.bbi.2007.07.009.

Koob, G.F., Volkow, N.D., 2016. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry 3 (8), 760–773. https://doi.org/10.1016/S2215-0366(16)00104-8.

Krasnova, I.N., Justinova, Z., Cadet, J.L., 2016. Methamphetamine addiction: involvement of CREB and neuroinflammatory signaling pathways. Psychopharmacology 233 (10), 1945–1962. https://doi.org/10.1007/s00213-016-4235-8.

Kraus, J., Börner, C., Giannini, E., Hickfang, K., Braun, H., Mayer, P., Höllt, V., 2001. Regulation of μ-opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site. J. Biol. Chem. 276 (47), 43901–43908. https://doi.org/10.1074/jbc.M107543200.

Kraus, J., Börner, C., Giannini, E., Höllt, V., 2003. The role of nuclear factor κB in tumor necrosis factor-regulated transcription of the human μ-opioid receptor gene. Mol. Pharmacol. 64 (4), 876–884. https://doi.org/10.1124/mol.64.4.876.

Kumar, V., 2019. Toll-like receptors in the pathogenesis of neuroinflammation. J. Neuroimmunol. 332, 16–30. https://doi.org/10.1016/j.jneuroim.2019.03.012.

Kurita, G.P., Sjøgren, P., Juel, K., Højsted, J., Ekholm, O., 2012. The burden of chronic pain: a cross-sectional survey focussing on diseases, immigration, and opioid use. PAIN® 153 (12), 2332–2338. https://doi.org/10.1016/j.pain.2012.07.023.

Ladenheim, B., Krasnova, I.N., Deng, X., Oyler, J.M., Polettini, A., Moran, T.H., Cadet, J. L., 2000. Methamphetamine-induced neurotoxicity is attenuated in transgenic mice with a null mutation for interleukin-6. Mol. Pharmacol. 58 (6), 1247–1256. https://doi.org/10.1124/mol.58.6.1247.

Landmark, T., Romundstad, P., Dale, O., Borchgrevink, P.C., Vatten, L., Kaasa, S., 2013. Chronic pain: one year prevalence and associated characteristics (the HUNT pain study. Scand. J. Pain. 4 (4), 182–187. https://doi.org/10.1016/j.sjpain.2013.07.022.

Langsdorf, E.F., Mao, X., Chang, S.L., 2011. A role for reactive oxygen species in endotoxin-induced elevation of MOR expression in the nervous and immune systems. J. Neuroimmunol. 236 (1–2), 57–64. https://doi.org/10.1016/j.jneuroim.2011.05.009.

Le Merrer, J., Becker, J.A., Befort, K., Kieffer, B.L., 2009. Reward processing by the opioid system in the brain. Physiol. Rev. 89 (4), 1379–1412. https://doi.org/10.1152/physrev.00005.2009.

Lewitus, G.M., Konefal, S.C., Greenhalgh, A.D., Pribiag, H., Augereau, K., Stellwagen, D., 2016. Microglial TNF-α suppresses cocaine-induced plasticity and behavioral sensitization. Neuron 90 (3), 483–491. https://doi.org/10.1016/j.neuron.2016.03.030.

Li, Q., Barres, B.A., 2018. Microglia and macrophages in brain homeostasis and disease. Nat. Rev. Immunol. 18 (4), 225–242. https://doi.org/10.1038/nri.2017.125.

Limiroli, E., Gaspani, L., Panerai, A.E., Sacerdote, P., 2002. Differential morphine tolerance development in the modulation of macrophage cytokine production in mice. J. Leukoc. Biol. 72 (1), 43–48. https://doi.org/10.1189/jlb.72.1.43.

Lin, S.L., Tsai, R.Y., Tai, Y.H., Cherng, C.H., Wu, C.T., Yeh, C.C., Wong, C.S., 2010. Ultra-low dose naloxone upregulates interleukin-10 expression and suppresses neuroinflammation in morphine-tolerant rat spinal cords. Behav. Brain Res. 207 (1), 30–36. https://doi.org/10.1016/j.bbr.2009.09.034.

Linker, K.E., Cross, S.J., Leslie, F.M., 2019. Glial mechanisms underlying substance use disorders. Eur. J. Neurosci. 50 (3), 2574–2589. https://doi.org/10.1111/ejn.14163.

Liu, J.F., Li, J.X., 2018. Drug addiction: a curable mental disorder. Acta Pharmacol. Sin. 39 (12), 1823–1829. https://doi.org/10.1038/s41401-018-0180-x.

Liu, L., Hutchinson, M.R., White, J.M., Somogyi, A.A., Coller, J.K., 2009. Association of IL-1B genetic polymorphisms with an increased risk of opioid and alcohol dependence. Pharm. Genom. 19 (11), 869–876. https://doi.org/10.1097/FPC.0b013e328331e68f.

Loftis, J.M., Janowsky, A., 2014. Neuroimmune basis of methamphetamine toxicity. In: International Review of Neurobiology, Vol. 118. Academic Press, pp. 165–197. https://doi.org/10.1016/B978-0-12-801284-0.00007-5.

Lorente, J.D., Cuitavi, J., Campos-Jurado, Y., Montón-Molina, R., González-Romero, J.L., Hipólito, L., 2022a. Kappa opioid receptor blockade in the nucleus accumbens shell prevents sex-dependent alcohol deprivation effect induced by inflammatory pain. Pain 163 (1), e137–e147. https://doi.org/10.1097/j.pain.0000000000002332.

Lorente, J.D., Cuitavi, J., Campos-Jurado, Y., Hipólito, L., 2022b. Pain-induced alterations in the dynorphinergic system within the mesocorticolimbic pathway: Implication for alcohol addiction. J. Neurosci. Res. 100 (1), 165–182. https://doi.org/10.1002/jnr.24703.

Ma, R., Kutchy, N.A., Hu, G., 2021. Astrocyte-derived extracellular vesicle–mediated activation of primary ciliary signaling contributes to the development of morphine tolerance. Biol. Psychiatry 90 (8), 575–585. https://doi.org/10.1016/j.biopsych.2021.06.009.

Machelska, H., Celik, M.Ö., 2020. Immune cell-mediated opioid analgesia. Immunol. Lett. 227, 48–59. https://doi.org/10.1016/j.imlet.2020.08.005.

Mahajan, S.D., Schwartz, S.A., Shanahan, T.C., Chawda, R.P., Nair, M.P., 2002. Morphine regulates gene expression of α-and β-chemokines and their receptors on astroglial cells via the opioid μ receptor. J. Immunol. 169 (7), 3589–3599. https://doi.org/10.4049/jimmunol.169.7.3589.

Mahajan, S.D., Schwartz, S.A., Aalinkeel, R., Chawda, R.P., Sykes, D.E., Nair, M.P., 2005. Morphine modulates chemokine gene regulation in normal human astrocytes. Clin. Immunol. 115 (3), 323–332. https://doi.org/10.1016/j.clim.2005.02.004.

Maher, D.P., Walia, D., Heller, N.M., 2019. Suppression of human natural killer cells by different classes of opioids. Anesth. Analg. 128 (5), 1013. https://doi.org/10.1213/ANE.0000000000004058.

Maher, D.P., Walia, D., Heller, N.M., 2020. Morphine decreases the function of primary human natural killer cells by both TLR4 and opioid receptor signaling. Brain Behav., Immun. 83, 298–302. https://doi.org/10.1016/j.bbi.2019.10.011.

Marcos, M., Pastor, I., González-Sarmiento, R., Laso, F.J., 2008. Interleukin-10 gene polymorphism is associated with alcoholism but not with alcoholic liver disease. Alcohol. Alcohol. 43 (5), 523–528. https://doi.org/10.1093/alcalc/agn026.

Matsuda, M., Huh, Y., Ji, R.R., 2019. Roles of inflammation, neurogenic inflammation, and neuroinflammation in pain. J. Anesth. 33 (1), 131–139. https://doi.org/10.1007/s00540-018-2579-4.

Maydych, V., 2019. The interplay between stress, inflammation, and emotional attention: relevance for depression. Front. Neurosci. *13*. https://doi.org/10.3389/fnins.2019.00384.

Mazahery, C., Benson, B.L., Cruz-Lebrón, A., Levine, A.D., 2020. Chronic methadone use alters the CD8+ T cell phenotype in vivo and modulates its responsiveness ex vivo to opioid receptor and TCR stimuli. J. Immunol. 204 (5), 1188–1200. https://doi.org/10.4049/jimmunol.1900862.

McNicol, E., Horowicz-Mehler, N., Fisk, R.A., Bennett, K., Gialeli-Goudas, M., Chew, P. W., Lau, J., Carr, D., 2003. Management of opioid side effects in cancer-related and chronic noncancer pain: a systematic review. J. Pain. 4 (5), 231–256. https://doi.org/10.1016/s1526-5900(03)00556-x.

Melief, E.J. Miyatake, M. Bruchas, M.R. Chavkin, C. (2010). Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc*eedings of the National Academy of Sciences, 107(25), 11608–11613. DOI: ⟨10.1073/pnas.1000751107⟩.

Merighi, S., Gessi, S., Varani, K., Fazzi, D., Stefanelli, A., Borea, P.A., 2013. Morphine mediates a proinflammatory phenotype via μ-opioid receptor–PKCε–Akt–ERK1/2

signaling pathway in activated microglial cells. Biochem. Pharmacol. 86 (4), 487–496. https://doi.org/10.1016/j.bcp.2013.05.027.
Meserve, J.R., Kaye, A.D., Prabhakar, A., Urman, R.D., 2014. The role of analgesics in cancer propagation. Best. Pract. Res. Clin. Anaesthesiol. 28 (2), 139–151. https://doi.org/10.1016/j.bpa.2014.04.004.
Metz, V.E., Jones, J.D., Manubay, J., Sullivan, M.A., Mogali, S., Segoshi, A., Comer, S.D., 2017. Effects of ibudilast on the subjective, reinforcing, and analgesic effects of oxycodone in recently detoxified adults with opioid dependence. Neuropsychopharmacology 42 (9), 1825–1832. https://doi.org/10.1038/npp.2017.70.
Milligan, E.D., Sloane, E.M., Langer, S.J., Hughes, T.S., Jekich, B.M., Frank, M.G., Mahoney, J.H., Levkoff, L.H., Maier, S.F., Cruz, P.E., Fotte, T.R., Johnson, K.W., Mahoney, M.M., Chavez, R.A., Leinwand, L.A., Watkins, L.R., 2006. Repeated intrathecal injections of plasmid DNA encoding interleukin-10 produce prolonged reversal of neuropathic pain. Pain 126 (1–3), 294–308. https://doi.org/10.1016/j.pain.2006.07.009.
Møller, T.C., Pedersen, M.F., van Senten, J.R., Seiersen, S.D., Mathiesen, J.M., Bouvier, M., Bräuner-Osborne, H., 2020. Dissecting the roles of GRK2 and GRK3 in μ-opioid receptor internalization and β-arrestin2 recruitment using CRISPR/Cas9-edited HEK293 cells. Sci. Rep. 10 (1), 1–13. https://doi.org/10.1038/s41598-020-73674-0.
Nakajima, A., Yamada, K., Nagai, T., Uchiyama, T., Miyamoto, Y., Mamiya, T., Seto, A., 2004. Role of tumor necrosis factor-α in methamphetamine-induced drug dependence and neurotoxicity. J. Neurosci. 24 (9), 2212–2225. https://doi.org/10.1523/JNEUROSCI.4847-03.2004.
Nicholas, M., Vlaeyen, J.W., Rief, W., Barke, A., Aziz, Q., Benoliel, R., Treede, R.D., 2019. The IASP classification of chronic pain for ICD-11: chronic primary pain. Pain 160 (1), 28–37. https://doi.org/10.1097/j.pain.0000000000001390.
Norlin, A.K., Walter, S., Icenhour, A., Keita, Å.V., Elsenbruch, S., Bednarska, O., Engström, M., 2021. Fatigue in irritable bowel syndrome is associated with plasma levels of TNF-α and mesocorticolimbic connectivity. Brain Behav. Immun. 92, 211–220. 10.1016/j.bbi.2020.11.035.
Paxinos, G., Watson, C., 2006. The Rat Brain in Stereotaxic Coordinates: Hard Cover Edition. Elsevier.
Peana, A.T., Sánchez-Catalán, M.J., Hipólito, L., Rosas, M., Porru, S., Bennardini, F., Acquas, E., 2017. Mystic acetaldehyde: the never-ending story on alcoholism. Front. Behav. Neurosci. 11, 81, 10.3389/fnbeh.2017.00081.
Peng, J., Pan, J., Wang, H., Mo, J., Lan, L., Peng, Y., 2022. Morphine-induced microglial immunosuppression via activation of insufficient mitophagy regulated by NLRX1. J. Neuroinflamm. 19 (1), 1–21. https://doi.org/10.1186/s12974-022-02453-7.
Peng, Y., Yang, J., Guo, D., Zheng, C., Sun, H., Zhang, Q., Candiotti, K.A., 2020. Sufentanil postoperative analgesia reduce the increase of T helper 17 (Th17) cells and FoxP3+ regulatory T (Treg) cells in rat hepatocellular carcinoma surgical model: a randomised animal study. BMC Anesthesiol. 20 (1), 1–9. https://doi.org/10.1186/s12871-020-01129-0.
Plein, L.M., Rittner, H.L., 2018. Opioids and the immune system–friend or foe. Br. J. Pharmacol. 175 (14), 2717–2725. https://doi.org/10.1111/bph.13750.
Popiolek-Barczyk, K., Makuch, W., Rojewska, E., Pilat, D., Mika, J., 2014. Inhibition of intracellular signaling pathways NF-κB and MEK1/2 attenuates neuropathic pain development and enhances morphine analgesia. Pharmacol. Rep. 66 (5), 845–851. https://doi.org/10.1016/j.pharep.2014.05.001.
Raghavendra, V., Tanga, F.Y., DeLeo, J.A., 2004. Attenuation of morphine tolerance, withdrawal-induced hyperalgesia, and associated spinal inflammatory immune responses by propentofylline in rats. Neuropsychopharmacology 29 (2), 327. https://doi.org/10.1038/sj.npp.1300315.
Raja, S.N., Carr, D.B., Cohen, M., Finnerup, N.B., Flor, H., Gibson, S., Vader, K., 2020. The revised international association for the study of pain definition of pain: concepts, challenges, and compromises. Pain. press. https://doi.org/10.1097/j.pain.0000000000001939.
Reid, K.J., Harker, J., Bala, M.M., Truyers, C., Kellen, E., Bekkering, G.E., Kleijnen, J., 2011. Epidemiology of chronic non-cancer pain in Europe: narrative review of prevalence, pain treatments and pain impact. Curr. Med. Res. Opin. 27 (2), 449–462. https://doi.org/10.1185/03007995.2010.545813.
Reiss, D., Maduna, T., Maurin, H., Audouard, E., Gaveriaux-Ruff, C., 2022. Mu opioid receptor in microglia contributes to morphine analgesic tolerance, hyperalgesia, and withdrawal in mice. J. Neurosci. Res. 100 (1), 203–219. https://doi.org/10.1002/jnr.24626.
Roy, S., Ninkovic, J., Banerjee, S., Charboneau, R.G., Das, S., Dutta, R., Barke, R.A., 2011. Opioid drug abuse and modulation of immune function: consequences in the susceptibility to opportunistic infections. J. Neuroimmun. Pharmacol. 6 (4), 442–465. https://doi.org/10.1007/s11481-011-9292-5.
Ruzicka, B.B., Thompson, R.C., Watson, S.J., Akil, H., 1996. Interleukin-1β-mediated regulation of μ-Opioid receptor mRNA in primary astrocyte-enriched cultures. J. Neurochem. 66 (1), 425–428. https://doi.org/10.1016/s0306-4522(96)00669-0.
Sá, K.N., Moreira, L., Baptista, A.F., Yeng, L.T., Teixeira, M.J., Galhardoni, R., de Andrade, D.C., 2019. Prevalence of chronic pain in developing countries: systematic review and meta-analysis. Pain. Rep. 4 (6) https://doi.org/10.1097/PR9.0000000000000779.
Sanna, M.D., Ghelardini, C., Galeotti, N., 2015. Activation of JNK pathway in spinal astrocytes contributes to acute ultra-low-dose morphine thermal hyperalgesia. Pain 156 (7), 1265–1275. https://doi.org/10.1097/j.pain.0000000000000164.
Santamaria, L.B., Schifilliti, D., La Torre, D., Fodale, V., 2010. Drugs of anaesthesia and cancer. Surg. Oncol. 19 (2), 63–81. https://doi.org/10.1016/j.suronc.2009.03.007.
Schmidlin, F. Déry, O. Bunnett, N.W. Grady, E.F. (2002). Heterologous regulation of trafficking and signaling of G protein-coupled receptors: β-arrestin-dependent interactions between neurokinin receptors. Proceedings of the National Academy of Sciences, 99(5), 3324–3329. DOI: (10.1073/pnas.052161299).
Schwarz, J.M., Bilbo, S.D., 2013. Adolescent morphine exposure affects long-term microglial function and later-life relapse liability in a model of addiction. J. Neurosci. 33 (3), 961–971. https://doi.org/10.1523/JNEUROSCI.2516-12.2013.
Sekandarzad, M.W., van Zundert, A.A., Lirk, P.B., Doornebal, C.W., Hollmann, M.W., 2017. Perioperative anesthesia care and tumor progression. Anesth. Analg. 124 (5), 1697–1708. https://doi.org/10.1213/ANE.0000000000001652.
Sharonov, G.V., Serebrovskaya, E.O., Yuzhakova, D.V., Britanova, O.V., Chudakov, D.M., 2020. B cells, plasma cells and antibody repertoires in the tumour microenvironment. Nat. Rev. Immunol. 20 (5), 294–307. https://doi.org/10.1038/s41577-019-0257-x.
Shavit, Y., Wolf, G., Goshen, I., Livshits, D., Yirmiya, R., 2005. Interleukin-1 antagonizes morphine analgesia and underlies morphine tolerance. Pain 115 (1–2), 50–59. https://doi.org/10.1016/j.pain.2005.02.003.
Shenoy, S.S.and Lui, F. (2018). Biochemistry, Endogenous Opioids. *StatPearls*. Bookshelf ID: NBK532899.
Shrivastava, P., Cabrera, M.A., Chastain, L.G., Boyadjieva, N.I., Jabbar, S., Franklin, T., Sarkar, D.K., 2017. Mu-opioid receptor and delta-opioid receptor differentially regulate microglial inflammatory response to control proopiomelanocortin neuronal apoptosis in the hypothalamus: effects of neonatal alcohol. J. Neuroinflamm. 14 (1), 1–19. https://doi.org/10.1186/s12974-017-0844-3.
Sil, S., Periyasamy, P., Guo, M.L., Callen, S., Buch, S., 2018. Morphine-mediated brain region-specific astrocytosis involves the ER stress-autophagy axis. Mol. Neurobiol. 55 (8), 6713–6733. https://doi.org/10.1007/s12035-018-0878-2.
Song, F.H., Liu, D.Q., Zhou, Y.Q., Mei, W., 2022. SIRT1: a promising therapeutic target for chronic pain. CNS Neurosci. Ther. https://doi.org/10.1111/cns.13838.
Song, P., Zhao, Z.Q., 2001. The involvement of glial cells in the development of morphine tolerance. Neurosci. Res. 39 (3), 281–286. https://doi.org/10.1016/s0168-0102(00)00226-1.
Souza, J.B.D., Grossmann, E., Perissinotti, D.M.N., Oliveira Junior, J.O.D., Fonseca, P.R. B.D., Posso, I.D.P., 2017. Prevalence of chronic pain, treatments, perception, and interference on life activities: Brazilian population-based survey. Pain. Res. Manag. 2017 https://doi.org/10.1155/2017/4643830.
Steingrímsdóttir, Ó.A., Landmark, T., Macfarlane, G.J., Nielsen, C.S., 2017. Defining chronic pain in epidemiological studies: a systematic review and meta-analysis. Pain 158 (11), 2092–2107. https://doi.org/10.1097/j.pain.0000000000001009.
Sun, J., Ramnath, R.D., Zhi, L., Tamizhselvi, R., Bhatia, M., 2008. Substance P enhances NF-κB transactivation and chemokine response in murine macrophages via ERK1/2 and p38 MAPK signaling pathways. Am. J. Physiol. -Cell Physiol. 294 (6), C1586–C1596. https://doi.org/10.1152/ajpcell.00129.2008.
Sung, C.S., Wen, Z.H., Chang, W.K., Chan, K.H., Ho, S.T., Tsai, S.K., Chang, Y.C., Wong, C.S., 2005. Inhibition of p38 mitogen-activated protein kinase attenuates interleukin-1β-induced thermal hyperalgesia and inducible nitric oxide synthase expression in the spinal cord. J. Neurochem. 94 (3), 742–752. https://doi.org/10.1111/j.1471-4159.2005.03226.x.
Tajerian, M., Leu, D., Zou, Y., Sahbaie, P., Li, W., Khan, H., Hsu, V., Kingery, W., Huang, T.T., Becerra, L., Clark, J.D., 2014. Brain neuroplastic changes accompany anxiety and memory deficits in a model of complex regional pain syndrome. Anesthesiol.: J. Am. Soc. Anesthesiol. 121 (4), 852–865. https://doi.org/10.1097/ALN.0000000000000403.
Taylor, A.M., Castonguay, A., Ghogha, A., Vayssiere, P., Pradhan, A.A., Xue, L., De Koninck, Y., 2016. Neuroimmune regulation of GABAergic neurons within the ventral tegmental area during withdrawal from chronic morphine. Neuropsychopharmacology 41 (4), 949–959. https://doi.org/10.1038/npp.2015.221.
Tekieh, E., Zaringhalam, J., Manaheji, H., Maghsoudi, N., Alani, B., Zardooz, H., 2011. Increased serum IL-6 level time-dependently regulates hyperalgesia and spinal mu opioid receptor expression during CFA-induced arthritis. EXCLI J. 10, 23. https://doi.org/10.17877/DE290R-8841.
Thompson, A.A., Liu, W., Chun, E., Katritch, V., Wu, H., Vardy, E., Huang, X., Trepella, C., Guerrini, R., Calo, G., Roth, B.L., Cherezov, V., Stevens, R.C., 2012. Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. Nature 485 (7398), 395. https://doi.org/10.1038/nature11085.
Tian, Y., Liu, M., Mao-Ying, Q.L., Liu, H., Wang, Z.F., Zhang, M.T., Wang, J., Li, Q., Liu, S. B., Mi, W.L., Ma, H.J., Wu, G.C., Wang, Y.Q., 2015. Early single Aspirin-triggered Lipoxin blocked morphine anti-nociception tolerance through inhibiting NALP1 inflammasome: involvement of PI3k/Akt signaling pathway. Brain Behav. Immun. 50, 63–77. https://doi.org/10.1016/j.bbi.2015.06.016.
Tsai, R.Y., Jang, F.L., Tai, Y.H., Lin, S.L., Shen, C.H., Wong, C.S., 2008. Ultra-low-dose naloxone restores the antinociceptive effect of morphine and suppresses spinal neuroinflammation in PTX-treated rats. Neuropsychopharmacology 33 (11), 2772. https://doi.org/10.1038/sj.npp.1301672.
Tsai, R.Y., Wang, J.C., Chou, K.Y., Wong, C.S., Cherng, C.H., 2016. Resveratrol reverses morphine-induced neuroinflammation in morphine-tolerant rats by reversal HDAC1 expression. J. Formos. Med. Assoc. 115 (6), 445–454. https://doi.org/10.1016/j.jfma.2015.05.010.
Vanderwall, A.G., Milligan, E.D., 2019. Cytokines in pain: harnessing endogenous anti-inflammatory signaling for improved pain management. Front. Immunol. 10, 3009. https://doi.org/10.3389/fimmu.2019.03009.
Vidal, E.L., Patel, N.A., Fiala, M., Chang, S.L., 1998. Interleukin-1 induces the expression of μ opioid receptors in endothelial cells. Immunopharmacology 38 (3), 261–266. https://doi.org/10.1016/s0162-3109(97)00085-4.
Vivier, E., Tomasello, E., Baratin, M., Walzer, T., Ugolini, S., 2008. Functions of natural killer cells. Nat. Immunol. 9 (5), 503–510. https://doi.org/10.1038/ni1582.

Vonder Haar, C., Ferland, J.M.N., Kaur, S., Riparip, L.K., Rosi, S., Winstanley, C.A., 2018. Cocaine self-administration is increased after frontal traumatic brain injury and associated with neuroinflammation. Eur. J. Neurosci. https://doi.org/10.1111/ejn.14123.

Wagley, Y., Hwang, C.K., Lin, H.Y., Kam, A.F., Law, P.Y., Loh, H.H., Wei, L.N., 2013. Inhibition of c-Jun NH2-terminal kinase stimulates mu opioid receptor expression via p38 MAPK-mediated nuclear NF-κB activation in neuronal and non-neuronal cells. Biochim. Et. Biophys. Acta (BBA)-Mol. Cell Res. 1833 (6), 1476–1488. https://doi.org/10.1016/j.bbamcr.2013.02.017.

Watkins, L.R., Maier, S.F., 2005. Immune regulation of central nervous system functions: from sickness responses to pathological pain. J. Intern. Med. 257 (2), 139–155. https://doi.org/10.1111/j.1365-2796.2004.01443.x.

Watkins, L.R., Hutchinson, M.R., Johnston, I.N., Maier, S.F., 2005. Glia: novel counter-regulators of opioid analgesia. Trends Neurosci. 28 (12), 661–669. https://doi.org/10.1016/j.tins.2005.10.001.

Watkins, L.R., Hutchinson, M.R., Ledeboer, A., Wieseler-Frank, J., Milligan, E.D., Maier, S.F., 2007. Glia as the "bad guys": implications for improving clinical pain control and the clinical utility of opioids. Brain Behav. Immun. 21 (2), 131–146. https://doi.org/10.1016/j.bbi.2006.10.011.

Willemen, H.L., Eijkelkamp, N., Wang, H., Dantzer, R., Dorn II, G.W., Kelley, K.W., Heijnen, C.J., Kavelaars, A., 2010. Microglial/macrophage GRK2 determines duration of peripheral IL-1β-induced hyperalgesia: contribution of spinal cord CX3CR1, p38 and IL-1 signaling. Pain 150 (3), 550–560. https://doi.org/10.1016/j.pain.2010.06.015.

Xiao, J., Zeng, S., Wang, X., Babazada, H., Li, Z., Liu, R., Yu, W., 2016. Neurokinin 1 and opioid receptors: relationships and interactions in nervous system. Transl. Perioper. Pain. Med. 1 (3), 11. https://doi.org/10.31480/2330-4871/039.

Xu, J.T., Zhao, J.Y., Zhao, X., Ligons, D., Tiwari, V., Atianjoh, F.E., Lee, C.Y., Liang, L., Zang, W., Njoku, D., Raja, S.N., Yaster, M., Tao, Y.X., 2014. Opioid receptor–triggered spinal mTORC1 activation contributes to morphine tolerance and hyperalgesia. J. Clin. Investig. 124 (2), 592–603. https://doi.org/10.1172/JCI70236.

Zalcman, S.S., 2001. Interleukin-2 potentiates novelty-and GBR 12909-induced exploratory activity. Brain Res. 899 (1–2), 1–9.

Zaringhalam, J., Tekieh, E., Manaheji, H., Akhtari, Z., 2013. Cellular events during arthritis-induced hyperalgesia are mediated by Interleukin-6 and p38 MAPK and their effects on the expression of spinal mu-opioid receptors. Rheumatol. Int. 33 (9), 2291–2299. https://doi.org/10.1007/s00296-013-2715-2.

Zhang, P., Yang, M., Chen, C., Liu, L., Wei, X., Zeng, S., 2020. Toll-like receptor 4 (TLR4)/opioid receptor pathway crosstalk and impact on opioid analgesia, immune function, and gastrointestinal motility. Front. Immunol. 11, 1455. https://doi.org/10.3389/fimmu.2020.01455.

Zheng, H., Chu, J., Zhang, Y., Loh, H.H., Law, P.Y., 2011. Modulating μ-opioid receptor phosphorylation switches agonist-dependent signaling as reflected in PKCε activation and dendritic spine stability. J. Biol. Chem. 286 (14), 12724–12733. https://doi.org/10.1074/jbc.M110.177089.

Zhou, J., Ma, R., Jin, Y., Fang, J., Du, J., Shao, X., Fang, J., 2021. Molecular mechanisms of opioid tolerance: From opioid receptors to inflammatory mediators. Exp. Ther. Med. 22 (3), 1–8. https://doi.org/10.3892/etm.2021.10437.

Zieglgänsberger, W., 2019. Substance P and pain chronicity. Cell Tissue Res. 375 (1), 227–241. https://doi.org/10.1007/s00441-018-2922-y.

CHAPTER 4

Objectives

The general aim of the present doctoral thesis is to study the relationship between Mu-Opioid receptors (MORs) and the immune system, and to investigate the role this relationship plays in pain-induced alcohol use disorders. With the aim of developing this main objective, and with the use of techniques of cellular and molecular biology, the following specific objectives are going to be tackled:

Objective 1. Assessing MORs role as neuroinflammation inducers in the mesocorticolimbic system (MCLS) through microglial activation and observing the effect of pain on it.

Objective 2. Observing if neuroinflammation modulates neuronal MOR activity, trafficking, and expression.

Objective 3. Studying the sex-dependent pain-induce alcohol relapse phenomenon and the role that the MOR-neuroinflammation relationship plays in it.

CHAPTER 5

Mu-Opioid Receptors: Neuroinflammation Drivers

Focal Mu-Opioid Receptor Activation Promotes Neuroinflammation and Microglial Activation in The Mesocorticolimbic System: Alterations Induced by Inflammatory Pain. Cuitavi J, Andrés-Herrera P, Meseguer D, Campos-Jurado Y, Lorente JD, Caruana H, and Hipólito L. *Glia, 2023*.

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RESEARCH ARTICLE

Focal mu-opioid receptor activation promotes neuroinflammation and microglial activation in the mesocorticolimbic system: Alterations induced by inflammatory pain

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Abstract

Microglia participates in the modulation of pain signaling. The activation of microglia is suggested to play an important role in affective disorders that are related to a dysfunction of the mesocorticolimbic system (MCLS) and are commonly associated with chronic pain. Moreover, there is evidence that mu-opioid receptors (MORs), expressed in the MCLS, are involved in neuroinflammatory events, although the way by which they do it remains to be elucidated. In this study, we propose that MOR pharmacological activation within the MCLS activates and triggers the local release of proinflammatory cytokines and this pattern of activation is impacted by the presence of systemic inflammatory pain. To test this hypothesis, we used in vivo microdialysis coupled with flow cytometry to measure cytokines release in the nucleus accumbens and immunofluorescence of IBA1 in areas of the MCLS on a rat model of inflammatory pain. Interestingly, the treatment with DAMGO, a MOR agonist locally in the nucleus accumbens, triggered the release of the IL1α, IL1β, and IL6 proinflammatory cytokines. Furthermore, MOR pharmacological activation in the ventral tegmental area (VTA) modified the levels of IBA1-positive cells in the VTA, prefrontal cortex, the nucleus accumbens and the amygdala in a dose-dependent way, without impacting mechanical nociception. Additionally, MOR blockade in the VTA prevents DAMGO-induced effects. Finally, we observed that systemic inflammatory pain altered the IBA1 immunostaining derived from MOR activation in the MSCLS. Altogether, our results indicate that the microglia–MOR relationship could be pivotal to unravel some inflammatory pain-induced comorbidities related to MCLS dysfunction.

KEYWORDS
cytokines, inflammatory pain, microglia, mu-opioid receptor, neuroinflammation

1 | INTRODUCTION

Microglial cells are macrophages that reside in the central nervous system. Among many other functions, they stand out for protecting neurons and other glial cells. However, under nonpathological circumstances, microglia also promotes both neuronal survival and death, participates in synaptogenesis and is constantly communicating with other cells (Li & Barres, 2018). Altogether, these functions tightly link microglia and pain, which makes microglia a target to consider when treating pain conditions (Hiraga et al., 2022). At the level of the spinal cord, pain induces an activated state in microglia, which triggers the release of neuroinflammatory mediators that modulate the response against the pain condition and, sometimes, may account for the chronicity of that pain condition (Hains & Waxman, 2006; Takeura et al., 2019; Taves et al., 2013). Nonetheless, very little is known of how the presence of a pain condition affects microglial activity within the different brain subareas. This is unfortunate since most of pain comorbidities, such as negative affect or drug use disorders, involve neuroplastic and neurochemical alterations in brain nuclei in which microglia play a pivotal role (Burkovetskaya et al., 2020; Cuitavi, Lorente, et al., 2021; Han et al., 2020; Jia et al., 2021; Melbourne et al., 2021; Taylor et al., 2015, 2016).

The mesocorticolimbic system (MCLS) is one of the most compromised systems in pain comorbidities (Koo et al., 2019; Nikolaus et al., 2019; Shi et al., 2021). The MCLS processes reward, aversion, and motivated behavior. Natural reinforcers activate this system to facilitate behaviors that lead to survival (Fields et al., 2007). To do so, it comprises brain areas that play a crucial role in reward management. Concretely, nucleus accumbens (NAc), prefrontal cortex (PFC), extended amygdala, ventral tegmental area (VTA), hippocampus and hypothalamus are the main brain structures that shape the neural reward circuitry. Among them, VTA stands out for being the origin of the dopaminergic neurons that project to all the others (Fields et al., 2007). Mu-opioid receptors (MORs) are metabotropic receptors involved in the modulation of the MCLS. In fact, one of their main roles in the VTA consists in controlling the firing rates of the resident dopamine neurons (Devine et al., 1993; Hipólito et al., 2011; Svingos et al., 2001).

MORs is tightly related to pain management (Cuitavi, Hipólito, & Canals, 2021). In fact, they also seem to be the key to unravel MCLS-based pain comorbidities (Campos-Jurado et al., 2019, 2022). Furthermore, there is growing evidence that MORs play a role in the regulation of immune mechanisms (Cuitavi et al., 2022; Zhang et al., 2020). Interestingly, microglial cells express MORs in the brain and the spinal cord (Coffey et al., 2022; Leduc-Pessah et al., 2017; Maduna et al., 2019), which might explain MORs control over inflammation under pain conditions (Machelska & Celik, 2020). However, the implication of MORs in MCLS-based pain comorbidities, although suggested, is far from being clear.

Chronic pain is a big burden for public health systems since approximately 18% of people in developed countries suffer from it (Sá et al., 2019). Moreover, there is a high prevalence of neuropsychiatric affections that comprise alterations in the MCLS in chronic pain patients (Foley et al., 2021), which difficult the adequate therapeutical management of pain itself but also the associated comorbid situations. Understanding how pain interacts with microglial cells that reside in the MCLS and how MORs modulate them alongside pro-inflammatory mediators will very possibly hint new targets to address these issues and improve patients' quality of life. In this article, we present how the pharmacological activation of MORs within the NAc core (NAcC) elicits the release of pro-inflammatory cytokines. In this sense, we suggest that this effect might be related to microglial activity since MORs activation in the VTA enhances ionized calcium-binding adapter molecule 1 (IBA1) expression in a dose-dependent manner. Furthermore, we describe how microglial cells in other areas of the MCLS with projections coming from VTA also modified their activity at the terminal level when a MOR agonist is administered intra-VTA. In addition to that, the existence of a pain condition alters these observed effects.

2 | MATERIALS AND METHODS

2.1 | Animals

Eighty-four male and female Sprague Dawley (Rattus norvegicus) rats (300–340 g) were used (Envigo®, Barcelona, Spain). All the animals were kept in light/dark (12/12 h) controlled cycles, temperature 23 ± 1°C, and 60% humidity. Animal were housed in standard plastic cages (42 × 27 × 18 cm^3) with food and tap water provided ad libitum throughout the experimental period. Rats were housed in the animal facilities of the SCSIE from the University of Valencia. Animal protocols followed in this work were approved by the Animal Care Committee of the University of Valencia, and were strictly adhered to in compliance with the EEC Council Directive 63/2010, Spanish laws (RD 53/2013) and animal protection policies.

2.2 | Surgeries

All surgeries were performed under isoflurane (1.5–2 minimum alveolar concentration, MAC) anesthesia under aseptic conditions. For the experiments rats were treated intra-VTA, animals were stereotaxically implanted (Stoelting) with a cannula (26G, Plastics One) targeting the posterior VTA (anteroposterior = −6.0 mm, mediolateral = 1.9 mm, dorsoventral = −8.1 mm) angled 10° from perpendicular axes (Campos-Jurado et al., 2019). Cannulae were implanted in a counterbalanced fashion for the hemisphere. For the microdialysis experiment, rats were stereotaxically implanted with bilateral vertical cannulae (CMA) into the NAcC (Males–anteroposterior = 1.4 mm, mediolateral = 1.5 mm and dorsoventral = 5.8 mm; Females–anteroposterior = 1.2 mm,

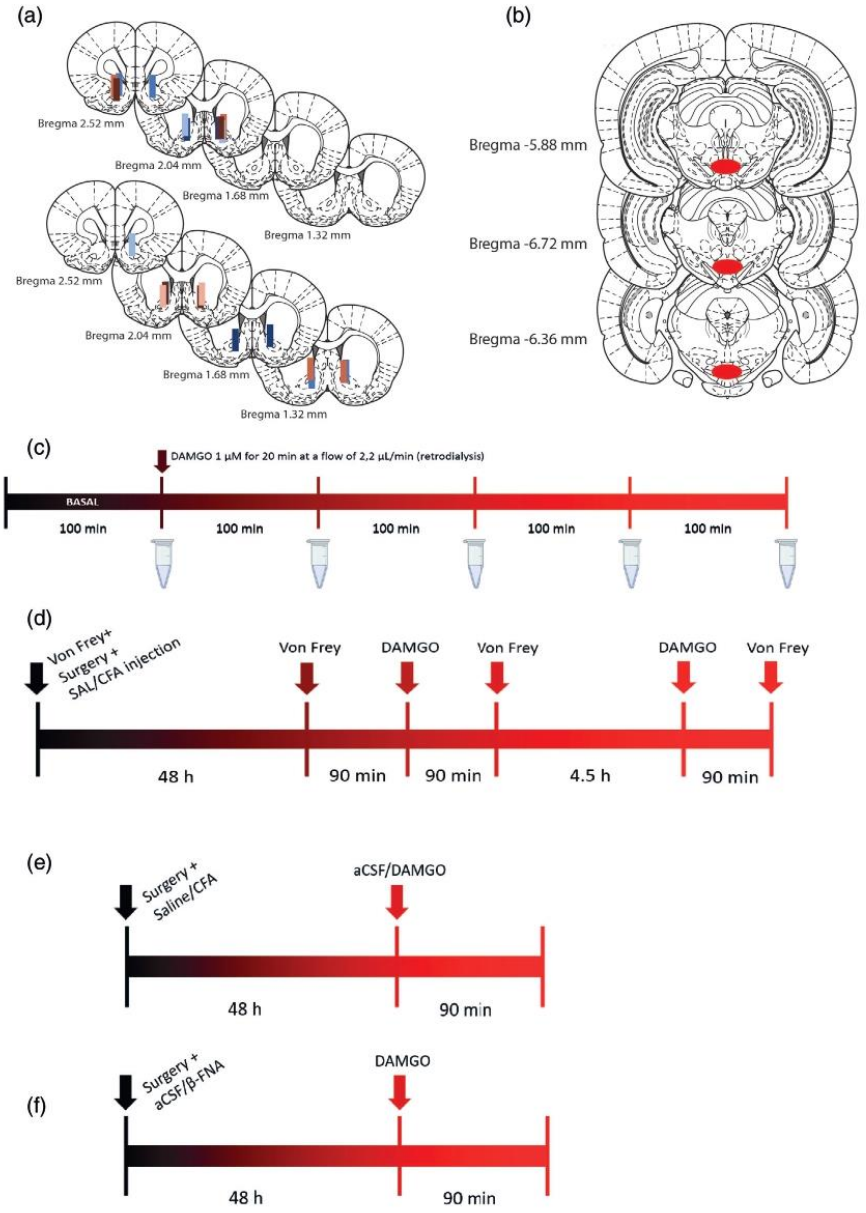

FIGURE 1 Cannulae placements and experimental designs. (a) Diagram of coronal sections indicating the placement of microdialysis probes. (b) Diagram of coronal sections indicating the placement of the tip of the injectors at the moment of the treatment. All the injectors were included in the red circle area. (c) Schematic of the microdialysis experiment design. (d) Schematic of the Von Frey experiment design. (e) Schematic of the IBA1 experiment design. (f) Schematic of the MOR blockade experiment design. aCSF, artificial cerebrospinal fluid; CFA, complete Freund adjuvant; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; SAL, saline; β-FNA, β-Funaltrexamine.

mediolateral = 1.4 mm and dorsoventral = 5.6 mm). The placement of all cannulae was assessed with a cresyl violet dying and represented in Figure 1a (microdialysis) and Figure 1b (microinjections).

2.3 | Experimental procedures

2.3.1 | DAMGO influence in cytokines production in the NAc

Five males and six females underwent a microdialysis experiment (Figure 1c). 48 h after the sterotaxical surgery animals were placed in Plexiglas bowls and concentric-style microdialysis probes containing 2 mm of active membrane (CMA; molecular cutoff 100,000 Da) was introduced through the cannulae, extending 3.0 mm below the tip of the cannulae. Then artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$ at pH = 6) containing 0.5 mg/mL BSA was introduced into the probe through a push-pull system, which consisted of an inlet tubing attached to a 2.5-mL syringe (Hamilton) mounted on a syringe pump (Harvard instruments) and an outlet tubing connected to a peristaltic pump (Ismatec®). Pumps were set at 2.2 μL/min. After a 1 h wash of the system, five dialysates were collected every 100 min. After the collection of the first dialysate, a treatment with 1 μm [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) was introduced through the microdialysis probe by retrodialysis for 20 min. The levels of IL4, IL1α, IL1β, and IL6 were measured in the dialysates by flow cytometry (Fortessa) using a customized multiplex approach (LEGENDplex™, BioLegend®).

2.3.2 | Intra-VTA DAMGO treatment influence in mechanical nociception

Eleven male rats underwent this experiment, the scheme of which is represented in Figure 1d. We selected the complete Freund adjuvant model (CFA) of inflammatory pain. CFA (Calbiochem) was prepared and injected as previously reported (Cuitavi, Lorente, et al., 2021; Lorente et al., 2022). Six rats were injected with CFA in the hind paw whereas five rats were injected with sterile saline as control. All the animals underwent VTA cannulae placement surgery the same day the CFA model was introduced.

Prior to the surgery, mechanical nociception thresholds were measured by the Von Frey test. The protocol started with a 20-min period of habituation of the behavioral boxes and the room where the test was performed. After this habituation period, we manually applied five filaments (Aesthesio, San José, CA) following a simplified up–down method, as previously described (Lorente et al., 2022). Results are expressed in grams (g). The 48 h after the surgery, the Von Frey test was repeated to assess basal nociception of saline and CFA rats before any treatment. Then all animals were microinjected. Intra-VTA microinjections were carried out with 33G stainless steel injectors, extending 1.0 mm below the tip of the cannulae. Six animals were treated with 7 ng DAMGO, whereas the resting five were microinjected with 14 ng DAMGO. Ninety minutes after the microinjections, the Von Frey test was repeated once again. Six hours after the first microinjections, new ones were carried out in a way that animals that previously received 7 ng DAMGO now received 14 ng DAMGO and vice versa in a latin-square design. The Von Frey test was then repeated 90 min after the microinjections and then animals were sacrificed with an isoflurane overdose.

2.3.3 | Intra-VTA DAMGO influence in IBA1 expression within the MCLS: Effect of inflammatory pain

Forty male rats underwent this procedure. Figure 1e shows the chronology of this experiment. All animals were implanted with cannulae in the VTA. We selected the complete CFA of inflammatory pain as in the previous section. The injection of CFA in the hind paw was performed at the same time of the surgery. Sterile saline was used as control.

Intra-VTA microinjections (aCSF, 7 ng DAMGO or 14 ng DAMGO) were carried out 48 h after the surgery with 33G stainless steel injectors, extending 1.0 mm below the tip of the cannulae. Microinjections were carried out as previously described (200 nL, flow rate of 0.6 mL/min) (Campos-Jurado et al., 2019). The saline/CFA injection and the intra-VTA treatment were the factors that defined our experimental groups: saline + aCSF ($n = 7$), CFA + aCSF ($n = 7$), saline + DAMGO 7 ng ($n = 5$), CFA + DAMGO 7 ng ($n = 5$), saline + DAMGO 14 ng ($n = 9$), and CFA + DAMGO 14 ng ($n = 7$).

Microglia proliferation was assessed by measuring IBA1 expression with an immunofluorescence assay (Cuitavi, Lorente, et al., 2021), since IBA1 is considered a marker for microglial activation (Lier et al., 2021). To do so, 90 min after the microinjections, animals were anesthetized by injecting pentobarbital and followed a procedure of cardiac perfusion with 200 mL paraformaldehyde 0.4% in phosphate buffer (PB) 0.1 M. Brains were extracted and kept in the same perfusion solution for 20 h at 4°C. After that, they were transferred to sucrose 30% in PB 0.1 M for 3 days. Consecutively, brains were cut in slices of 40 μm on a freezing microtome and were stored at −80°C in sucrose 30% in PB 0.1 M until their use. Immunofluorescence was performed as previously described (Cuitavi, Lorente, et al., 2021). The rabbit IgG anti-IBA1 (1:2000, Wako) primary antibody and the donkey IgG anti-rabbit Alexa Fluor® 594 (1:1000, Abcam) secondary antibody was used.

Images from VTA and its projection areas, NAcC, NAc shell (NAcS), hippocampal CA1 region, basolateral amygdala (BLA), central amygdala (CeA), cingulate cortex (CgL), infralimbic cortex (IL), prelimbic cortex (PrL) were obtained with a 20× objective (Leica Biosystems, Gemany; Images size 441 × 330 μm). We obtained four images per area, counted the total number of IBA1-positive cells and expressed the results as the average of those four images. Primary motor cortex (M1) was used as control since it is a nonprojection area from VTA, and it does not play a role in pain management. Then we counted the number of IBA1-positive cells and normalize the data as percentage of the saline-aCSF group.

2.3.4 | Effect of pharmacological blockade of MORs in the VTA on DAMGO-induced IBA1 alterations

Twelve male rats were used for this experiment. Figure 1f shows the chronology of this experiment. All the animals were implanted

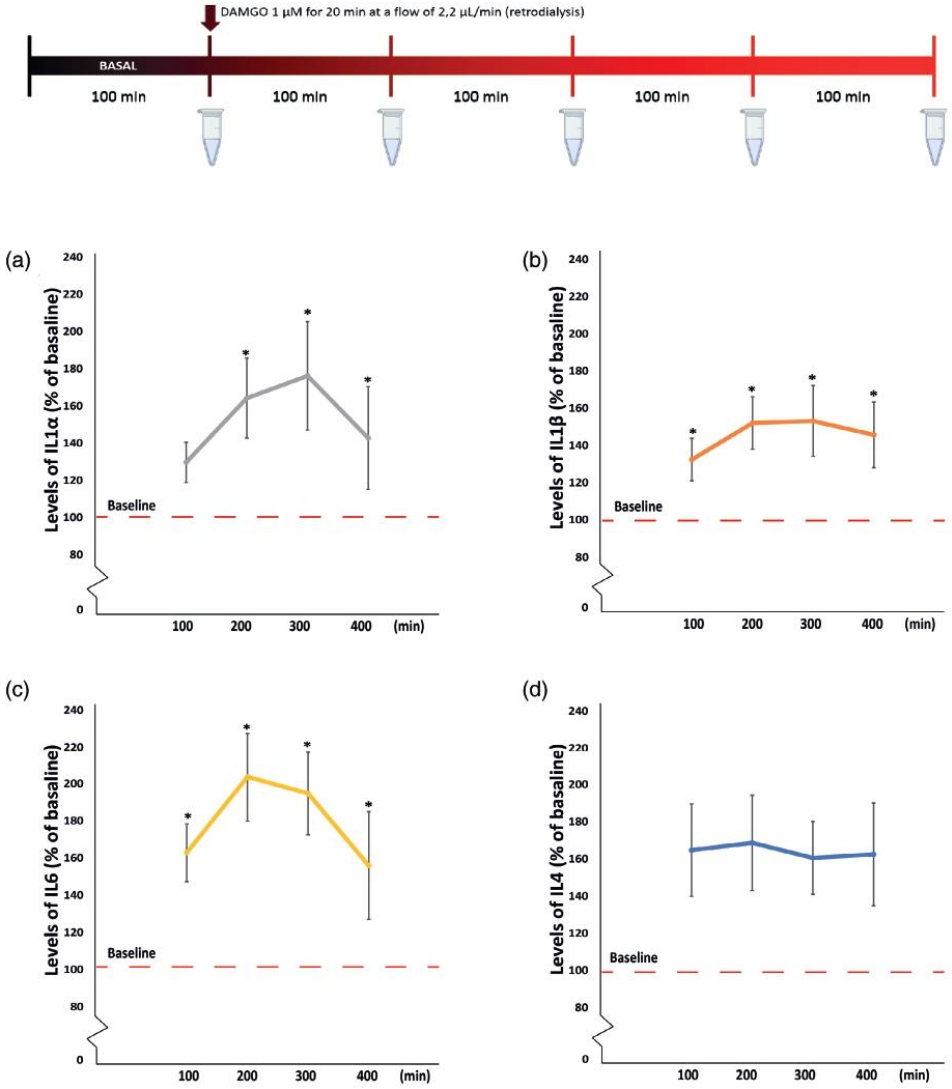

FIGURE 2 DAMGO intra-NAc increases cytokines local extracellular levels. Levels of the measured cytokines (Expressed in % of baseline) along time post DAMGO administration by retrodialysis. Data are expressed as mean ± SEM. *$p < .05$ (ANOVA repeated measures followed by Dunnett). (a) IL1α; (b) IL1β; (c) IL6; (d) IL4. DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; IL, interleukin.

with cannulae in the VTA. Intra-VTA microinjections of aCSF ($n = 6$) or 1.251 nmol β-Funaltrexamine (β-FNA; $n = 6$) were carried out right after the surgery with 33G stainless steel injectors, extending 1.0 mm below the tip of the cannulae. Forty-eight hours after, new Intra-VTA microinjections of 7 ng DAMGO were performed. Ninety minutes after, these animals were sacrificed and IBA1 levels measured in the VTA following the processes explained in the previous section.

2.4 | Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). To perform the statistical analysis, the 26.0 version of the SPSS program was used. The Kolmogorov–Smirnov test and Levene's test were performed to assess the normality and the homogeneity of the variance of the data. Then, for the microdialysis and the Von Frey experiments a repeated measures, ANOVA was performed. This repeated

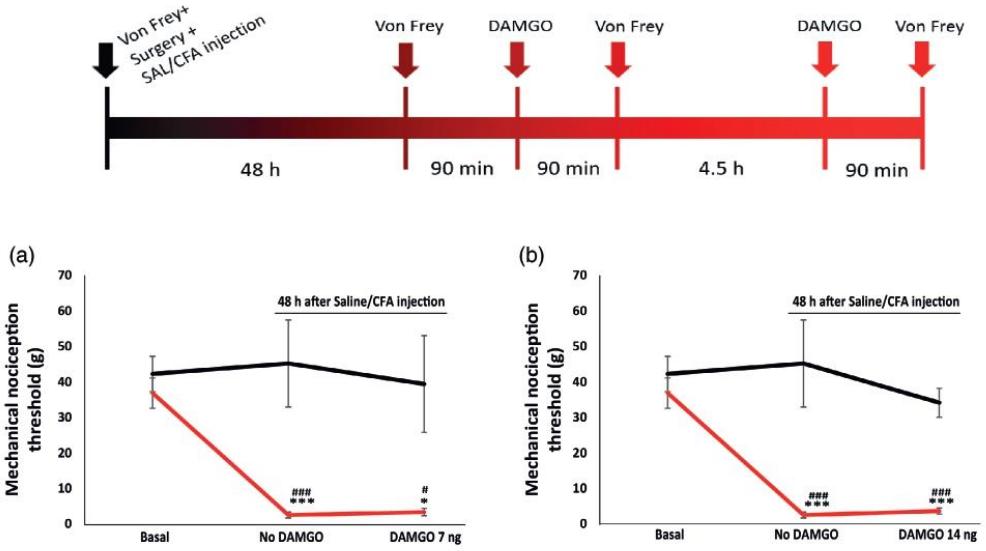

FIGURE 3 DAMGO administered into the VTA does not alter mechanical nociception. Data are expressed as mean ± SEM of g ($n = 5–6$/group). Data were collected at three different time points: basal (prior to the SAL/CFA injections), No DAMGO (48 h after the SAL/CFA injections), DAMGO (90 min after DAMGO administration). Black lines represent animals injected with SAL whereas red lines represent animals injected with CFA. *Differences with basal. #Differences SAL-CFA. *$p < .05$, ***$p < .005$ (Repeated measures ANOVA followed by Bonferroni multiple comparisons). (a) 7 ng DAMGO; (b) 14 ng DAMGO. CFA, complete Freund adjuvant; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; SAL, saline.

measures ANOVA was followed by Dunnett in the microdialysis experiment to compare intrasubjects post-treatment effects with baseline when significant differences were found, and by Bonferroni in the Von Frey experiments. For IBA1 measurement, a two-way ANOVA test was performed followed by Bonferroni multiple comparisons when significant differences in the main effects (pain; treatment) or in their interaction were detected. The only exception was in the experiment involving β-FNA, for which a Student's *t* test for independent samples was used. In all cases a 95%, confidence level was set.

3 | RESULTS

3.1 | DAMGO induces pro-inflammatory cytokines release in the NAcC

Microdialysis and flow cytometry were used to assess cytokine levels over time after a treatment with DAMGO in the NAcC. Figure 2 shows the graphs for the levels of IL4, IL1α, IL1β, and IL6. Interestingly, no significant differences were found between males and females, thus the data shown is the combination of both sexes. The repeated measures ANOVA performed for each cytokine showed that only pro-inflammatory cytokines significantly increased their levels over baseline after a treatment with DAMGO (Figure 2a, IL1α $F(1,18) = 2.983$, $p = .024$; Figure 2b, IL1β $F(1,18) = 3.303$, $p = .015$; Figure 2c, IL6 $F(1,18) = 5.264$, $p = .001$) whereas the anti-inflammatory cytokine IL4 did not show any statistically significant changes (Figure 2d, $F(1,18) = 2.714$, $p = .117$). It is important to note that levels of pro-inflammatory cytokines increased in the first dialysates and then decreased in the last ones meaning that the pharmacological effect of DAMGO over MORs is limited.

3.2 | Intra-VTA 7 and 14 ng DAMGO does not alter mechanical nociception

Von Frey test was used to measure possible changes induced by DAMGO in mechanical nociception. Figure 3 shows the graphs for the mechanical nociception threshold before and after the saline and CFA injections, and the DAMGO microinjections. The repeated measures ANOVA performed showed differences in the mechanical nociception threshold along time between both groups when animals were microinjected with 7 ng DAMGO (Figure 3a, $F(1,9) = 83.064$, $p = .0001$) and 14 ng DAMGO (Figure 3b, $F(1,9) = 79.485$, $p = .0001$). Post hoc tests revealed that there is a decrease in the mechanical nociception threshold after the CFA injection, which does not occur with the animals injected with saline. Moreover, both doses of DAMGO fail to alter mechanical nociception when microinjected intra-VTA.

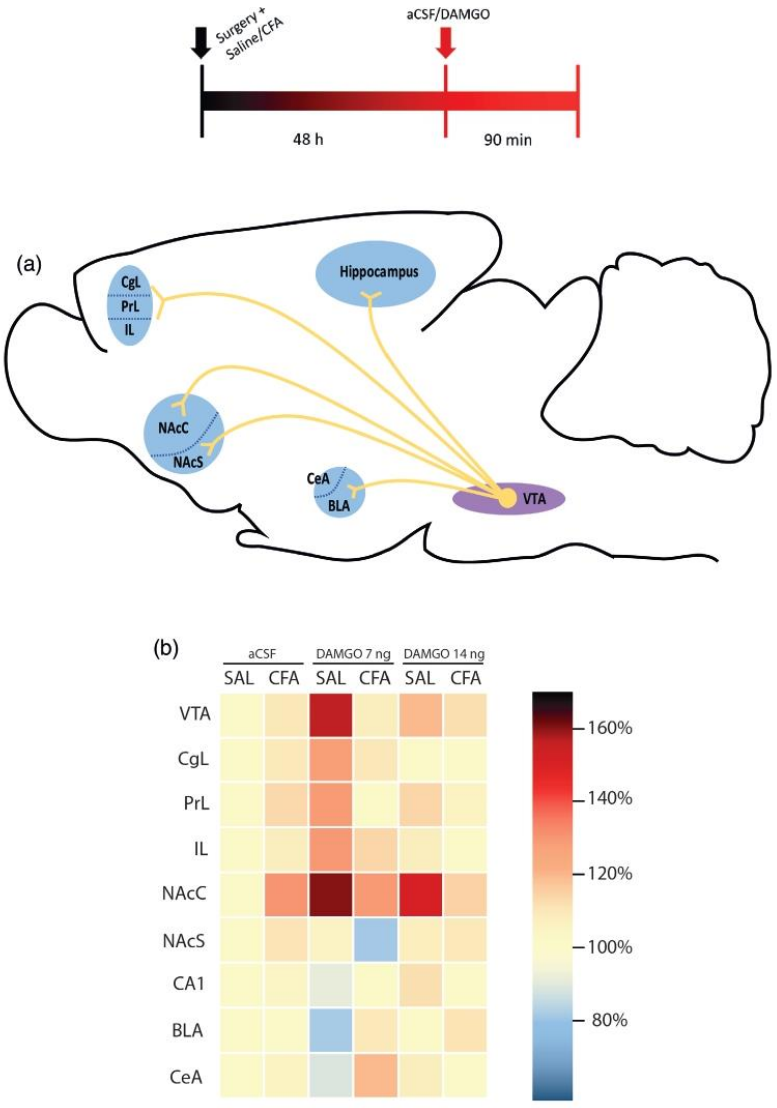

FIGURE 4 DAMGO administered into the VTA produces dose-dependent and area-dependent alterations in the number of IBA1-positive cells. (a) Schematic of the VTA projection areas in the MCLS analyzed in this study. (b) Heatmap indicating the levels of IBA1-positive cells (Expressed in % of the SAL-aCSF group) in each brain area after the microinjections and the SAL/CFA injection. Data are expressed as mean ± SEM of IBA1-positive cells ($n = 5–9$/group). aCSF, artificial cerebrospinal fluid; BLA, basolateral amygdala; CeA, central amygdala; CFA, complete Freund adjuvant; CgL, cingulate cortex; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; IBA1, ionized calcium-binding adapter molecule 1; IL, infralimbic cortex; MCLS, mesocorticolimbic system; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; PrL, prelimbic cortex; SAL, Saline; VTA, ventral tegmental area.

3.3 | MORs pharmacological activation alters the number of IBA-1 positive cells in the area of injection and its areas of projection in a dose-dependent manner: Difference between saline and CFA animals

Figure 4 shows a schematic representation of VTA and its projection areas (Figure 4a) and a heatmap created to compare differences between groups in an easier way (Figure 4b). Figure 5 shows representative pictures of the IBA1 immunostaining in each brain area analyzed whereas Figure 6 contains the graphs of the results obtained from quantifying all these images. Values of the F and p for the main factor (pain and treatment), and the interaction of those factors obtained from the two-way ANOVA analysis are summarized in Table 1.

3.3.1 | DAMGO increases the number of IBA-1 positive cells in VTA in a dose-dependent way and inflammatory pain impacts this effect

To assess changes in microglial activation within the VTA under our experimental conditions we used the immunohistochemistry

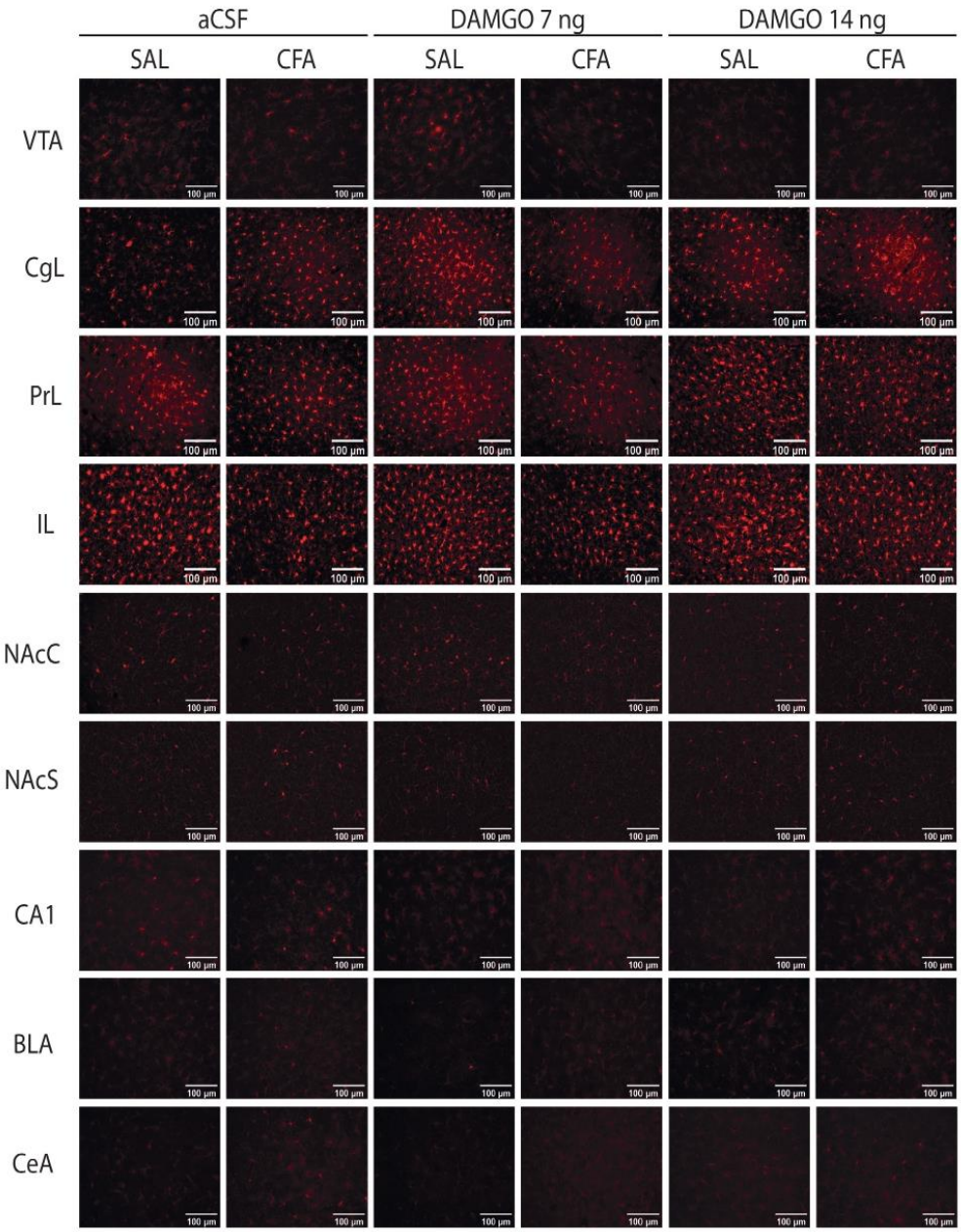

FIGURE 5 Representative images of the alterations of IBA1-positive cells in each brain area after the microinjections and the SAL/CFA injection. White scale bars represent 100 µm. aCSF, artificial cerebrospinal fluid; BLA, basolateral amygdala; CeA, central amygdala; CFA, Complete Freund Adjuvant; CgL, cingulate cortex; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; IBA1, ionized calcium-binding adapter molecule 1IL, infralimbic cortex; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; PrL, prelimbic cortex; SAL, Saline; VTA, ventral tegmental area.

technique with IBA1 as our maker of choice. The two-way ANOVA performed showed significant differences in the interaction of the pain condition and the treatment (Table 1). Figure 6 (VTA) shows that DAMGO 7 ng induced microglial activation whereas DAMGO 14 ng

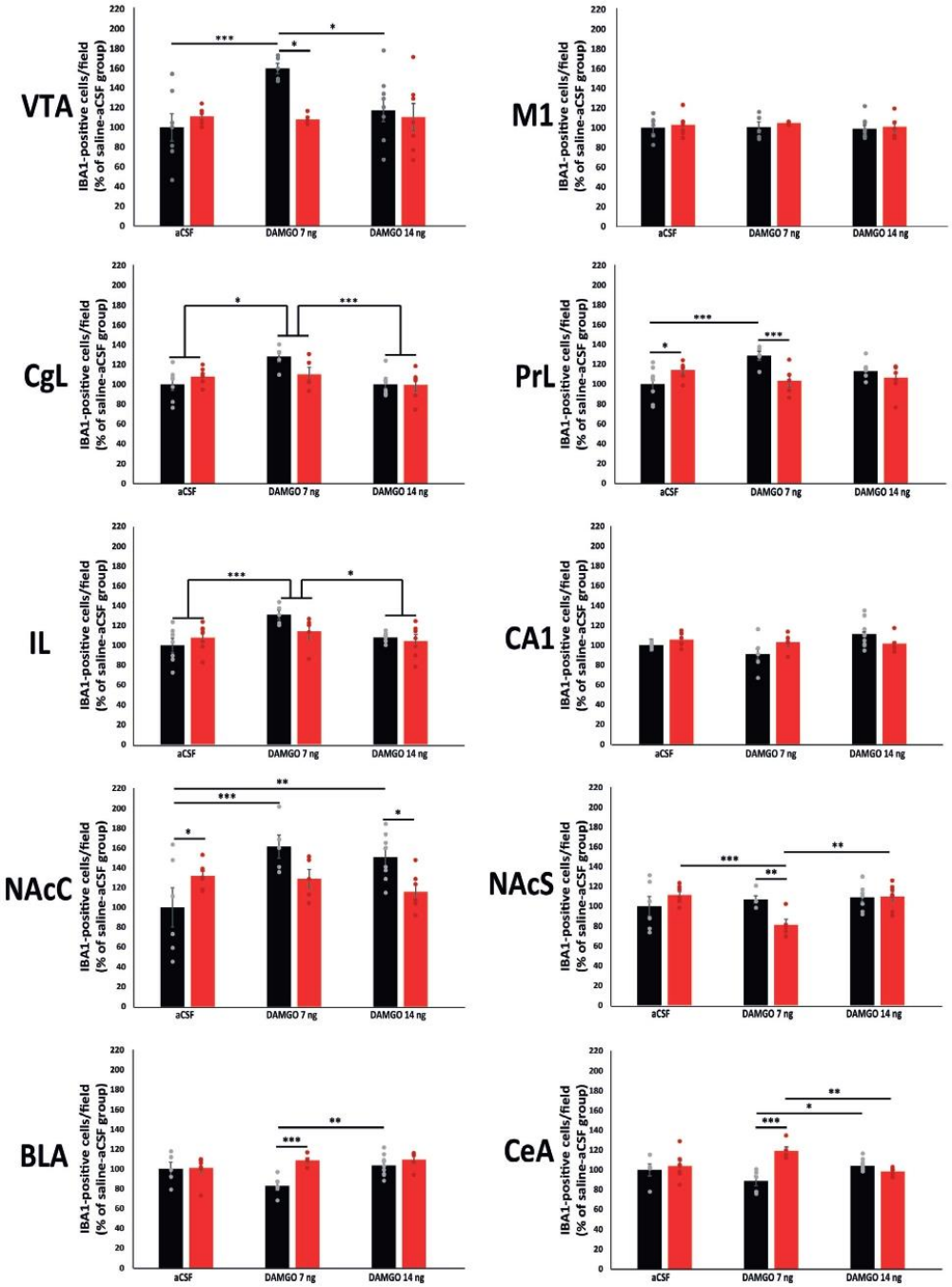

FIGURE 6 Legend on next page.

TABLE 1 Statistical analysis for Figure 6 (two-way ANOVA).

Brain area	Factor name	*F*-value	*p*-value
VTA	Pain	$F(1,5) = 2.889$	.990
	Treatment	$F(2,5) = 2.961$	.660
	Interaction	$F(2,5) = 3.614$	.038*
M1	Pain	$F(1,5) = 0.729$	.400
	Treatment	$F(2,5) = 0.159$	.853
	Interaction	$F(2,5) = 0.028$	.972
CgL	Pain	$F(1,5) = 0.591$	.448
	Treatment	$F(2,5) = 6.563$	.004*
	Interaction	$F(2,5) = 2.769$	.078
PrL	Pain	$F(1,5) = 1.932$	.174
	Treatment	$F(2,5) = 1.468$	.245
	Interaction	$F(2,5) = 6.926$	.003*
Il	Pain	$F(1,5) = 0.900$	.350
	Treatment	$F(2,5) = 6.030$	.006*
	Interaction	$F(2,5) = 2.355$	.111
CA1	Pain	$F(1,5) = 0.485$	.492
	Treatment	$F(2,5) = 1.960$	.159
	Interaction	$F(2,5) = 2.733$	.082
NAcC	Pain	$F(1,5) = 1.721$	.199
	Treatment	$F(2,5) = 3.435$	.045*
	Interaction	$F(2,5) = 6.281$	.005*
NAcS	Pain	$F(1,5) = 0.876$	.356
	Treatment	$F(2,5) = 3.611$	.039*
	Interaction	$F(2,5) = 4.944$	.013*
BLA	Pain	$F(1,5) = 8.018$	.008*
	Treatment	$F(2,5) = 2.554$	.095
	Interaction	$F(2,5) = 3.565$	.041*
CeA	Pain	$F(1,5) = 7.401$	.011*
	Treatment	$F(2,5) = 0.198$	.821
	Interaction	$F(2,5) = 9.082$	.001*

Abbreviations: BLA, basolateral amygdala; CeA, central amygdala; CgL, cingulate cortex; IL, infralimbic cortex; M1, primary motor cortex; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; PrL, prelimbic cortex; VTA, ventral tegmental area.
*$p < 0.050$.

failed to do so. In fact, the *post-hoc* analysis performed showed that microglial activation was significantly higher in DAMGO 7 ng than in aCSF and DAMGO 14 ng. Interestingly, the presence of inflammatory pain did not change microglial activation per se in the absence of a treatment with DAMGO in the brain areas studied. However, in the DAMGO 7 ng group, inflammatory pain impaired the increase observed in saline animals. In fact, according to the Bonferroni *post-hoc*, CFA-treated animals showed significant lower levels of microglial activation when treated with DAMGO 7 ng than saline-treated rats.

3.3.2 | The number of IBA1-positive cells within PFC is increased with an intra-VTA DAMGO 7 ng treatment

Prefrontal cortex is tightly connected with the VTA through neurons that release dopamine and other collateral neurotransmitter (Morales & Margolis, 2017). In this sense, the statistical analysis carried out for the three PFC subregions (CgL, PrL, and IL) revealed that there were significant differences when comparing our experimental groups and that changes in all three subareas followed a similar pattern (Table 1). Figure 6 (CgL, PrL, and IL) shows changes in microglial activation in those subareas.

On the one hand, the two-way ANOVA analysis and the posterior *post-hoc* applied showed that DAMGO 7 ng significantly increased microglia activation in both CgL and IL when compared to the aCSF group. As shown in VTA in the previous section, DAMGO 14 ng failed to induce this activation. However, opposite to what happened in VTA, according to the two-way ANOVA performed, inflammatory pain did not interfere with the increase in the number of IBA1-positive cells observed when animals were treated with DAMGO 7 ng in CgL and IL. On the other hand, inflammatory pain did elicit an effect in microglial activation in PrL. First, the *post-hoc* test showed that inflammatory pain significantly increased microglial activation in animals that followed the aCSF control treatment. Second, as in the other PFC subregions, the intra-VTA treatment with DAMGO 7 ng induced microglial activation in PrL. However, the statistical analysis did show that inflammatory pain modified this effect. Finally, DAMGO 14 ng failed to induce any significant alteration in microglial activation in this subregion.

3.3.3 | Microglia in the hippocampal CA1 region is altered by neither an intra-VTA DAMGO treatment nor the presence of inflammatory pain

The hippocampus plays a crucial role in the MCLS due to its function in the memory circuitry. Thereby, VTA-based dopaminergic neurons also project to this brain region (Morales & Margolis, 2017). The

FIGURE 6 Alterations of IBA1-positive cells (Expressed in % of the SAL-aCSF group) in each brain area after the microinjections and the SAL/CFA injection. Data are expressed as mean ± SEM of IBA1-positve cells ($n = 5–9$/group). Black bars represent animals injected with SAL whereas red bars represent animals injected with CFA. Points represent the individual data from each animal within each group. *$p < .05$, **$p < .01$, ***$p < .005$ (Two-way ANOVA followed by Bonferroni multiple comparisons). aCSF, artificial cerebrospinal fluid; BLA, basolateral amygdala; CeA, central amygdala; CFA, complete Freund adjuvant; CgL, cingulate cortex; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; IBA1, ionized calcium-binding adapter molecule 1; IL, infralimbic cortex; M1, primary motor cortex; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; PrL, prelimbic cortex; SAL, saline; VTA, ventral tegmental area.

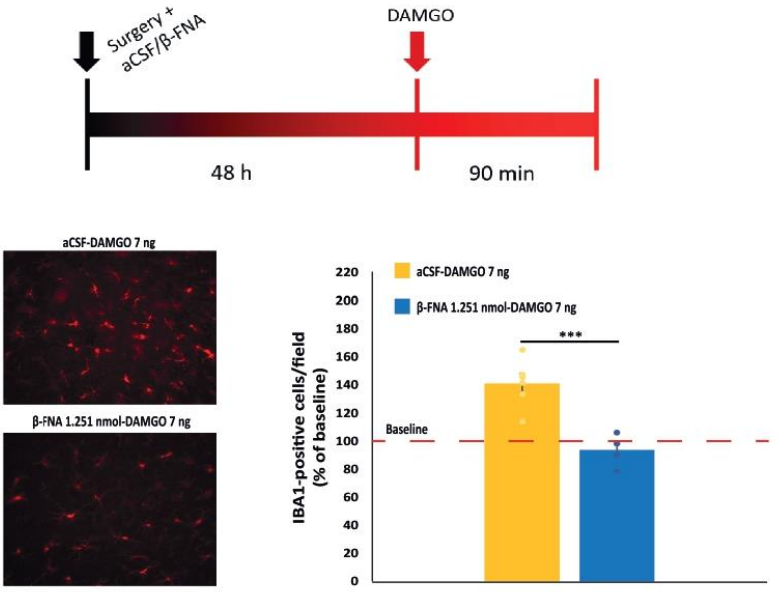

FIGURE 7 Alterations of IBA1-positive cells (Expressed in % of the saline-aCSF group) in the VTA after the microinjections aCSF/β-FNA–DAMGO. Data are expressed as mean ± SEM of IBA1-positive cells ($n = 6$/group). The red line corresponds to the baseline data average (saline-aCSF group). The yellow bar represents the aCSF-DAMGO group whereas the blue bar represents the β-FNA-DAMGO group. Points represent the individual data from each animal within each group. ***$p < .005$ (Student's t-test for independent samples). aCSF, Artificial cerebrospinal fluid; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; IBA1, ionized calcium-binding adapter molecule 1; VTA, ventral tegmental area; β-FNA, β-funaltrexamine.

hippocampal CA1 region is tightly related to reward processing (Takamiya et al., 2021) and directly receives the dopaminergic input from VTA (Ghanbarian & Motamedi, 2013). Regardless of these premises, the two-way ANOVA performed in the data obtained from our IBA1 immunohistochemistry results did not show any statistical differences in this specific hippocampal subregion (Table 1; Figure 6, CA1).

3.3.4 | Ventral striatal IBA1 expression is altered after the pharmacological activation of VTA MORs, and inflammatory pain modifies this alteration

The NAc regulates many behaviors among which we can find depression and addiction. To do so, NAcS gets projections from neurons belonging to the MCLS whereas NAcC is also connected to the motor system (Xu et al., 2020). Furthermore, the dopaminergic connection between VTA and NAc has been widely studied and proved (Salgado & Kaplitt, 2015).

The two-way ANOVA tests performed (Table 1) showed different patterns of alterations between our experimental groups in NAcC and NAcS as it can be observed in Figure 6 (NAcC and NAcS). On the one hand, the post hoc test carried out in NAcC revealed that inflammatory pain significantly increased microglial activation in our control animals like in the PrL region. Moreover, the Bonferroni post hoc showed that both doses of DAMGO significantly increased microglial activation in NAcC when animals were treated with them intra-VTA. Interestingly, inflammatory pain blunted that increase. On the other hand, none of the DAMGO doses used intra-VTA managed to change microglial activation in NAcS. However, the post hoc test performed showed a significant decrease in microglial activation when treated with DAMGO 7 ng intra-VTA.

3.3.5 | Inflammatory pain induced an increase in the number IBA1-positive cells in the amygdala when treated with DAMGO 7 ng intra-VTA

The main role of the amygdala in the MCLS is assessing the emotional value of a specific reinforcer that has activated the system (Šimić et al., 2021). Both BLA and CeA are especially connected to pain-related memories (Neugebauer, 2015). However, dopaminergic afferent projections from VTA mainly arrive to BLA (Vitay & Hamker, 2014).

The statistics applied to our results showed significant differences in BLA and CeA (Table 1). As shown in Figure 6 (BLA and CeA), the post hoc test performed revealed that animal treated with DAMGO 14 ng intra-VTA had significantly higher levels of microglial activation in the areas of the amygdala tested than those treated with DAMGO 7 ng. However, no differences were found when comparing those groups to the aCSF-treated group. Moreover, the presence of inflammatory pain significantly increased microglial activation in the DAMGO 7 ng-treated animals when compared to pain-free animals with the same treatment.

3.4 | MOR pharmacological blockade in the VTA prevents 7 ng DAMGO-induced increase in IBA1-positive cells

An intra-VTA pretreatment with β-FNA was used to assess if the increase in IBA1-positive cells that occurs in the VTA when microinjected with 7 ng DAMGO (Figure 6), is directly dependent on MOR activation. Interestingly, Figure 7 confirms that the intra-VTA administration of 7 ng DAMGO increases the number of IBA1-positive cells

in the VTA over baseline (% of saline-aCSF group) which was blunted by the pretreatment with 1.251 nmol β-FNA (Student's t test, $p = .001$).

4 | DISCUSSION

In this article, we show how MORs pharmacological activation in the NAcC induces the release of pro-inflammatory cytokines. Moreover, mechanical nociception is not altered by intra-VTA treatments of DAMGO 7 and 14 ng. Our data also reveal that an intra-VTA treatment with a MOR agonist elicits changes in the basal levels of microglial activation in a dose-dependent way, not only in the very VTA, but also in several areas of projection with a different pattern of alteration. We have also shown how inflammatory pain per se can trigger microglial activation in some brain areas belonging to the MCLS. Furthermore, CFA-treated animals had different patterns of microglial activation when treated with DAMGO intra-VTA in nearly all the areas analyzed. However, there were many discrepancies between the MCLS areas tested regarding how the variables inflammatory pain and intra-VTA treatment affect microglial activation. Finally, the blockade of MOR within the VTA prevents the 7 ng DAMGO-induced increase in IBA1-postive cells of this very brain area.

Whether MOR activation triggers neuroinflammatory processes is unclear, although some researchers suggest that this might be the case (Cuitavi et al., 2022; Gessi et al., 2016; Merighi et al., 2013; Shrivastava et al., 2017). However, to the best of our knowledge, this has mainly been tested in vitro. Thereby, we aimed to approach this matter in an in vivo microdialysis experiment and, very interestingly, our results evidence that MORs activation induces the release of the proinflammatory cytokines IL1α, IL1β, and IL6. Additionally, these cytokines participate in many pain comorbidities that involve alterations within the MCLS, among which opioid and alcohol use disorders stand out for acting through MOR (Karimi-Haghighi et al., 2022; Moura et al., 2022).

It is important to notice that, even if our data point towards the implication of MORs activation in promoting neuroinflammation, the cell type or cell types carrying out this process remains unknown and/or the impact of a systemic pain condition in the observed effects. Nonetheless, it has been previously reported that MORs are expressed in microglial cells within the VTA (Maduna et al., 2019). With this in mind, and after testing that intra-VTA DAMGO does not alter mechanical nociception, we proceeded to test the effect of MOR activation in the VTA over microglial cells in rats with and without inflammatory pain. In fact, we show how DAMGO 7 ng induces microglial activation within the VTA and that the pretreatment with a MOR antagonist prevents this phenomenon, thus further confirming the connection between MORs and neuroinflammation. However, DAMGO 14 ng failed to elicit the same effect, which reveals that the opioidergic system within the neuroimmune system holds a great level of complexity. Interestingly, our team previously described this dose-dependent effect of DAMGO on neuronal MORs (Campos-Jurado et al., 2019). DAMGO binding to MORs results in rapid internalization rates, above all when comparing with other opioids (Koch & Höllt, 2008). In this sense, high doses of DAMGO could be quickly internalizing these receptors and, thereby, reducing the effect observed. Additionally, our results also showed how inflammatory pain reverted to the increase in the microgliosis in the DAMGO 7 ng group. This is a complex matter since the study of MORs on glial cells remains a hardly explored subject. However, there is evidence of a decrease in the availability of MORs in neuronal cell in presence of a pain condition (Campos-Jurado et al., 2022; Thompson et al., 2018). If that was also the case for microglial MORs, we hypothesize that DAMGO would not be able to activate them and, thus, microglial activation would not occur, which would be interesting as a subject of study in future research.

The MCLS dopaminergic, glutamatergic, and GABAergic projections together with other neurotransmitters perfectly regulate neurons of this system with a pivotal role in reward within the different brain regions that belong to the system. The VTA is the origin of most of the dopaminergic projections and holds their neuronal bodies (Fields et al., 2007; Morales & Margolis, 2017). The intricacy of this system is possibly the answer to the heterogeneous results that we have obtain for microglial activation in the VTA projection areas tested, although they still raise some questions. First, we must respond how microglial activation can be spread from its area of origin to other areas in such a short time span (1 h and 30 min after DAMGO administration). Interestingly, previous research point to dopamine as a neuroimmune modulator, although if it enhances or reduces neuroinflammation remains a subject of debate (Dominguez-Meijide et al., 2017). Moreover, a VTA-treatment with DAMGO induces neuronal activity in areas of projection in a dose- and area-dependent manner, probably due to dopamine release (Campos-Jurado et al., 2019). Therefore, the different patterns observed in between brain areas could be explained if dopamine was to trigger microglial activation. Second, as previously mentioned, pain desensitizes MOR within the MCLS (Hipólito et al., 2015), thus, reducing VTA dopaminergic neurons firing rates. This might be the reason why PFC and NAc subareas also reduce microglial activation in the groups where an intra-VTA DAMGO treatment was increasing it. Finally, microglial cells in BLA and CeA modulate their activation in the presence of the different treatments and inflammatory pain in a different way to other areas. A possible explanation for this could be that dopamine in these areas conducts a reduction in neuroinflammation, probably because of a different availability of dopamine receptors on microglia within the amygdala when compared to other MCLS areas, or other mediators involved in microglia activation. However, there is a gap in the literature regarding this matter and future investigations will have to be carried out to shed light on this issue.

Finally, as previously mentioned, microglia within the spinal cord and chronic pain are strongly related (Chen et al., 2018). Nonetheless, not many articles focus on how pain influences microglial states in the brain. Herein, we have shown that in PrL and NAcC there is a significant increase in microglial activation in CFA-treated animals. Moreover, other brain areas such as the rest of subregions of the PFC and

the NAc also show a nonstatistically significant increase in microglial activation as it can be perceived in the heatmap shown in Figure 4b. Interestingly, the differences between brain areas might be partially attributed to the temporality of the CFA model. In fact, Chen et al. (2018) reviewed that spinal microgliosis, although present in short-term pain conditions, is usually associated with chronic pain. If this is also true for microglial cells resident in the MCLS, that longer periods of CFA-induced inflammatory pain might increase the effect observed. Moreover, since the model of choice in this article consists of an arthritis-like inflammatory pain, animals present a peripheral inflammation. Therefore, pro-inflammatory cytokines such as TNFα, IL1β, and INFγ could be trespassing the blood brain barrier and then activating microglia within the brain as previously described (Perry, 2010). Interestingly, microglial activation in the MCLS mediates a myriad of pain-derived diseases by changing neuron signaling patterns due to their role in synapses and by altering the environment surrounding local neurons (Hinwood et al., 2012; Miguel-Hidalgo, 2009; Yirmiya et al., 2015).

All in all, in the present article, we highlight the role of MORs as modulators of microglial activation in the MCLS. This research, alongside future complementary investigations, might serve to put MOR–microglia interactions in the spotlight to use them as a target for possible treatments when addressing pain comorbidities related to MCLS dysfunction.

AUTHOR CONTRIBUTIONS

Javier Cuitavi, Lucía Hipólito: Conceptualization. **Javier Cuitavi, David Meseguer, Yolanda Campos-Jurado, Lucía Hipólito:** Methodology. **Javier Cuitavi, David Meseguer, Jesús D. Lorente, Hannah Caruana, Lucía Hipólito:** Formal analysis. **Javier Cuitavi, Paula Andrés-Herrera, David Meseguer:** Investigation. **Javier Cuitavi:** Writing – original draft. **Lucía Hipólito:** Resources. **Lucía Hipólito:** Supervision. **Javier Cuitavi, Paula Andrés-Herrera, David Meseguer, Yolanda Campos-Jurado, Jesús D. Lorente, Hannah Caruana, Lucía Hipólito:** Writing – review and editing. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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REFERENCES

Burkovetskaya, M. E., Small, R., Guo, L., Buch, S., & Guo, M. L. (2020). Cocaine self-administration differentially activates microglia in the mouse brain. *Neuroscience Letters*, *728*, 134951. https://doi.org/10.1016/j.neulet.2020.134951

Campos-Jurado, Y., Cuitavi, J., Landsberg, N., Lorente, J. D., & Hipólito, L. (2022). Inflammatory and neuropathic pain impact on the opioid function in the mesocorticolimbic system. In *The neurobiology, physiology, and psychology of pain* (pp. 91–102). Academic Press. https://doi.org/10.1016/B978-0-12-820589-1.00045-2

Campos-Jurado, Y., Igual-López, M., Padilla, F., Zornoza, T., Granero, L., Polache, A., Agustín-Pavón, C., & Hipólito, L. (2019). Activation of MORs in the VTA induces changes on cFos expression in different projecting regions: Effect of inflammatory pain. *Neurochemistry International*, *131*, 104521. https://doi.org/10.1016/j.neuint.2019.104521

Chen, G., Zhang, Y. Q., Qadri, Y. J., Serhan, C. N., & Ji, R. R. (2018). Microglia in pain: Detrimental and protective roles in pathogenesis and resolution of pain. *Neuron*, *100*(6), 1292–1311. https://doi.org/10.1016/j.neuron.2018.11.009

Coffey, K. R., Lesiak, A. J., Marx, R. E., Vo, E. K., Garden, G. A., & Neumaier, J. F. (2022). A cAMP-related gene network in microglia is inversely regulated by morphine tolerance and withdrawal. *Biological Psychiatry Global Open Science*, *2*(2), 180–189. https://doi.org/10.1016/j.bpsgos.2021.07.011

Cuitavi, J., Hipólito, L., & Canals, M. (2021). The life cycle of the mu-opioid receptor. *Trends in Biochemical Sciences*, *46*(4), 315–328. https://doi.org/10.1016/j.tibs.2020.10.002

Cuitavi, J., Lorente, J. D., Campos-Jurado, Y., Polache, A., & Hipólito, L. (2021). Neuroimmune and mu-opioid receptor alterations in the mesocorticolimbic system in a sex-dependent inflammatory pain-induced alcohol relapse-like rat model. *Frontiers in Immunology*, *12*, 689453. https://doi.org/10.3389/fimmu.2021.689453

Cuitavi, J., Torres-Pérez, J. V., Lorente, J. D., Campos-Jurado, Y., Andrés-Herrera, P., Polache, A., Agustín-Pavón, C., & Hipólito, L. (2022). Cross-talk between mu-opioid receptors and neuroinflammation: Consequences for drug addiction and pain. *Neuroscience & Biobehavioral Reviews*, *145*, 105011. https://doi.org/10.1016/j.neubiorev.2022.105011

Devine, D. P., Leone, P., Pocock, D., & Wise, R. A. (1993). Differential involvement of ventral tegmental mu, delta and kappa opioid receptors in modulation of basal mesolimbic dopamine release: In vivo microdialysis studies. *Journal of Pharmacology and Experimental Therapeutics*, *266*(3), 1236–1246.

Dominguez-Meijide, A., Rodriguez-Perez, A. I., Diaz-Ruiz, C., Guerra, M. J., & Labandeira-Garcia, J. L. (2017). Dopamine modulates astroglial and microglial activity via glial renin-angiotensin system in cultures. *Brain, Behavior, and Immunity*, *62*, 277–290. https://doi.org/10.1016/j.bbi.2017.02.013

Fields, H. L., Hjelmstad, G. O., Margolis, E. B., & Nicola, S. M. (2007). Ventral tegmental area neurons in learned appetitive behavior and positive

reinforcement. *Annual Review of Neuroscience*, *30*, 289–316. https://doi.org/10.1146/annurev.neuro.30.051606.094341

Foley, H. E., Knight, J. C., Ploughman, M., Asghari, S., & Audas, R. (2021). Association of chronic pain with comorbidities and health care utilization: A retrospective cohort study using health administrative data. *Pain*, *162*(11), 2737–2749. https://doi.org/10.1097/j.pain.0000000000002264

Gessi, S., Borea, P. A., Bencivenni, S., Fazzi, D., Varani, K., & Merighi, S. (2016). The activation of μ-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia. *FEBS Letters*, *590*(17), 2813–2826. https://doi.org/10.1002/1873-3468.12313

Ghanbarian, E., & Motamedi, F. (2013). Ventral tegmental area inactivation suppresses the expression of CA1 long term potentiation in anesthetized rat. *PLoS One*, *8*(3), e58844. https://doi.org/10.1371/journal.pone.0058844

Hains, B. C., & Waxman, S. G. (2006). Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *Journal of Neuroscience*, *26*(16), 4308–4317. https://doi.org/10.1523/JNEUROSCI.0003-06.2006

Han, Y. Y., Jin, K., Pan, Q. S., Li, B., Wu, Z. Q., Gan, L., Yang, L., & Long, C. (2020). Microglial activation in the dorsal striatum participates in anxiety-like behavior in Cyld knockout mice. *Brain, Behavior, and Immunity*, *89*, 326–338. https://doi.org/10.1016/j.bbi.2020.07.011

Hinwood, M., Morandini, J., Day, T. A., & Walker, F. R. (2012). Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex. *Cerebral Cortex*, *22*(6), 1442–1454. https://doi.org/10.1093/cercor/bhr229

Hipólito, L., Martí-Prats, L., Sánchez-Catalán, M. J., Polache, A., & Granero, L. (2011). Induction of conditioned place preference and dopamine release by salsolinol in posterior VTA of rats: Involvement of μ-opioid receptors. *Neurochemistry International*, *59*(5), 559–562. https://doi.org/10.1016/j.neuint.2011.04.014

Hipólito, L., Wilson-Poe, A., Campos-Jurado, Y., Zhong, E., Gonzalez-Romero, J., Virag, L., Whittington, R., Comer, S. D., Carlton, S. M., Walker, B. M., Bruchas, M. R., & Morón, J. A. (2015). Inflammatory pain promotes increased opioid self-administration: Role of dysregulated ventral tegmental area μ opioid receptors. *Journal of Neuroscience*, *35*(35), 12217–12231. https://doi.org/10.1523/JNEUROSCI.1053-15.2015

Hiraga, S. I., Itokazu, T., Nishibe, M., & Yamashita, T. (2022). Neuroplasticity related to chronic pain and its modulation by microglia. *Inflammation and Regeneration*, *42*(1), 1–10. https://doi.org/10.1186/s41232-022-00199-6

Jia, X., Gao, Z., & Hu, H. (2021). Microglia in depression: Current perspectives. *Science China. Life Sciences*, *64*(6), 911–925. https://doi.org/10.1007/s11427-020-1815-6

Karimi-Haghighi, S., Chavoshinezhad, S., Mozafari, R., Noorbakhsh, F., Borhani-Haghighi, A., & Haghparast, A. (2022). Neuroinflammatory response in reward-associated psychostimulants and opioids: A review. *Cellular and Molecular Neurobiology*, *43*, 649–682. https://doi.org/10.1007/s10571-022-01223-6

Koch, T., & Höllt, V. (2008). Role of receptor internalization in opioid tolerance and dependence. *Pharmacology & Therapeutics*, *117*(2), 199–206. https://doi.org/10.1016/j.pharmthera.2007.10.003

Koo, J. W., Chaudhury, D., Han, M. H., & Nestler, E. J. (2019). Role of mesolimbic brain-derived neurotrophic factor in depression. *Biological Psychiatry*, *86*(10), 738–748. https://doi.org/10.1016/j.biopsych.2019.05.020

Leduc-Pessah, H., Weilinger, N. L., Fan, C. Y., Burma, N. E., Thompson, R. J., & Trang, T. (2017). Site-specific regulation of P_2X_7 receptor function in microglia gates morphine analgesic tolerance. *Journal of Neuroscience*, *37*(42), 10154–10172. https://doi.org/10.1523/JNEUROSCI.0852-17.2017

Li, Q., & Barres, B. A. (2018). Microglia and macrophages in brain homeostasis and disease. *Nature Reviews Immunology*, *18*(4), 225–242. https://doi.org/10.1038/nri.2017.125

Lier, J., Streit, W. J., & Bechmann, I. (2021). Beyond activation: Characterizing microglial functional phenotypes. *Cell*, *10*(9), 2236. https://doi.org/10.3390/cells10092236

Lorente, J. D., Cuitavi, J., Campos-Jurado, Y., Montón-Molina, R., González-Romero, J. L., & Hipólito, L. (2022). Kappa opioid receptor blockade in the nucleus accumbens shell prevents sex-dependent alcohol deprivation effect induced by inflammatory pain. *Pain*, *163*(1), e137–e147. https://doi.org/10.1097/j.pain.0000000000002332

Machelska, H., & Celik, M. Ö. (2020). Opioid receptors in immune and glial cells—Implications for pain control. *Frontiers in Immunology*, *11*, 300. https://doi.org/10.3389/fimmu.2020.00300

Maduna, T., Audouard, E., Dembélé, D., Mouzaoui, N., Reiss, D., Massotte, D., & Gaveriaux-Ruff, C. (2019). Microglia express mu opioid receptor: Insights from transcriptomics and fluorescent reporter mice. *Frontiers in Psychiatry*, *9*, 726. https://doi.org/10.3389/fpsyt.2018.00726

Melbourne, J. K., Chandler, C. M., Van Doorn, C. E., Bardo, M. T., Pauly, J. R., Peng, H., & Nixon, K. (2021). Primed for addiction: A critical review of the role of microglia in the neurodevelopmental consequences of adolescent alcohol drinking. *Alcoholism: Clinical and Experimental Research*, *45*(10), 1908–1926. https://doi.org/10.1111/acer.14694

Merighi, S., Gessi, S., Varani, K., Fazzi, D., Stefanelli, A., & Borea, P. A. (2013). Morphine mediates a pro-inflammatory phenotype via μ-opioid receptor–PKCε–Akt–ERK1/2 signaling pathway in activated microglial cells. *Biochemical Pharmacology*, *86*(4), 487–496. https://doi.org/10.1016/j.bcp.2013.05.027

Miguel-Hidalgo, J. J. (2009). The role of glial cells in drug abuse. *Current Drug Abuse Reviews*, *2*(1), 76–82. https://doi.org/10.2174/1874473710902010076

Morales, M., & Margolis, E. B. (2017). Ventral tegmental area: Cellular heterogeneity, connectivity and behaviour. *Nature Reviews Neuroscience*, *18*(2), 73–85. https://doi.org/10.1038/nrn.2016.165

Moura, H. F., Hansen, F., Galland, F., Silvelo, D., Rebelatto, F. P., Ornell, F., Massuda, R., Scherer, J. N., Schuch, F., Kessler, F. H., & von Diemen, L. (2022). Inflammatory cytokines and alcohol use disorder: Systematic review and meta-analysis. *Brazilian Journal of Psychiatry*, *44*, 548–556. https://doi.org/10.47626/1516-4446-1893

Neugebauer, V. (2015). Amygdala pain mechanisms. *Handbook of Experimental Pharmacology*, *227*, 261–284. https://doi.org/10.1007/978-3-662-46450-2_13

Nikolaus, S., Mamlins, E., Hautzel, H., & Müller, H. W. (2019). Acute anxiety disorder, major depressive disorder, bipolar disorder and schizophrenia are related to different patterns of nigrostriatal and mesolimbic dopamine dysfunction. *Reviews in the Neurosciences*, *30*(4), 381–426. https://doi.org/10.1515/revneuro-2018-0037

Perry, V. H. (2010). Contribution of systemic inflammation to chronic neurodegeneration. *Acta neuropathologica*, *120*, 277–286. https://doi.org/10.1007/s00401-010-0722-x

Sá, K. N., Moreira, L., Baptista, A. F., Yeng, L. T., Teixeira, M. J., Galhardoni, R., & de Andrade, D. C. (2019). Prevalence of chronic pain in developing countries: Systematic review and meta-analysis. *Pain Reports*, *4*(6), e779. https://doi.org/10.1097/PR9.0000000000000779

Salgado, S., & Kaplitt, M. G. (2015). The nucleus accumbens: A comprehensive review. *Stereotactic and Functional Neurosurgery*, *93*(2), 75–93. https://doi.org/10.1159/000368279

Shi, Z., Jagannathan, K., Padley, J. H., Wang, A. L., Fairchild, V. P., O'Brien, C. P., Childress, A. R., & Langleben, D. D. (2021). The role of withdrawal in mesocorticolimbic drug cue reactivity in opioid use disorder. *Addiction Biology*, *26*(4), e12977. https://doi.org/10.1111/adb.12977

Shrivastava, P., Cabrera, M. A., Chastain, L. G., Boyadjieva, N. I., Jabbar, S., Franklin, T., & Sarkar, D. K. (2017). Mu-opioid receptor and delta-opioid receptor differentially regulate microglial inflammatory response to control proopiomelanocortin neuronal apoptosis in the hypothalamus: Effects of neonatal alcohol. *Journal of Neuroinflammation*, *14*(1), 1–19. https://doi.org/10.1186/s12974-017-0844-3

Šimić, G., Tkalčić, M., Vukić, V., Mulc, D., Španić, E., Šagud, M., Olucha-Bordonau, F. E., Vukšić, M., & Hof, P. R. (2021). Understanding emotions: Origins and roles of the amygdala. *Biomolecules*, *11*(6), 823. https://doi.org/10.3390/biom11060823

Svingos, A. L., Garzón, M., Colago, E. E., & Pickel, V. M. (2001). μ-Opioid receptors in the ventral tegmental area are targeted to presynaptically and directly modulate mesocortical projection neurons. *Synapse*, *41*(3), 221–229. https://doi.org/10.1002/syn.1079

Takamiya, S., Shiotani, K., Ohnuki, T., Osako, Y., Tanisumi, Y., Yuki, S., Manabe, H., Hirokawa, J., & Sakurai, Y. (2021). Hippocampal CA1 neurons represent positive feedback during the learning process of an associative memory task. *Frontiers in Systems Neuroscience*, *12*, 689453. https://doi.org/10.3389/fnsys.2021.718619

Takeura, N., Nakajima, H., Watanabe, S., Honjoh, K., Takahashi, A., & Matsumine, A. (2019). Role of macrophages and activated microglia in neuropathic pain associated with chronic progressive spinal cord compression. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-52234-1

Taves, S., Berta, T., Chen, G., & Ji, R. R. (2013). Microglia and spinal cord synaptic plasticity in persistent pain. *Neural Plasticity*, *2013*, 1–10. https://doi.org/10.1155/2013/753656

Taylor, A. M., Castonguay, A., Ghogha, A., Vayssiere, P., Pradhan, A. A., Xue, L., Mehrabani, S., Wu, J., Levitt, P., Olmstead, M. C., De Koninck, Y., Evans, C. J., & Cahill, C. M. (2016). Neuroimmune regulation of GABAergic neurons within the ventral tegmental area during withdrawal from chronic morphine. *Neuropsychopharmacology*, *41*(4), 949–959. https://doi.org/10.1038/npp.2015.221

Taylor, A. M., Castonguay, A., Taylor, A. J., Murphy, N. P., Ghogha, A., Cook, C., Olmstead, M. C., De Koninck, Y., Evans, C. J., & Cahill, C. M. (2015). Microglia disrupt mesolimbic reward circuitry in chronic pain. *Journal of Neuroscience*, *35*(22), 8442–8450. https://doi.org/10.1523/JNEUROSCI.4036-14.2015

Thompson, S. J., Pitcher, M. H., Stone, L. S., Tarum, F., Niu, G., Chen, X., Kiesewetter, D. O., Schweinhardt, P., & Bushnell, M. C. (2018). Chronic neuropathic pain reduces opioid receptor availability with associated anhedonia in rat. *Pain*, *159*(9), 1856–1866. https://doi.org/10.1097/j.pain.0000000000001282

Vitay, J., & Hamker, F. H. (2014). Timing and expectation of reward: A neuro-computational model of the afferents to the ventral tegmental area. *Frontiers in Neurorobotics*, *8*, 4. https://doi.org/10.3389/fnbot.2014.00004

Xu, L., Nan, J., & Lan, Y. (2020). The nucleus accumbens: A common target in the comorbidity of depression and addiction. *Frontiers in Neural Circuits*, *14*, 37. https://doi.org/10.3389/fncir.2020.00037

Yirmiya, R., Rimmerman, N., & Reshef, R. (2015). Depression as a microglial disease. *Trends in Neurosciences*, *38*(10), 637–658. https://doi.org/10.1016/j.tins.2015.08.001

Zhang, P., Yang, M., Chen, C., Liu, L., Wei, X., & Zeng, S. (2020). Toll-like receptor 4 (TLR4)/opioid receptor pathway crosstalk and impact on opioid analgesia, immune function, and gastrointestinal motility. *Frontiers in Immunology*, *11*, 1455. https://doi.org/10.3389/fimmu.2020.01455

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CHAPTER 6

Microglial Control Over Mu-Opioid Receptors Signalling and Activation

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Effect of Neuroinflammation on Neuronal Mu-Opioid Receptor Expression and Signalling

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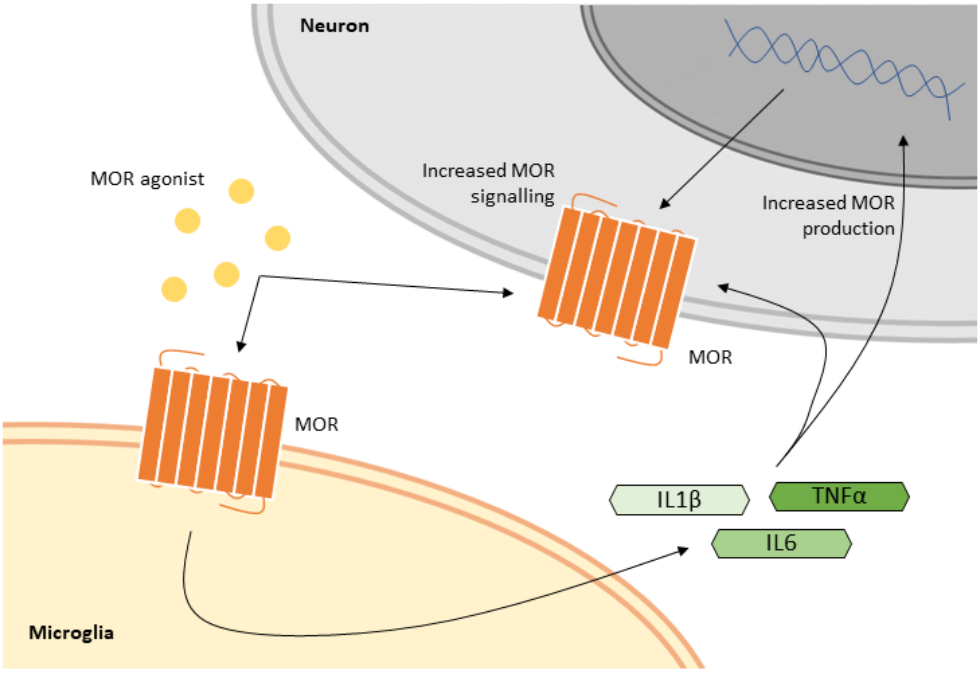
Abstract

Chronic pain is a burden for many healthcare systems and the use of opioids to treat it has contributed to "the opioid epidemic", which affects many patients that now suffer from opioid use disorders (OUDs). Mu-Opioid receptors (MORs) play a pivotal role in both pain processing and opioid-driven reward. Additionally, neuroinflammation seems to be tightly related to MORs and exert a control over pain through the modulation of these receptors. Nonetheless, the intricacies of the relationship between neuroinflammation and MOR function remain to be elucidated. Here, we investigated the role of the activated microglia secretome in neuronal MOR expression and signalling. Our results show that microglial secretome increases neuronal MOR expression through the action of proinflammatory cytokines. Moreover, microglia-derived cytokines also enhance the [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO)-induced MOR activation. Finally, we show

that DAMGO-induced neuroinflammation increases neuronal MOR expression, activation, and trafficking. All these new data shed light on the microglia-neuroinflammation-MOR relationship, which is revealed as a target to consider when treating chronic pain-induced OUDs.

Keywords: Microglia, neuroinflammation, Mu-Opioid receptors, DAMGO, SH-SY5Y

Graphical abstract



Introduction

Chronic pain, alongside its comorbidities, is a burden for many healthcare systems, worsens the quality of life of patients worldwide, and has a high prevalence in the world, with epidemiologic data showing that it affects around 18% of people in developed countries (Reid et al., 2011; Sá et al., 2019). In addition, opioid use disorders (OUDs) are tightly related to chronic pain conditions. In countries such as the United States of America, Canada, the United Kingdom, and the Netherlands, opioids have been prescribed in great quantities to ease pain conditions (Giraudon et al., 2013; King et al., 2014; Fischer et al., 2016; Kalkman et al., 2019), leading to what is now known as "the opioid epidemic" (King et al., 2014). Up to 26% of opioid-treated chronic pain patients develop an OUD (Chou et al., 2015), and it has been reported that pharmaceutical opioids are involved in 61% of drug overdoses in the United States of America (Rudd et al., 2016; Volkow and Collins, 2017). The Mu-Opioid receptors (MORs) are G protein-coupled receptors (GPCRs) that play a pivotal role in pain management and reinforcement and are therefore suggested targets to treat pain-induced OUDs (Cuitavi et al., 2021a). However, while MOR antagonists (such as naloxone and naltrexone) and agonists (such as methadone) are widely used to address OUDs (Bell and Strang, 2020), treatments with MOR agents alone are not always successful.

Recently, the relationship between the MOR and neuroinflammation has been suggested as a potential therapeutic target for OUDs (Cuitavi et al., 2023a). On the one hand, there is evidence that MORs within the central nervous system act as promoters of neuroinflammation (Johnston et al., 2004; Bianchi et al., 2008; Lin et al., 2010; Tekieh et al., 2011; Merighi et al., 2013; Bai et al., 2014; Tian et al., 2015; Gessi et al., 2016; Tsai et al., 2016; Shrivastava et al., 2017; Cuitavi et al., 2023b). On the other hand, additional evidence shows that proinflammatory cytokines such as

IL1β, IL6 and TNFα increase the expression of the *OPRM1* gene in several cell types (Ruzicka et al., 1996; Vidal et al., 1998; Kraus et al., 2003; Börner et al., 2004), including the astrocytoma cell line U97 MG, where the levels of both messenger RNA and the protein for MOR were upregulated upon treatment with IL1β (Byrne et al., 2012). Interestingly, Langsdorf et al. (2011) proposed that reactive oxygen species (ROS) also played a role in the regulation of the *OPRM1* gene transcription in SH-SY5Y neuron-like cells. Despite these reports, it is still unclear whether neuroinflammation modifies neuronal MOR signalling. Here, we investigated the relationship between microglia and MOR with a particular focus on how proinflammatory cytokines regulate neuronal MOR expression, activation, and trafficking, with the aim of understanding how alterations induced within neuroinflammatory processes can affect the action of this important therapeutic target. Interestingly, we found that cytokines released by microglia up-regulate the expression and activity of the MOR.

Materials and methods

Animals

10 female and 10 male C57BL6/J mice (6-8 weeks old; Jackson Laboratories), and 5 males and 6 females Sprague Dawley rats (9-12 weeks old; Envigo®) were used. All the animals were kept in light/dark (12/12h) controlled cycles, temperature 23±1ºC, and 60% humidity. Animals were housed in standard plastic cages (mice: 42×26×14 cm^3; rats:42×27×18 cm^3) with food and tap water provided *ad libitum* throughout the experimental period. Animals were housed in the animal facilities of the SCSIE from the University of Valencia. Animal protocols followed in this work were approved by the Animal Care Committee of University of Valencia, and were strictly

adhered to, in compliance with the EEC Council Directive 63/2010, Spanish laws (RD 53/2013) and animal protection policies.

Primary microglial cell cultures, treatment, and cytokines production assessment

Microglial cell cultures were performed as detailed in Bohlen et al. (2019). Briefly, male and female mouse brains were extracted, and the cerebellum and the olfactory bulbs were removed. Then the tissue was mixed and homogenised, first mechanically with a scalpel and then chemically with a gentleMACS™ Dissociator. After chemical digestion, tissue was filtered with 40 µm filters. This was followed by an immunoprecipitation step using CD11b MicroBeads and MS Columns from Miltenyi Biotec to discard non-expressing CD11b cells, CD11b being a marker of microglia. Finally, microglial cells were counted and cultured in 6-well plates (50,000 cells/plate) coated with Poly-D-Lysine (PDL). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium with Nutrient Mixture F12 (DMEM/F12) without phenol red supplemented with 1% Penicillin-Streptomycin, 1% L-Glutamine, N-Acetylcysteine (5 µg/mL), Sodium Selenite (100 pg/mL), Apo-Transferrin (100 µg/mL), CSF-1 (10 pg/mL), TGF-β2 (2 pg/mL), Heparan Sulphate (1 µg/mL).

A week after isolation, cells were treated with either 10 ng/mL lipopolysaccharide (LPS) for 24 h or the same relative volume of sterile water (control). Then, the cell culture medium was collected and filtered with 0.22 µm filters. Cells were also collected for the characterisation of the cytokine production profile. The medium was profiled by using a Proteome Profiler™ Mouse Cytokine Array Panel A (R&D systems) following the manufacturer's guidelines, whereas cells underwent a real time quantitative polymerase chain reaction (RT-qPCR). For the latter, microglial cells were lysed with RLT plus buffer and the RNA was extracted with the RNeasy Plus

Micro Kit (QIAGEN) following the manufacturer's instructions. The RNA obtained was then quantified using the Qubit_RNA HS Assay Kit (Thermo Fisher) in a Qubit Fluorometer (Thermo Fisher). Then RNA was retrotranscribed to cDNA using the PrimeScriptTM RT-PCR Kit (Clontech) according to the manufacturer's instructions. Gene expression analysis was assessed using 10-20 ng of cDNA, specific Taqman probes (Applied Biosystems) and the Premix Ex TaqTM (Probe qPCR) Kit (Clontech). RT-qPCR was performed in a Step One Plus PCR device (Applied Biosystems). The expression level of each gene was obtained by relative quantification (2(–ΔΔCt)) using constitutive expression of GAPDH and 18S genes as housekeeping endogenous controls.

Taqman probes:

- 18S TaqMan_ Probe Thermo Fisher Cat#4331182; Hs99999901_s1
- GAPDH TaqMan_ Probe Thermo Fisher Cat#4331182; Mm99999915_g1
- TNFα TaqMan_ Probe Thermo Fisher Cat#4331182; Mm00443258_m1
- IL1β TaqMan_ Probe Thermo Fisher Cat#4331182; Mm00434228_m1
- IL6 TaqMan_ Probe Thermo Fisher Cat#4331182; Mm00446190_m1

Protein fraction isolation

Cell culture media from primary microglia cell cultures underwent a process of separation by centrifuging twice and collecting the supernatant (3000xg for 15 min, and 16000xg for 20 min). Samples were then filtered by using 0.22 µm filters and concentrated with an Amicon® Ultra-4 tube (Merck). 12 fractions of 100 µL were obtained by using a 20 µm pore size column (Merck) loaded with 1mL of stacked Sepharose CL-2B (Sigma Aldrich), using PBS as elution buffer. The protein

concentration of every fraction was measured using a Micro BCA protein assay kit (Thermo Fisher Scientific). Fractions 9-12 were pooled as the most enriched protein fractions. Finally, samples were taken to the pre-concentration volume so that all the proteins returned to their original concentrations.

Microdialysis

Rats underwent a microdialysis experiment to obtain *in vivo* samples of [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO)-induced extracellular release of cytokines. Surgeries were performed under isoflurane (1.5-2 minimum alveolar concentration, MAC) anaesthesia under aseptic conditions. Rats were stereotaxically implanted (Stoelting) with bilateral vertical guide cannulae (CMA 12) into the nucleus accumbens core (NacC; Males – anteroposterior = 1.4 mm, mediolateral = 1.5 mm, and dorsoventral = 5.8 mm; Females – anteroposterior = 1.2 mm, mediolateral = 1.4 mm and dorsoventral = 5.6 mm). 48 h after surgery animals were placed in Plexiglas bowls and concentric-style microdialysis probes containing 2 mm of active membrane (CMA 12 HighCo; molecular *cutoff* 100,000 Da) were introduced through the cannulae, extending 3.0 mm below the tip of the cannulae. Then, artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$ at pH = 6) containing 0.5 mg/ml BSA was perfused into the probe through a push-pull system, which consisted of an inlet tubing attached to a 2.5-mL syringe (Hamilton) mounted on a syringe pump (Harvard instruments) and an outlet tubing connected to a peristaltic pump (Ismatec®). Pumps were set at 2.2 µL/min. After a 1 h wash of the system, 5 dialysates were collected every 100 min. After the collection of the first dialysate a treatment with 1 µM DAMGO was administered by retrodialysis for 20 min. These dialysates were analysed in Cuitavi et al., (2023b) where the production of proinflammatory cytokines was characterised by flow cytometry (Fortessa) using a customised multiplex approach (LEGENDplex™, BioLegend®).

SH-SY5Y cell cultures and treatments

SH-SY5Y (ATCC HTB-11) cells were maintained in DMEM/F12 supplemented with 10% heat inactivated foetal calf serum (FCS) and 2 mM L-Glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Transient transfections were performed using FuGENE® HD Transfetion Reagent (Promega) at a DNA:FuGENE® ratio of 1:3 according to the manufacturer's guidelines. Cells were transfected with different amounts of DNA depending on the experiment:

- MOR bioluminescence for MOR expression: 1 µg of FLAG-mMOR-Nluc, and 4 µg of pcDNA3.1. 1 µg of D2-Nluc was used as control.
- βγ release for G protein activation: 2 µg of $G\alpha_{i2}$, 1 µg of Venus155-239–$G\beta_1$, 1 µg of Venus1-155–$G\gamma_2$, 1 µg of masGRK3ct-Rluc8, and 1 µg of SNAP-mMOR.
- β-Arrestin 2 recruitment: 1 µg of FLAG-mMOR-Nluc, 1 µg of GRK2, and 4 µg of β-Arrestin 2–Venus.

Cells were replated into PDL–coated 96-well plates 24 hours after transfection and allowed to adhere for 4 hours. Then the medium was replaced by DMEM/F12 supplemented with and 2 mM L-Glutamine without FCS, which contains cytokines and other inflammatory mediators, and then cells were treated with:

- Medium from microglia treated with either vehicle or LPS.
- Protein fraction of the medium coming from microglia treated with either vehicle or LPS.
- TNFα (500 pg/mL), IL6 (1800 pg/mL), IL1β (60 pg/mL) or vehicle. (reported to be present in the media of a primary microglial cell culture after a 24 h treatment with 10 ng/mL LPS (He et al., 2021)).

- The rat brain dialysate pre- and post- DAMGO treatment.

MOR expression by bioluminescence

Cells transfected with FLAG-mMOR-Nluc underwent the treatments stated above. After 24h incubation, Coelenterazine h (NanoLight Technologies, AZ) was added at a final concentration of 5 µM. Luminescence was measured immediately in a PHERAstar Omega plate reader (BMG LABTECH, optic module, 475 ± 30). This experiment was performed three times in duplicate. Data is expressed as percentage of the vehicle-treated group. Cells transfected with D2-Nluc were used as a negative control.

Bioluminescence Resonance Energy Transfer (BRET)

βγ release and β-Arrestin 2 recruitment were performed 24 h after the treatments in cells transfected as described above. Cells are taken out of the incubator and washed with DPBS. Then, they were incubated for 30 min with 5 mM glucose in DPBS at 37ºC. Coelenterazine h (NanoLight Technologies, AZ) was used as a substrate for the luciferase constructs at a final concentration of 5 µM and after its addition cells were incubated for 5 min in the dark at 37ºC. Vehicle and 10 µM DAMGO were added 10 min before dual fluorescence/luminescence measurement in a PHERAstar Omega plate reader (BMG LABTECH). The BRET signal was calculated as the ratio of light emitted by Venus at 530 nm (optic module 535 ± 30) over the light emitted by NLuc or Rluc8 at 430 nm (optic module, 475 ± 30). We repeated BRET experiments three times in duplicate. A fold-over basal ratio was obtained by dividing DAMGO-treated BRET ratio by the vehicle-treated BRET ratio.

Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). To perform the statistical analysis, the 26.0 version of the SPSS program was used. The Kolmogorov–Smirnov test and Levene's test were performed to assess the normality and the homogeneity of the variance of the data. For the RT-qPCR analysis and the experiments where the treatments were either microglial cell media, the protein fraction of that media, or dialysates a T-test for independent samples was used. When cells were treated with proinflammatory cytokines a one-way ANOVA, followed by either Bonferroni or Games-Howell when appropriate, was used. In all the statistical tests, a 95% confidence level was set.

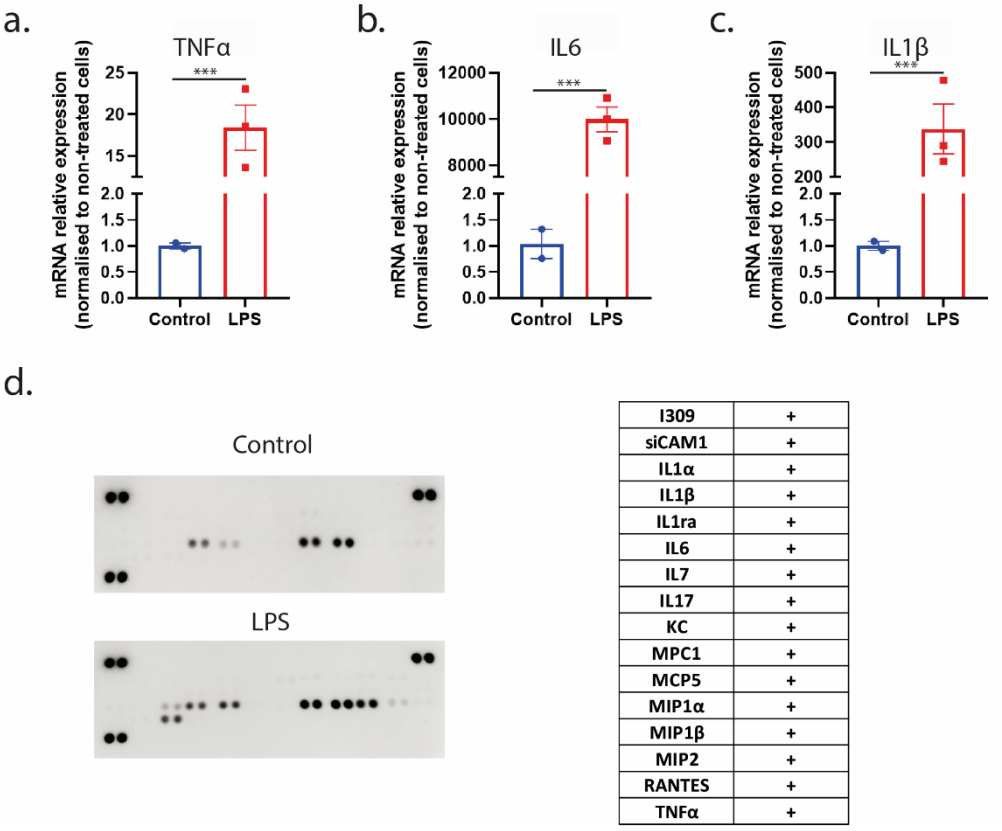
Results

MICROGLIAL CONTROL OVER NEURONAL MOR EXPRESSION AND SIGNALLING

Assessment of the production of proinflammatory mediators by microglia

The production and release of proinflammatory mediators by microglial primary cell cultures treated with either vehicle or LPS (10 ng/mL) for 24 h was assessed by RT-qPCR and proteome cytokine arrays (Fig.1). The mRNA levels of three proinflammatory cytokines (TNFα, IL6, and IL1β) were measured by RT-qPCR. The mRNA levels for these three cytokines were significantly higher in the LPS-treated cells than in the vehicle-treated cells (Fig.1a, TNFα, p=0.0001; Fig.1b, IL6, p=0.0001; Fig.1c, IL1β, p=0.0001). On the other hand, the proteome cytokine arrays revealed the release of several proinflammatory mediators upon LPS treatment (Fig.1d). Indeed, this qualitative analysis showed increased content of I309, siCAM1, IL1α, IL1β, IL1ra, IL6, IL7, IL17, KC, MPC1, MCP5, MIP1α, MIP1β, MIP2, RANTES, and TNFα in the LPS-treated microglia secretome in comparison to the vehicle-treated one. However, we also detected unchanged levels of some inflammatory mediators (BCA-

1, C5a, G-CSF, GM-CSF, eotaxin, INFγ, IL2, IL3, IL4, IL5, IL10, IL12, IL13, IL16, IL23, IL27, IP10, I-TAC, M-CSF, MCP-1, MCP-5, MIG, SDF-1, TARC, TIMP1, and TREM-1).



I309	+
siCAM1	+
IL1α	+
IL1β	+
IL1ra	+
IL6	+
IL7	+
IL17	+
KC	+
MPC1	+
MCP5	+
MIP1α	+
MIP1β	+
MIP2	+
RANTES	+
TNFα	+

Figure 1. Cytokine production assessment in microglia secretome*. a-c: RT-qPCR (n=2-3/group; a: TNFα, b: IL6, c: IL1β). d: Cytokine Array Panel image and data (+ indicates higher intensity versus vehicle). Data are expressed as mean ± SEM. *p<0.05, ***p<0.005 (T-test for independent samples). Lipopolysaccharide (LPS).*

Detection of MOR expression upon treatment with proinflammatory mediators

The bioluminescence of the Nluc tag fused to the C-terminus of MOR was used to determine the total amount of MOR protein expressed in SH-SY5Y cells when treated with activated microglial secretome, the protein fraction of that secretome or

proinflammatory cytokines for 24 h (Fig. 2). Interestingly, the expression of MOR protein was significantly increased when SH-SY5Y cells were exposed to all cytokine-enriched media (Fig. 2a, Microglial secretome ($p=0.023$), Fig. 2b, Protein fraction ($p=0.033$) and Fig. 2c, cytokines $F(3,11)=50.714$ $p=0.0001$, Games-Howell Vh-TNFα $p=0.019$, Vh-IL6 $p=0.026$, Vh-IL1β $p=0.037$). To rule out that such increases were not due to effects on the activity of the CMV promoter of the pcDNA3.1 plasmid carrying the tagged-MOR cDNA, we transfected the cells with a D2R-Nluc construct in the same pcDNA3.1 plasmid. None of the treatments did alter D2R expression on SH-SY5Y cells as shown in Fig.2d-f (Microglial secretome, $p=0.181$; Protein fraction, $p=0.725$; Cytokines, $F(3,11)=3.480$ $p=0.070$).

Measurement of G protein activation and β-arrestin 2 recruitment by MOR upon treatment with proinflammatory mediators

To assess MOR-induced G protein activation, the βγ release BRET assay (Gillis et al., 2020) was performed in transfected SH-SY5Y cells. In this assay, activation of MOR by 10 μM DAMGO for 10 min is detected as the release of the βγ subunits from the activated Gαi subunit of the G protein. The released βγ subunit has high affinity for the lipid-modified reporter peptide GRK3ct (masGRK3ct-Nluc) and such interaction is measured through BRET. We observed that cells exposed to media obtained from LPS-treated microglial cells or to the protein fraction of those media had a significantly higher DAMGO-induced G protein activation than those exposed to medium or protein recovered from vehicle-treated microglia (Fig.3a-c). The three tested cytokines had a similar inducing effect (Fig.3a-c) ($p=0.039$, $p=0.005$, and one-way ANOVA $F(3,11)=28.517$ $p=0.0001$; Bonferroni multiple comparisons test Vh-TNFα $p=0.001$, Vh-IL6 $p=0.001$ Vh-IL1β $p=0.0001$, respectively) (Fig.3a-c).

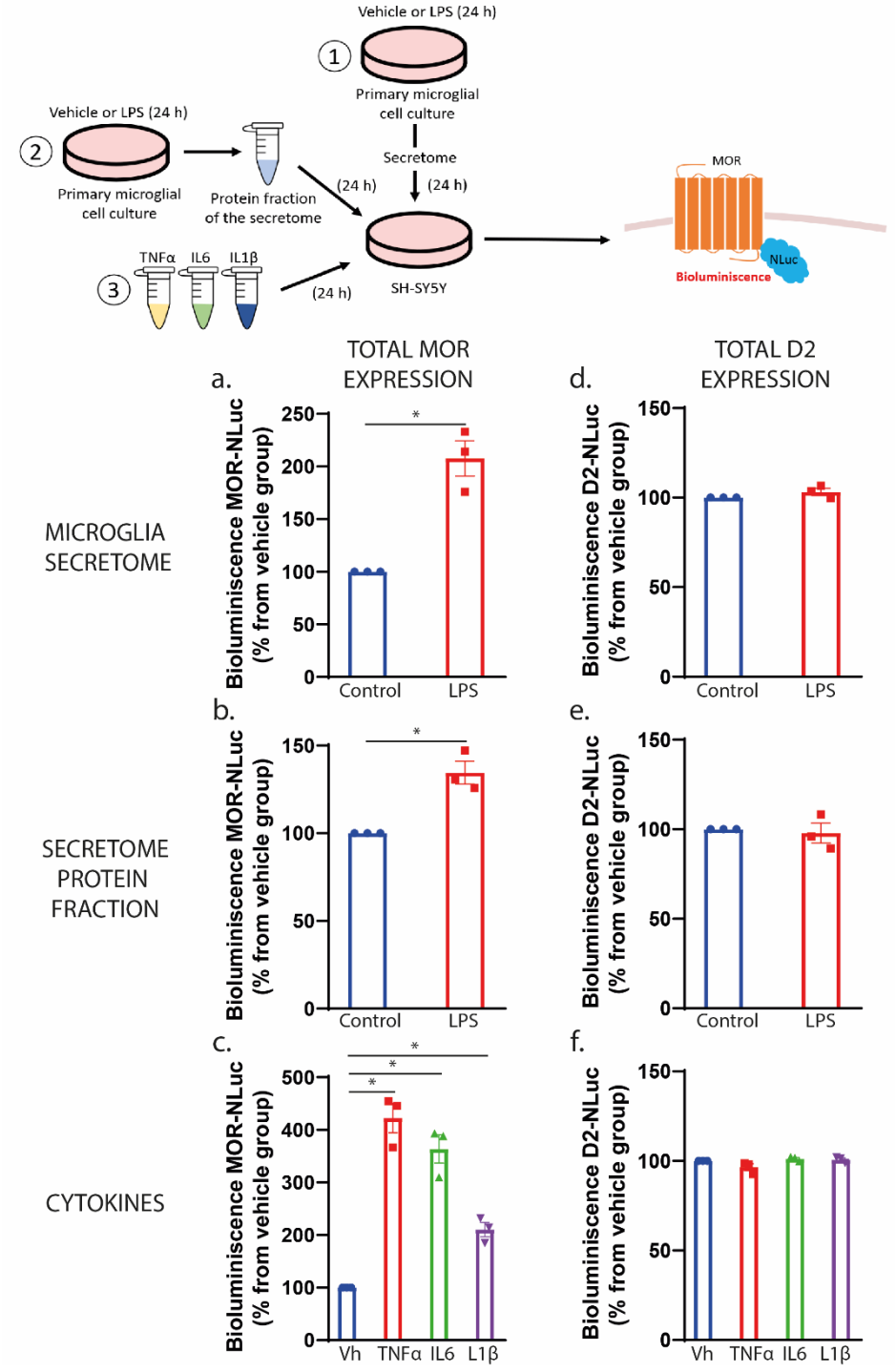

Vehicle or LPS (24 h)
1
Primary microglial cell culture
Secretome
(24 h)
(24 h)
Vehicle or LPS (24 h)
2
Primary microglial cell culture
Protein fraction of the secretome
TNFα IL6 IL1β
3
(24 h)
SH-SY5Y
MOR
NLuc
Bioluminiscence
TOTAL MOR EXPRESSION
TOTAL D2 EXPRESSION
a.
d.
MICROGLIA SECRETOME
Bioluminiscence MOR-NLuc (% from vehicle group)
Bioluminiscence D2-NLuc (% from vehicle group)
Control
LPS
b.
e.
SECRETOME PROTEIN FRACTION
c.
f.
CYTOKINES
Vh
TNFα
IL6
L1β

Figure 2. MOR and D2 protein expression in SH-SY5Y cells treated with microglial secretome, its protein fraction, or proinflammatory cytokines*. a-c: MOR (n=3/group; a: secretome, b: protein fraction, c: cytokines). d-f: D2 (n=3/group; d: secretome, e: protein fraction, f: cytokines). Data are expressed as mean ± SEM. *p<0.05 (T-test for independent samples or one-way ANOVA followed by Games-Howell when appropriate). Lipopolysaccharide (LPS), mu-opioid receptor (MOR), dopamine D2 receptor (D2), nano luciferase (NLuc), vh (vehicle).*

Upon activation, phosphorylated MORs recruit β-arrestins to mediate receptor internalisation. We thus assessed whether the increases in G protein activation were also manifested when β-Arrestin 2 recruitment to the MOR was monitored (Gillis et al., 2020). For this, the BRET between β-Arrestin 2-Venus and MOR-Nluc was measured upon activation of the MOR with 10 µM DAMGO for 10 min. Fig.3d-f show how, although β-Arrestin 2 was being recruited upon activation of the receptor with DAMGO, no differences were found between groups in any of our analysis for microglial secretome, protein fraction of the secretome and cytokines (Microglial secretome, p=0.799; Protein fraction, p=0.838; Cytokines, F(3,11)=0.926 p=0.471).

ACTIVATION OF MOR ON RAT NAcC RELEASES FACTORS THAT CONTROL MOR EXPRESSION AND SIGNALLING IN NEURONAL CELLS

Alongside other authors (Johnston et al., 2004; Bianchi et al., 2008; Lin et al., 2010; Tekieh et al., 2011; Merighi et al., 2013; Bai et al., 2014; Tian et al., 2015; Gessi et al., 2016; Tsai et al., 2016; Shrivastava et al., 2017), we have previously demonstrated that MOR activation can trigger neuroinflammation (Cuitavi et al., 2023b). Therefore, we assessed whether neuroinflammatory mediators released upon MOR pharmacological activation can also regulate neuronal MOR expression and signalling.

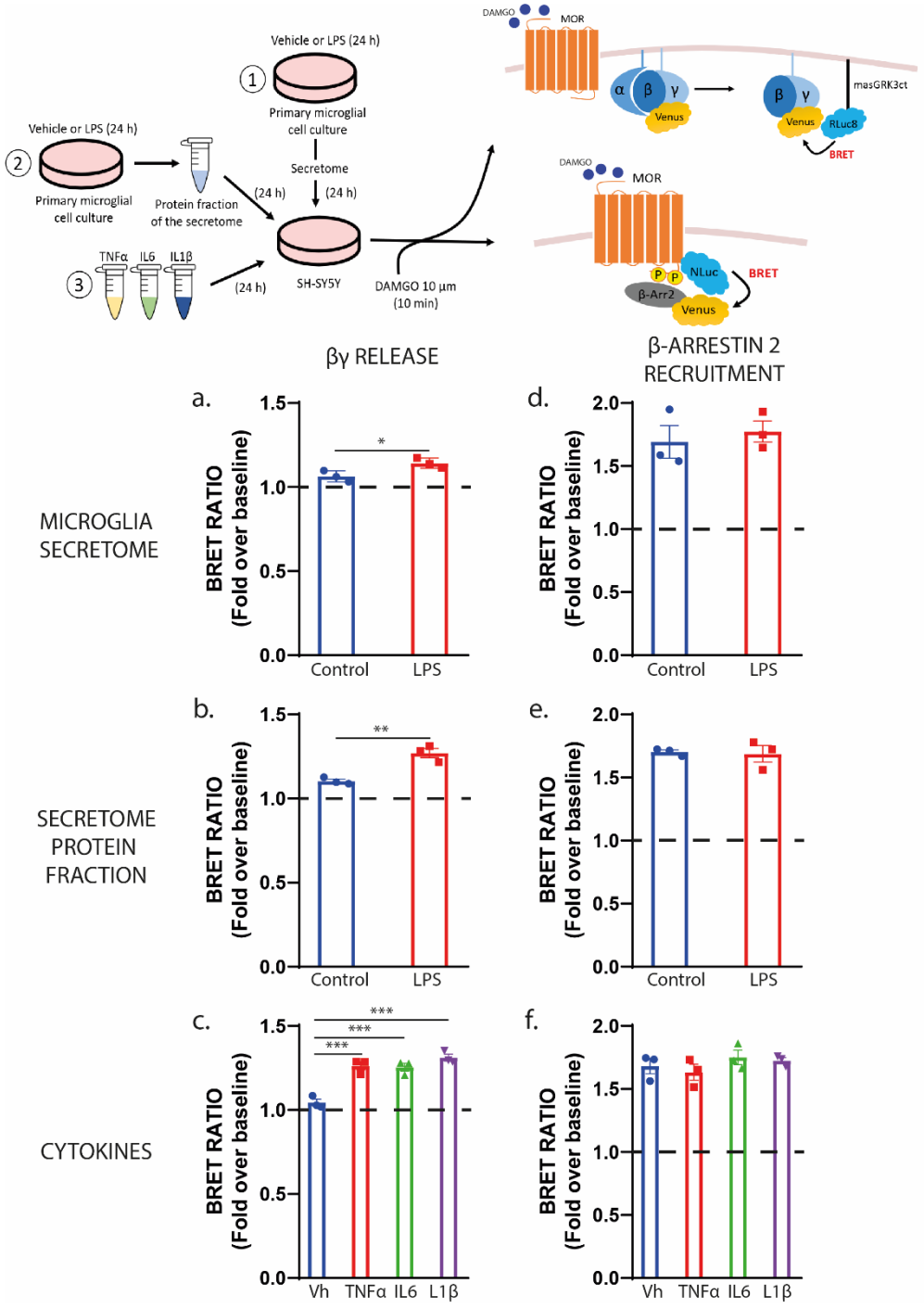

Vehicle or LPS (24 h)
1
Primary microglial cell culture
Secretome
(24 h)
(24 h)
Vehicle or LPS (24 h)
2
Primary microglial cell culture
Protein fraction of the secretome
TNFα IL6 IL1β
3
(24 h)
SH-SY5Y
DAMGO 10 µm (10 min)
DAMGO
MOR
α β γ
Venus
β γ
masGRK3ct
Venus
RLuc8
BRET
DAMGO
MOR
P P
NLuc
BRET
β-Arr2
Venus
βγ RELEASE
β-ARRESTIN 2 RECRUITMENT
MICROGLIA SECRETOME
SECRETOME PROTEIN FRACTION
CYTOKINES
a.
b.
c.
d.
e.
f.
BRET RATIO (Fold over baseline)
Control
LPS
Vh
TNFα
IL6
L1β
*
**
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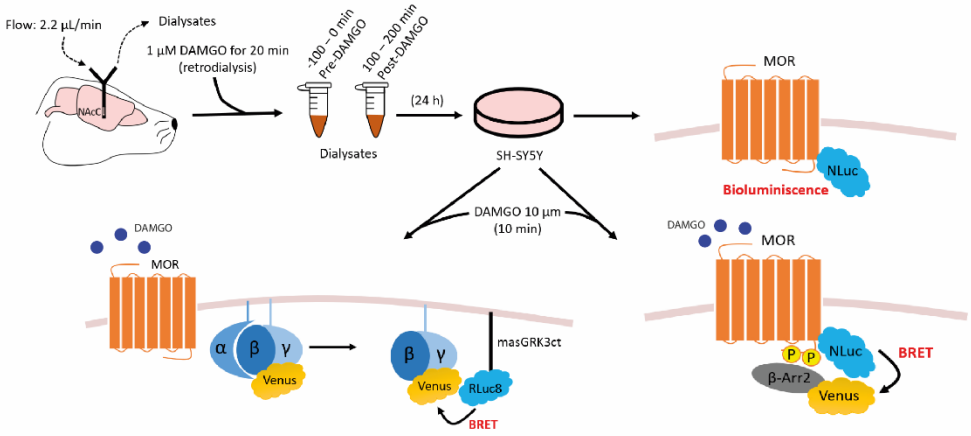
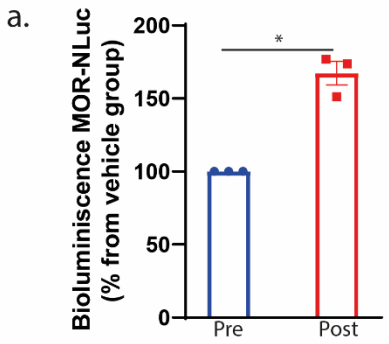
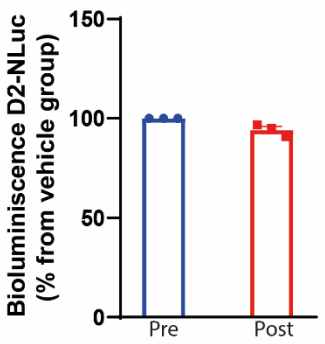
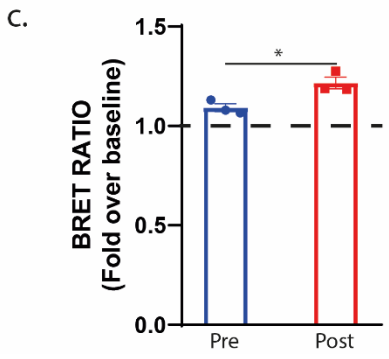
Figure 3. G protein activation and ß-arrestin 2 recruitment to MOR in cells treated with microglial secretome, its protein fraction, or proinflammatory cytokines. *a-c: βγ release (n=3/group; a: secretome, b: protein fraction, c: cytokines). d-f: ß-arrestin 2 recruitment (n=3/group; d: secretome, e: protein fraction, f: cytokines). Dotted lines indicate baseline. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.005 (T-test for independent samples or one-way ANOVA followed by Bonferroni when appropriate). Bioluminescence resonance energy transfer (BRET), lipopolysaccharide (LPS), mu-opioid receptor (MOR), nano luciferase (NLuc), Renilla luciferase 8 (RLuc8), ß-arrestin 2 (ß-Arr2), vh (vehicle), [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO).*

To do so, we carried out a microdialysis experiment in rats in which we obtained dialysates from NacC every 100 min. The first dialysate was used as basal whereas the next four were cytokine-enriched dialysates that followed a retrodialysis administration of 1 µM DAMGO at a 2.2 µL/min flow rate for 20 min (see Cuitavi et al., 2023b for more details). Fig.4 shows how treatment of SH-SY5Y with dialysates from *in vivo* DAMGO-induced neuroinflammation significantly increased MOR protein levels (Fig.4a, p=0.014) without altering D2 levels (Fig.4b, p=0.080). Moreover, dialysates enriched with DAMGO-induced cytokines significantly increased MOR-mediated βγ release when compared to cells treated dialysates obtained prior to the administration of DAMGO to the rats (Fig.4c, p=0.025). Interestingly, cells treated with dialysates enriched with DAMGO-induced cytokines show significant higher levels of β-Arrestin 2 recruitment than when treated with pre-DAMGO-exposed dialysates (Fig.4d, p=0.026), which did not occur in the experiments involving microglial cells (Fig.3).


Flow: 2.2 µL/min
Dialysates
1 µM DAMGO for 20 min
(retrodialysis)
NAcC
-100 – 0 min
Pre-DAMGO
100 – 200 min
Post-DAMGO
Dialysates
(24 h)
SH-SY5Y
MOR
NLuc
Bioluminiscence
DAMGO 10 µm
(10 min)
DAMGO
MOR
α
β
γ
Venus
masGRK3ct
RLuc8
BRET
DAMGO
MOR
P
P
NLuc
BRET
β-Arr2
Venus


a.
Bioluminiscence MOR-NLuc
(% from vehicle group)
200
150
100
50
0
*
Pre
Post


b.
Bioluminiscence D2-NLuc
(% from vehicle group)
150
100
50
0
Pre
Post


c.
BRET RATIO
(Fold over baseline)
1.5
1.0
0.5
0.0
*
Pre
Post

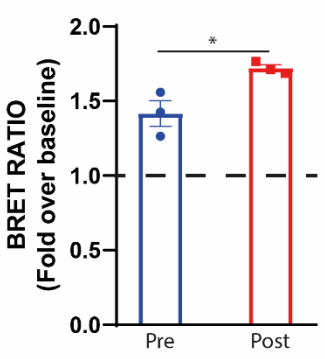

d.
BRET RATIO
(Fold over baseline)
2.0
1.5
1.0
0.5
0.0
*
Pre
Post

Figure 4. MOR and D2 protein expression, G protein activation, and ß-arrestin 2 recruitment in cells treated with DAMGO-induced cytokines-enriched dialysates from rat NAcC *(n=3/group; a: MOR protein expression, b: D2 protein expression, c: βγ release, d: ß-arrestin 2 recruitment). Data are expressed as mean ± SEM. *p<0.05 (T-test for independent samples) Bioluminescence resonance energy transfer (BRET), mu-opioid receptor (MOR), dopamine D2 receptor (D2), nano luciferase (NLuc), Renilla luciferase 8 (RLuc8), ß-arrestin 2 (ß-Arr2), vh (vehicle), [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), nucleus accumbens core (NAcC).*

Discussion

Here, we present how the activation of microglial cells can alter MOR expression and signalling in a neuron-like cell through the release of proinflammatory cytokines. We also show that *in vivo* MOR-mediated cytokine release accounts for an increase in neuronal MOR expression and signalling, which hints the existence of a positive feedback in MOR activation. Altogether our results and previous published data strongly support that glial MORs might impact on neuronal MORs functioning through neuroimmune events.

Several reports have shown an increase in the expression of the MOR mRNA induced by proinflammatory cytokines in different cell types (Ruzicka et al., 1996; Vidal et al., 1998; Kraus et al., 2003; Börner et al., 2004). Of these, only Börner et al. (2004) used neuron-like cells (SH-SY5Y) to show an IL6-induced increase in the *OPRM1* expression. Langsdorf et al. (2011), using an approach similar to the one described here, showed that the medium from macrophage-like cells activated with LPS also increased *OPRM1* expression in SH-SY5Y cells. Our results confirm the role of TNFα, IL6, IL1β and as MOR upregulators in SH-SY5Y cells. Furthermore, our data suggest that the upregulation occurs at the level of the mRNA since the MOR detected in our

experiments is transcribed from an exogenous plasmid the promoter of which is unaffected by proinflammatory cytokines exposure. We also report that LPS-induced microglial activation can increase MOR expression in neuron-like cells through the release of proteins, including but not limited to TNFα, IL6, and IL1β. Our results also show a microglial cytokines-induced increase in the activity of the MORs in the SH-SY5Y cells. This is likely a direct consequence of the increase in MOR expression, although further concentration-response curves of MOR activity will reveal whether the effect observed results from increased potency and/or efficacy of DAMGO at the MOR.

As neuronal MOR activity is tightly related to pain processing (Fields, 2004) and microglia activation-driven neuroinflammation plays a role in the chronicity of pain (Matsuda et al., 2019) our results provide mechanistic insight with regards to the relationship between neuroinflammatory processes and MOR anti-nociceptive activity among other related roles of these receptors. Indeed, microglial cells and MORs play important roles in reward and thus, in substance use disorders (Cuitavi et al., 2021a), specially under pain conditions (Hipólito et al., 2015; Cuitavi et al., 2021b). Therefore, this mechanistic insight between microglial activation and neuronal MORs activity might be used for future research aiming to find new therapies focusing on pain analgesia while limiting the development of OUDs or other substance use disorders. Of note, DAMGO-induced β-Arrestin 2 recruitment by MORs remains unaffected by cytokines exposure, which might indicate that neuroinflammation does not alter MOR internalisation. However, as β-Arrestin 2 recruitment is a less amplified process than G protein activation when measured by βγ release, it cannot be ruled out that the lack of effect in β-Arrestin 2 recruitment is related to assay sensitivity. Other different orthogonal approaches might need to be used to shed light onto this matter. Interestingly, opposite to the results obtained

with the microglial secretome, DAMGO-induced β-Arrestin 2 recruitment is increased in SH-SY5Y cells after a treatment with the cytokines-enriched dialysates derived from DAMGO-treated animals. Although we have previously shown in Cuitavi et al. (2023b) that MOR activation in areas of the MCLS triggers cytokines release and neuroinflammation, it is possible that dialysates contain other mediators that may influence MOR activity in a manner that β-Arrestin 2 recruitment to the receptor becomes more apparent.

Altogether our and others' data suggest that microglia-neuron communication plays a key role in chronic pain, opioid tolerance, and opioid-induced hyperalgesia. On the one hand, chronic pain has a very important inflammatory component that is present in different areas that process pain (Hore and Denk, 2019), which would participate in local MOR desensitisation. On the other hand, there is evidence that opioids induce neuroinflammation (Cahill and Taylor, 2017), which might be partially produced due to their interaction with MORs (Cuitavi et al., 2023b). Therefore, opioid drugs might be promoting tolerance, hyperalgesia, OUD through proinflammatory cytokines, a process enhanced in the presence of pain. Through the work presented here we suggest a relationship between microglial activation, cytokine release, and neuronal MOR expression and activation. Future research is required to further clarify the nature of this relationship, its temporal dynamics and its behavioural consequences. Moreover, we suggest a new mechanism by which pain might become chronic and opioids might be inducing tolerance, hyperalgesia and alterations in opioid reward through its indirect impact on neuronal MOR signalling and expression mediated by microglia. In this context, further experiments with opioids used in clinical settings, such as morphine and fentanyl, to induce microglial and MOR activation would also be interesting to elucidate if they replicate the results obtained with the reference agonist DAMGO.

References

Bai, L. Zhai, C. Han, K. Li, Z. Qian, J. Jing, Y. Zhang, W., Xu, J. T. (2014). Toll-like receptor 4- mediated nuclear factor-κB activation in spinal cord contributes to chronic morphine-induced analgesic tolerance and hyperalgesia in rats. *Neuroscience bulletin, 30*(6), 936-948. https://doi.org/10.1007/s12264-014-1483-7

Bell, J., Strang, J. (2020). Medication treatment of opioid use disorder. *Biological psychiatry*, *87*(1), 82-88. https://doi.org/10.1016/j.biopsych.2019.06.020

Bianchi, M. Franchi, S. Ferrario, P. Sotgiu, M. L., Sacerdote, P. (2008). Effects of the bisphosphonate ibandronate on hyperalgesia, substance P, and cytokine levels in a rat model of persistent inflammatory pain. *European journal of pain, 12*(3), 284-292. https://doi.org/10.1016/j.ejpain.2007.06.005

Bohlen, C. J., Bennett, F. C., Bennett, M. L. (2019). Isolation and culture of microglia. *Current protocols in immunology*, *125*(1), e70. https://doi.org/10.1002/cpim.70

Börner, C. Kraus, J. Schröder, H. Ammer, H., Höllt, V. (2004). Transcriptional regulation of the human μ-opioid receptor gene by interleukin-6. Molecular pharmacology, 66(6), 1719-1726. https://doi.org/10.1124/mol.104.003806

Byrne, L. S. Peng, J. Sarkar, S., Chang, S. L. (2012). Interleukin-1 beta-induced up-regulation of opioid receptors in the untreated and morphine-desensitized U87 MG human astrocytoma cells. *Journal of neuroinflammation, 9*(1), 252. https://doi.org/10.1186/1742-2094-9-252

Cahill, C. M., Taylor, A. M. (2017). Neuroinflammation—a co-occurring phenomenon linking chronic pain and opioid dependence. *Current opinion in behavioral sciences*, *13*, 171-177. https://doi.org/10.1016/j.cobeha.2016.12.003

Chou, R., Turner, J. A., Devine, E. B., Hansen, R. N., Sullivan, S. D., Blazina, I., Dana, T., Bougatsos, C., Deyo, R. A. (2015). The effectiveness and risks of long-term opioid therapy for chronic pain: a systematic review for a National Institutes of Health Pathways to Prevention Workshop. *Annals of internal medicine*, *162*(4), 276-286. https://doi.org/10.7326/M14-2559

Cuitavi, J., Andrés-Herrera, P., Meseguer, D., Campos-Jurado, Y., Lorente, J. D., Caruana, H., & Hipólito, L. (2023). Focal mu-opioid receptor activation promotes neuroinflammation and microglial activation in the mesocorticolimbic system: Alterations induced by inflammatory pain. *Glia*. https://doi.org/10.1002/glia.24374

Cuitavi, J., Hipólito, L., Canals, M. (2021a). The life cycle of the mu-opioid receptor. *Trends in Biochemical Sciences*, *46*(4), 315-328. https://doi.org/10.1016/j.tibs.2020.10.002

Cuitavi, J., Lorente, J. D., Campos-Jurado, Y., Polache, A., & Hipólito, L. (2021b). Neuroimmune and Mu-Opioid Receptor Alterations in the Mesocorticolimbic System in a Sex-Dependent Inflammatory Pain-Induced Alcohol Relapse-Like Rat Model. *Frontiers in immunology*, 3722. https://doi.org/10.3389/fimmu.2021.689453

Cuitavi, J., Torres-Pérez, J. V., Lorente, J. D., Campos-Jurado, Y., Andrés-Herrera, P., Polache, A., Agustín-Pavón, C., Hipólito, L. (2023a). Crosstalk between Mu-Opioid receptors and neuroinflammation: Consequences for drug addiction and pain. *Neuroscience & Biobehavioral Reviews*, 105011. https://doi.org/10.1016/j.neubiorev.2022.105011

Fields, H. (2004). State-dependent opioid control of pain. *Nature Reviews Neuroscience*, *5*(7), 565-575. https://doi.org/10.1038/nrn1431

Fischer, B., Kurdyak, P., Goldner, E., Tyndall, M., Rehm, J. (2016). Treatment of prescription opioid disorders in Canada: looking at the 'other epidemic'?. *Substance abuse treatment, prevention, and policy*, *11*(1), 12. https://doi.org/10.1186/s13011-016-0055-4

Gessi, S. Borea, P. A. Bencivenni, S. Fazzi, D. Varani, K., Merighi, S. (2016). The activation of μ-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia. *FEBS letters, 590*(17), 2813-2826. https://doi.org/10.1002/1873-3468.12313

Gillis, A., Gondin, A. B., Kliewer, A., Sanchez, J., Lim, H. D., Alamein, C., ... & Canals, M. (2020). Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. *Science Signaling*, *13*(625), eaaz3140. https://doi.org/10.1126/scisignal.aaz3140

Giraudon, I., Lowitz, K., Dargan, P. I., Wood, D. M., Dart, R. C. (2013). Prescription opioid abuse in the UK. *British journal of clinical pharmacology*, *76*(5), 823-824. https://doi.org/10.1111/bcp.12133

He, Y., Taylor, N., Yao, X., Bhattacharya, A. (2021). Mouse primary microglia respond differently to LPS and poly (I: C) in vitro. *Scientific reports*, *11*(1), 1-14. https://doi.org/10.1038/s41598-021-89777-1

Hipólito, L., Wilson-Poe, A., Campos-Jurado, Y., Zhong, E., Gonzalez-Romero, J., Virag, L., ... & Morón, J. A. (2015). Inflammatory pain promotes increased opioid self-administration: role of dysregulated ventral tegmental area μ opioid receptors. *Journal of Neuroscience*, *35*(35), 12217-12231. https://doi.org/10.1523/JNEUROSCI.1053-15.2015

Hore, Z., Denk, F. (2019). Neuroimmune interactions in chronic pain–an interdisciplinary perspective. *Brain, behavior, and immunity*, *79*, 56-62. https://doi.org/10.1016/j.bbi.2019.04.033

Johnston, I. N. Milligan, E. D. Wieseler-Frank, J. Frank, M. G. Zapata, V. Campisi, J. Langer, S. Martin, D. Green,P. Fleshner, M. F. Leinwand, L, Maier, S. F., Watkings, L. R. (2004). A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. *Journal of Neuroscience, 24*(33), 7353-7365. https://doi.org/10.1523/JNEUROSCI.1850-04.2004

Kalkman, G. A., Kramers, C., van Dongen, R. T., van den Brink, W., Schellekens, A. (2019). Trends in use and misuse of opioids in the Netherlands: a retrospective, multi-source database study. *The Lancet Public Health*, *4*(10), e498-e505. https://doi.org/10.1016/S2468-2667(19)30128-8

King, N. B., Fraser, V., Boikos, C., Richardson, R., Harper, S. (2014). Determinants of increased opioid-related mortality in the United States and Canada, 1990–2013: a systematic review. *American journal of public health*, *104*(8), e32-e42. https://doi.org/10.2105/AJPH.2014.301966

Kraus, J. Börner, C. Giannini, E., Höllt, V. (2003). The role of nuclear factor κB in tumor necrosis factor-regulated transcription of the human μ-opioid receptor gene. *Molecular pharmacology, 64*(4), 876-884. https://doi.org/10.1124/mol.64.4.876

Langsdorf, E. F., Mao, X., Chang, S. L. (2011). A role for reactive oxygen species in endotoxin-induced elevation of MOR expression in the nervous and immune systems. *Journal of neuroimmunology*, *236*(1-2), 57-64. https://doi.org/10.1016/j.jneuroim.2011.05.009

Lin, S. L. Tsai, R. Y. Tai, Y. H. Cherng, C. H. Wu, C. T. Yeh, C. C., Wong, C. S. (2010). Ultra-low dose naloxone upregulates interleukin-10 expression and suppresses neuroinflammation in morphine-tolerant rat spinal cords. *Behavioural brain research, 207*(1), 30-36. https://doi.org/10.1016/j.bbr.2009.09.034

Matsuda, M., Huh, Y., Ji, R. R. (2019). Roles of inflammation, neurogenic inflammation, and neuroinflammation in pain. *Journal of anesthesia*, *33*(1), 131-139. https://doi.org/10.1007/s00540-018-2579-4

Merighi, S. Gessi, S. Varani, K. Fazzi, D. Stefanelli, A., Borea, P. A. (2013). Morphine mediates a proinflammatory phenotype via μ-opioid receptor–PKCε–Akt–ERK1/2 signaling pathway in activated microglial cells. *Biochemical pharmacology, 86*(4), 487-496. https://doi.org/10.1016/j.bcp.2013.05.027

Reid, K. J., Harker, J., Bala, M. M., Truyers, C., Kellen, E., Bekkering, G. E., Kleijnen, J. (2011). Epidemiology of chronic non-cancer pain in Europe: narrative review of prevalence, pain treatments and pain impact. *Current medical research and opinion*, *27*(2), 449-462. https://doi.org/10.1185/03007995.2010.545813

Rudd, R. A., Seth, P., David, F., Scholl, L. (2016). Increases in drug and opioid-involved overdose deaths—United States, 2010–2015. *Morbidity and mortality weekly report*, *65*(50 & 51), 1445-1452. https://doi.org/10.15585/mmwr.mm655051e1

Ruzicka, B. B. Thompson, R. C. Watson, S. J., Akil, H. (1996). Interleukin-1β-Mediated Regulation of μ-Opioid Receptor mRNA in Primary Astrocyte-Enriched Cultures. *Journal of neurochemistry, 66*(1), 425-428. https://doi.org/10.1016/s0306-4522(96)00669-0

Sá, K. N., Moreira, L., Baptista, A. F., Yeng, L. T., Teixeira, M. J., Galhardoni, R., & de Andrade, D. C. (2019). Prevalence of chronic pain in developing countries: systematic review and meta-analysis. *Pain reports*, *4*(6), e779.

Shrivastava, P., Cabrera, M. A., Chastain, L. G., Boyadjieva, N. I., Jabbar, S., Franklin, T., Sarkar, D. K. (2017). Mu-opioid receptor and delta-opioid receptor differentially regulate microglial inflammatory response to control proopiomelanocortin neuronal apoptosis in the hypothalamus: effects of neonatal alcohol. *Journal of neuroinflammation*, *14*(1), 1-19. https://doi.org/10.1186/s12974-017-0844-3

Tekieh, E. Zaringhalam, J. Manaheji, H. Maghsoudi, N. Alani, B., Zardooz, H. (2011). Increased serum IL-6 level time-dependently regulates hyperalgesia and spinal mu opioid receptor expression during CFA-induced arthritis. *EXCLI journal, 10*, 23. https://doi.org/10.17877/DE290R-8841

Tian, Y. Liu, M. Mao-Ying, Q. L. Liu, H. Wang, Z. F. Zhang, M. T. Wang, J. Li, Q. Liu, S. B. Mi, W. L. Ma. H. J. Wu, G. C., Wang, Y. Q. (2015). Early single Aspirin-triggered Lipoxin blocked morphine anti-nociception tolerance through inhibiting NALP1 inflammasome: involvement of PI3k/Akt signaling pathway. Brain, behavior, and immunity, 50, 63-77. https://doi.org/10.1016/j.bbi.2015.06.016

Tsai, R. Y. Wang, J. C. Chou, K. Y. Wong, C. S., Cherng, C. H. (2016). Resveratrol reverses morphine-induced neuroinflammation in morphine-tolerant rats by reversal HDAC1 expression. *Journal of the Formosan Medical Association, 115*(6), 445-454. https://doi.org/10.1016/j.jfma.2015.05.010

Vidal, E. L. Patel, N. A. Fiala, M., Chang, S. L. (1998). Interleukin-1 induces the expression of μ opioid receptors in endothelial cells. *Immunopharmacology, 38*(3), 261-266. https://doi.org/10.1016/s0162-3109(97)00085-4

Volkow, N. D., Collins, F. S. (2017). The role of science in addressing the opioid crisis. *New England Journal of Medicine*, *377*(4), 391-394. https://doi.org/10.1056/NEJMsr1706626

Statements and declarations

Data availability statement

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

Compliance with ethical standards

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.

The animal study was reviewed and approved by the Ethics Committee in Experimentation and Animal Welfare of the University of Valencia and the local Government of Valencia (Conselleria d'Agricultura, Desenvolupament Rural, Emergéncia Climática i Transició Ecológica).

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CHAPTER 7

Pain-Induced Alcohol Relapse: Role of Mu-Opioid Receptors and Neuroinflammation

Neuroimmune and Mu-Opioid Receptor Alterations in the Mesocorticolimbic System in a Sex-Dependent Inflammatory Pain-Induced Alcohol Relapse-Like Rat Model. Cuitavi J, Lorente JD, Campos-Jurado Y, Polache A, and Hipólito L. *Frontiers in Immunology, 2021*.

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Neuroimmune and Mu-Opioid Receptor Alterations in the Mesocorticolimbic System in a Sex-Dependent Inflammatory Pain-Induced Alcohol Relapse-Like Rat Model

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Evidence concerning the role of alcohol-induced neuroinflammation in alcohol intake and relapse has increased in the last few years. It is also proven that mu-opioid receptors (MORs) mediate the reinforcing properties of alcohol and, interestingly, previous research suggests that neuroinflammation and MORs could be related. Our objective is to study neuroinflammatory states and microglial activation, together with adaptations on MOR expression in the mesocorticolimbic system (MCLS) during the abstinence and relapse phases. To do so, we have used a sex-dependent rat model of complete Freund's adjuvant (CFA)-induced alcohol deprivation effect (ADE). Firstly, our results confirm that only CFA-treated female rats, the only experimental group that showed relapse-like behavior, exhibited specific alterations in the expression of phosphorylated NFκB, iNOS, and COX2 in the PFC and VTA. More interestingly, the analysis of the IBA1 expression revealed a decrease of the microglial activation in PFC during abstinence and an increase of its expression in the relapse phase, together with an augmentation of this activation in the NAc in both phases that only occur in female CFA-treated rats. Additionally, the expression of IL1β also evidenced these dynamic changes through these two phases following similar expression patterns in both areas. Furthermore, the expression of the cytokine IL10 showed a different profile than that of IL1β, indicating anti-inflammatory processes occurring only during abstinence in the PFC of CFA-female rats but neither during the reintroduction phase in PFC nor in the NAc. These data indicate a downregulation of microglial activation and pro-inflammatory processes during abstinence in the PFC, whereas an upregulation can be observed in the NAc during abstinence that is maintained during the reintroduction phase only in CFA-female rats. Secondly, our data reveal a correlation between the alterations observed in IL1β, IBA1 levels, and MOR levels in the PFC and NAc of CFA-treated female rats. Although premature, our data suggest that neuroinflammatory processes, together with neural

adaptations involving MOR, might play an important role in alcohol relapse in female rats, so further investigations are warranted.

Keywords: mu-opioid receptor, alcohol, pain, alcohol deprivation effect, microglia, neuroinflammation

1 INTRODUCTION

Chronic alcohol intake is the third cause of death in developed countries (1, 2), and it is related to many medical conditions, since it is one of the most harmful drugs (3). Alcohol use disorder (AUD) is a recurrent pathological condition that is characterized by repeated relapse episodes after periods of prolonged abstinence (4). Nowadays, pharmacotherapeutic strategies to prevent alcohol relapse have not always shown a great rate of success, probably because there are different aspects (i.e., stress, co-occurrence of other pathologies) that can be developed during abstinence and might impact the efficacy of the anti-relapse pharmacological treatments (5–7). This point is crucial because a better knowledge of neurochemical adaptations occurring during the abstinence and the relapse phases in the presence of different factors (i.e., gender, genetic, contextual, environmental) might help us to develop better therapeutical strategies tailored to the characteristics of the patients.

Mu-opioid receptor (MOR) antagonists (naltrexone and nalmefene) are one of the selected anti-relapse treatments. These medications help reduce the risk of relapse and promote less hazardous drinking (8). Their use is based on the involvement of these receptors in the rewarding and reinforcing properties of alcohol. MORs located in different areas of the mesocorticolimbic system (MCLS) such as the ventral tegmental area (VTA), the nucleus accumbens (NAc), and the prefrontal cortex (PFC) are activated by alcohol-induced endorphin release (9) or alcohol metabolism derivatives (10) to indirectly increase dopaminergic neurons activity and, finally, drive some of the behavioral consequences of alcohol administration. Very interestingly, a variety of literature has revealed a relationship between MOR activation and neuroinflammatory events. On the one hand, MORs on microglia seem to enhance the release of neuroinflammatory mediators, cytokines, and chemokines after their activation (11, 12). On the other hand, pro-inflammatory cytokines such as IL1β, IL6, and TNFα can regulate MOR expression on some immune cells and neurons (13–16). Although this relationship is still not fully understood, cross talk between Toll-like receptor 4 (TLR4) and MORs at the intracellular level seems to participate, as has been recently explained in a revision (17, 18).

Alcohol has also shown to trigger neuroinflammatory events through the TLR4 pathway. In fact, Guerri's laboratory has shown in the last decade that alcohol intermittent administration induces proinflammatory cytokine release through the activation of TLR4, promoting neuronal adaptations (19–23). The reported results by this and other groups have shown that neuroinflammatory events appeared during alcohol administration and early abstinence but might also play an important role in alcohol relapse. In this sense, Ezquer and colleagues have shown very recently that the prevention of alcohol-induced oxidative stress and neuroinflammation in key brain areas of the MCLS through the intranasal administration of exosomes from human mesenquimal cells decreased alcohol intake and blunted alcohol relapse-like binge drinking in female rats bred as alcohol consumers (24).

We hypothesize that, during the abstinence and relapse phases, specific adaptations of the neuroinflammatory state and changes in MOR expression can be developed in selected brain areas of the MCLS of the rats showing relapse-like behavior. To further investigate this hypothesis, we selected a sex-dependent inflammatory pain-induced alcohol deprivation effect (ADE) rat model developed recently by our laboratory based on the complete Freund's adjuvant (CFA) (25). In this model, inflammatory pain could act as a risk factor toward alcohol relapse only in female rats, which were the only group that manifested the ADE (25). It is interesting to note that other animal models to investigate the relapse phenomenon induce alcohol-relapse-like behavior *per se* (i.e., four-bottle choice ADE paradigm), making the investigation of the biochemical adaptation occurring during abstinence in relapsing and non-relapsing individuals difficult. The use of the model proposed here allows us to do this since males and control females do not develop ADE, and the only group showing a significant increase of alcohol intake is the female rats suffering from inflammatory pain. Our objective here, by using this model, is to explore neuroinflammation (measuring phosphorylated NFkB, iNOS, COX2 expression), the activation of microglia (through the expression of IBA1), and cytokine (IL1β and IL10) expression in the abstinence and the reintroduction relapse phases in males and females with or without inflammatory pain in parallel to the expression of the MORs in selected areas of the MCLS.

2 METHODS

2.1 Animals

One hundred fourteen Sprague Dawley adult rats, females and males, were used (Envigo®, Barcelona, Spain). All the animals were kept in inverted light/dark (12/12 h, light on at 22:00) controlled cycles, temperature 23 ± 1°C, and 60% humidity. Each animal was individually housed in a standard plastic cage (42 × 27 × 18 cm^3) with food and tap water provided *ad libitum* throughout the experimental period. Rats were housed in the animal facilities of the University of Valencia. Animal protocols followed in this work were approved by the Animal Care Committee of University of Valencia and were strictly adhered to in compliance with the EEC Council Directive 63/2010 and Spanish laws (RD 53/2013).

2.2 Ethanol Intermittent Access Model and Pain Induction

In this procedure, 40 males and 52 females (total n = 92 rats) followed the classical ethanol intermittent access (IA) model (26) shown in **Figure 1A** in combination with a CFA-based inflammatory pain model (27–29), as we have previously described (25). Rats had free access to 20% ethanol solution and water on Monday, Wednesday, and Friday during 24 h for 8 weeks. After this acquisition period, alcohol was removed for 3 weeks to force a period of abstinence. On the first day of the third week of abstinence, animals received 0.1 ml of CFA (Calbiochem), or sterile saline, in the plantar surface of the hindpaw. The intraplantar injection was made alternately in the right or left hindpaw of the animals in a counterbalance fashion. At the end of 3 weeks of forced abstinence, alcohol was reintroduced following the same IA procedure for five more sessions. Twenty-four hours after the last alcohol session, animals were sacrificed by either isoflurane when the brain was freshly removed or pentobarbital overdose when animals were perfused with paraformaldehyde. Rats belonging to the abstinence groups were sacrificed in the same way after completing 3 weeks of abstinence, on the day when alcohol was supposed to be reintroduced. It is important to notice that brain tissue obtained from all males and 31 females following this protocol were obtained from rats used in a previous study (25). This decision was taken to reduce the number of animals used in this study in compliance with the 3Rs and animal care regulations. Nonetheless, to prove the reproducibility in the animal model first described by Lorente and collaborators (25), a new batch of 21 females was run. Therefore, we only include the alcohol consumption data from these females on this paper (see *Inflammatory Pain Induces Alcohol Relapse in Females* and **Figure 1**). The alcohol consumption data from the males and from the rest of the females can be found in Lorente et al. (2021) (25).

In addition to the rats that followed IA, a control group for the semiquantitative techniques (Western blot and immunofluorescence) composed of 10 females and 12 males

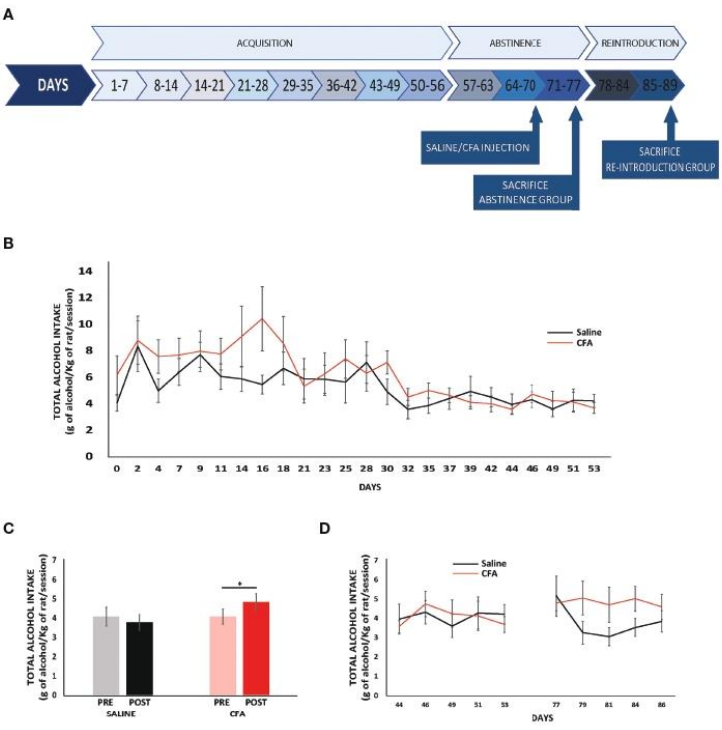

FIGURE 1 | Inflammatory pain induces alcohol relapse in female rats. **(A)** Schematic of the alcohol and inflammatory pain experimental design. **(B)** Alcohol intake during the acquisition period. Data are expressed as mean ± SEM of each consumption day (n = 10–11/group) (ANOVA for repeated measures, $p > 0.05$). **(C)** Mean ± SEM (left saline-female and right CFA-female) of total alcohol intake (g/kg/day) of the 5-day pre- (basal, lighter bar) and post-abstinence (darker bars) shown in gray/black for the saline-treated group (n = 10/group) and in pink/red for the CFA-treated group (n = 11/group) (ANOVA for repeated measures followed by Bonferroni multiple comparisons, $^*p < 0.05$). **(D)** Mean ± SEM of total alcohol intake (g/kg/day) of the last 5 consumption days pre-abstinence and the 5 consumption days post-abstinence (n = 10–11/group); saline-females in black and CFA-females in red (ANOVA for repeated measures, $p > 0.05$).

(n = 22), only having access to water, was run at the same time. Half of the animals from this group were sacrificed by isoflurane when the brain was freshly removed whereas the other half were sacrificed by pentobarbital overdose when animals were perfused with paraformaldehyde.

Therefore, 10 experimental groups were organized by sex:

1. Male (n = 52): control group (rats that had access only to water) and four groups that followed the IA protocol, SAL-A (rats without pain, sacrificed during abstinence), SAL-R (rats without pain sacrificed after reintroduction), CFA-A (rats in pain sacrificed during abstinence), and CFA-R (rats in pain sacrificed after reintroduction);
2. Female (n = 62): control group and four groups that followed the IA protocol, SAL-A, SAL-R, CFA-A, and CFA-R.

2.3 Western Blot

This technique was used to measure the expression levels of phosphorylated-NFκB, iNOS, COX2, IL1β, IL10, and MOR, in different brain areas from control and IA animals sacrificed by isoflurane overdose. Freshly removed brains from 30 females (n = 6/condition) and 30 males (n = 6/condition) were immediately frozen in dry ice and stored at -80°C until the Western blot experiment was performed. Then, PFC, NAc, and VTA were dissected in both hemispheres, and the tissues were homogenized in cold lysis buffer (1% IGEPAL CA-630, 20 mM Tris–HCl pH 8, 130 mM NaCl, 10 mM NaF, and 1% protease inhibitor cocktail, Sigma, St. Louis), using 0.5 ml of lysis buffer each 250 mg of tissue. The homogenate extracts were kept in ice for 30 min. Afterward, samples were immediately centrifuged at 15,000 g for 15 min at 4°C; the supernatant was collected to determine the protein concentration by using a Bradford protein assay kit (Bio-Rad). This procedure was adapted from one previously used (30, 31).

Western blot was used to determine the expression levels of the abovementioned proteins in the homogenate extracts. To do so, we followed a previously used protocol described by Lorente and collaborators (25). The following primary antibodies were used: rabbit IgG anti-MOR (1:1000, Abcam ab134054) (32), rabbit IgG anti-phosphorylated-NFκB p65 (1:1000, Abcam ab86299) (33), rabbit IgG anti-iNOS (1:500, Abcam ab204017) (34), rabbit IgG anti-COX2 (1:1000, Abcam ab52237) (35), rabbit IgG anti-IL1β (1:2500, Invitrogen PA5-79485), and rabbit anti-IL10 (1:2500, Abcam ab9969) (36). Goat IgG anti-rabbit (1:1000, Bio-Rad 1706515) was used as a secondary antibody. Mouse IgG anti-GAPDH conjugated with HRP (1:1000, Invitrogen MA5-15738-HRP) (37) was used to detect GAPDH as a protein loading control. When the membranes were incubated with more than one primary antibody, before probing with the second or third antibody, they were treated with Restore™ Western Blot Stripping Buffer (Thermo Fisher) for 15 min. Finally, the intensity of the bands was expressed as arbitrary units and normalized to GAPDH band intensity. Relative protein levels to control were determined by setting the control group to 100% and calculating the respective percentages for each band. All samples (20 μg) were run in duplicate, obtaining an average of the % from control for each sample. A representative image obtained from each group included in the Western blot analysis is shown on **Supplementary Figure 1**.

2.4 Immunofluorescence

Microglial activation was assessed by measuring ionized calcium-binding adapter molecule 1 (IBA1) expression with an immunofluorescence assay (38). To do so, control and IA animals were used. Thirty-two females (control: n = 4; SAL-A, SAL-R, CFA-A, CFA_R: n = 7/condition) and 22 males (control: n = 6; SAL-A, SAL-R, CFA-A, CFA-R: n = 4/condition) were anesthetized by injecting pentobarbital and followed a procedure of cardiac perfusion with 200 ml paraformaldehyde 0.4% in phosphate buffer (PB) 0.1 M. Brains were extracted and kept in the same perfusion solution for 20 h at 4™C. After that, they were transferred to sucrose 30% in PB 0.1 M for 3 days. Following this, 40-μm brain slices were obtained in four series on a freezing microtome and were stored at -80°C in sucrose 30% in PB 0.1 M until their use. Immunofluorescence was performed as previously described (25). The rabbit IgG anti-IBA1 (1:2000, Wako 019-19741) primary antibody (39) and the donkey IgG anti-rabbit Alexa Fluor® 488 (1:1000, Invitrogen A32790) secondary antibody were used.

Images from PFC, NAc, and VTA were obtained with a ×20 objective (Leica Biosystems, Germany; images size 441 × 330 μm). We obtained six to eight images (from both hemispheres) per area, and mean gray intensity (MGI) was analyzed by means of FIJI software. Results were expressed in percentage of the control group. A representative microphotography of the DAPI and IBA1 staining is shown in **Supplementary Figures 1** and **2**.

2.5 Statistical Analysis

Results are shown as mean ± standard error of the mean (SEM). To perform the statistical analysis, the 26.0 version of the SPSS program was used. The Kolmogorov–Smirnov test and Levene's test were performed to assess the normality and the homogeneity of the variance of the data. When an experimental variable (i.e., alcohol consumption) was continuously measured (i.e., along the experimental procedure), an ANOVA for repeated measures was applied followed by Bonferroni multiple comparisons when appropriate. For Western blot and immunofluorescence, the control group (non-treated rats not exposed to alcohol) was used to allow us to compare the rest of the groups by normalizing them to control in a percentage. For these experiments, the two-way ANOVA test was performed followed by Bonferroni multiple comparisons when significant differences in the main effects (pain; abstinence) or in the interaction were detected. In all the statistical tests, a 95% confidence level was set.

3 RESULTS

3.1 Inflammatory Pain Induces Alcohol Relapse in Female Rats

Figure 1B shows the total alcohol intake rate (g/kg/session) of the new batch of 21 females run in this study along the days of

the acquisition period. Very importantly, no significant differences were found in alcohol consumption before the forced abstinence period between females that were afterward injected with CFA and the ones injected with saline (**Figure 1B**; ANOVA repeated measures, $F(1,19) = 1.048$ $p = 0.319$). **Figure 1C** shows the averages of the alcohol intake levels during the last 5 days of acquisition and the 5 days of reintroduction. Interestingly, the repeated-measure ANOVA showed significant differences ($F(1,19) = 4.615$ $p = 0.045$). In fact, the *post-hoc* test revealed that CFA-female rats significantly increased their consumption levels regarding the basal levels whereas animals injected with saline did not change their consumption levels (**Figure 1D** shows single-day data from the last 5 days of acquisition and the 5 days of reintroduction. Repeated-measure ANOVA showed that there are no differences in the ethanol intake between saline and CFA female rats through time ($F(1,19) = 0.858$ $p = 0.366$) but, as can be observed in the figure, CFA female rats presented higher levels of ethanol consumption than saline ones after reintroduction every testing day.

3.2 Biochemical Analysis of Neuroinflammatory Mediators, IBA-1, IL1β, IL10, and MOR Expression in PFC, NAc, and VTA of Saline and CFA-Treated Male and Female Rats in Abstinence and Reintroduction Phases

Statistical analysis and values of the F and p for the main effects *pain* and *abstinence/re-introduction* and the interaction are summarized in the table of the **Supplementary Material**.

3.2.1 Alcohol-Induced Neuroinflammation in PFC, NAc, and VTA of CFA-Treated Females Present Specific Alterations During Abstinence and Reintroduction Phases

As has previously been shown in the literature, during chronic alcohol administration and early abstinence (24 h), neuroinflammatory pathway is activated. Thereby, the levels of transcriptional factors (as pNFκB) and neuroinflammatory mediators (such as iNOS and COX2) are increased (20, 40). To investigate the neuroinflammatory pathway activation in NAc, PFC, and VTA of saline and CFA rats during abstinence and relapse, we measured by Western blot the levels of phosphorylated NFκB, iNOS, and COX2.

The two-way ANOVA performed found statistically significant differences in main variables and/or its interaction in PFC from saline- and CFA-treated females and males. On the one hand, regarding female rats, pNFκB levels were significantly increased after alcohol reintroduction regardless of the presence of inflammatory pain (**Figure 2A**: SAL_A *vs.* SAL_R, p= 0.03; CFA_A *vs.* CFA_R, $p = 0.0001$). When analyzing iNOS, we observe the opposite phenomenon since its levels were significantly decreased after alcohol reintroduction, regardless of the presence of inflammatory pain (**Figure 2D**: SAL_A *vs.* SAL_R, $p = 0.014$; CFA_A *vs.* CFA_R, $p = 0.036$). Very interestingly, COX2 was the only neuroinflammatory mediator that significantly increased after reintroduction only in CFA-females, indicating a specific change derived from the development of pain during abstinence (**Figure 2G**, CFA_A *vs.* CFA_R, $p = 0.0001$). On the other hand, regarding male rats, no significant differences were observed when analyzing the levels of pNFκB (**Figure 2J**). However, iNOS and COX2 show statistically significant alterations between groups. Interestingly, both proteins are altered only in CFA-males during abstinence since they have significantly higher levels of those proteins during abstinence than after reintroduction, which could be a direct consequence of the presence of inflammatory pain (**Figures 2M, P**).

We also observed significant alterations in VTA from females in our animal model when analyzing pNFκB and iNOS, but we did not observe any significant changes for COX2 (**Figure 2I**). Interestingly, inflammatory pain alters pNFκB levels in a different pattern during abstinence and after alcohol reintroduction in comparison with saline-females. Indeed, CFA-females have higher pNFκB levels than SAL-females during abstinence ($p = 0.0001$). Nonetheless, SAL-females increase their pNFκB levels after alcohol reintroduction ($p = 0.0001$), whereas CFA-females decrease them ($p = 0.007$) (**Figure 2C**). The two-way ANOVA also found differences for the main effect pain when analyzing iNOS expression in the VTA of female rats (**Figure 2F**). In this case, iNOS expression increased only in CFA-females after alcohol reintroduction which was only significant when compared with SAL-females ($p = 0.016$).

Finally, the two-way ANOVA tests performed failed to find any significant differences between groups in NAc for both females and males when analyzing pNFκB, iNOS, and COX2 (**Figure 2B**: pNFκB; **Figures 2E, N**: iNOS, **Figures 2H, Q**: COX2). Additionally, we found no significant differences when comparing groups in VTA from males (**Figures 2L, O, R**).

3.2.2 IBA1 Expression Is Decreased During Abstinence and Increased After Reintroduction of the Alcohol Beverages in the PFC Whereas Opposed Alterations Are Observed in the NAc of Only CFA-Treated Female Rats

Neuroinflammatory processes within the brain are partially regulated by glial cells. Microglia are the immune cells per excellence in the brain. Therefore, we assessed microglial activation with IBA1 immunofluorescence in brain areas of the mesocorticolimbic system, as shown in **Figure 3**.

The two-way ANOVA tests performed showed significant differences when comparing groups in female rats for PFC and NAc, but not for VTA IBA1 staining (**Figure 3E**). Regarding PFC, CFA-females show significantly lower levels during abstinence than after reintroduction ($p = 0.004$) and when compared to SAL-females ($p = 0.009$) during the same period (**Figure 3A**). Interestingly, alterations produced by pain in the NAc were not dependent on the abstinence or relapse-like phase, indicating a specific change for CFA-treated female rats (**Figure 3B**). In fact, inflammatory pain induces microglial activation in both periods since its expression is significantly

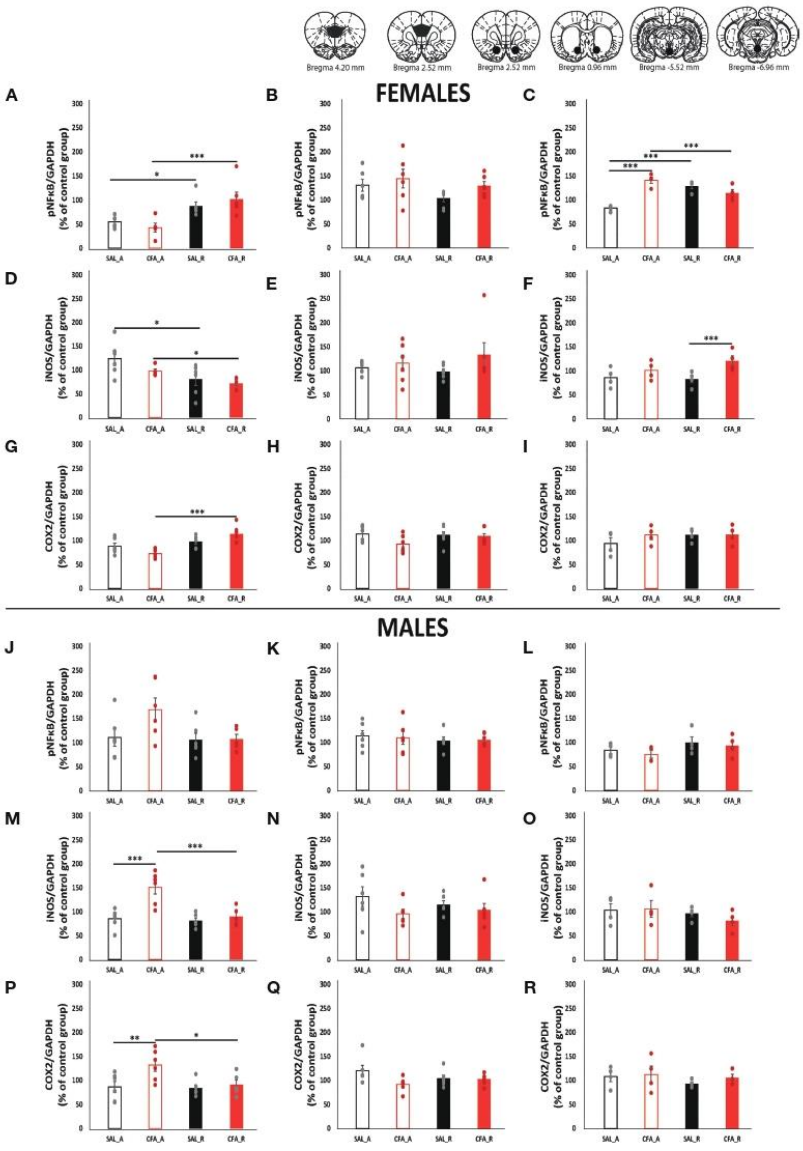

FIGURE 2 | Alterations of inflammatory mediators during the abstinence and the alcohol reintroduction periods in the presence or absence of inflammatory pain in female and male rats. Data are expressed as mean ± SEM of protein levels in % relative to control (n = 4–6/group). Black and red bars represent saline and CFA-females, respectively, during abstinence (empty bars) and after abstinence (filled bars) periods. Points represent the individual data from each animal from the group. On top, schematic representations of the punched brain areas harvested to perform the Western blots. Graphs **(A–I)** gather the protein analysis of female rats and graphs **(J–R)** those from male rats; PFC, NAc, and VTA protein analyses are represented in the following order, PFC: **(A**, **D**, **G**, **J**, **M**, **P)**; NAc: **(B**, **E**, **H**, **K**, **N**, **Q)**; VTA: **(C**, **F**, **I**, **L**, **O**, **R)**. The proteins analyzed are pNFκB **(A–C**, **J–L)**, iNOS **(D–F**, **M–O)**, and COX2 **(G–I**, **P–R)**. Asterisks mark statistically significant differences in the Bonferroni multiple-comparison test applied when the two-way ANOVA detected significant differences in the main effects or in the interaction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$). CFA, complete Freund adjuvant; SAL, saline; A, abstinence period; R, reintroduction period; PRC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; pNFκB, phosphorylated nuclear factor κB; iNOS, inducible nitric oxide synthase and COX2, cyclooxygenase 2.

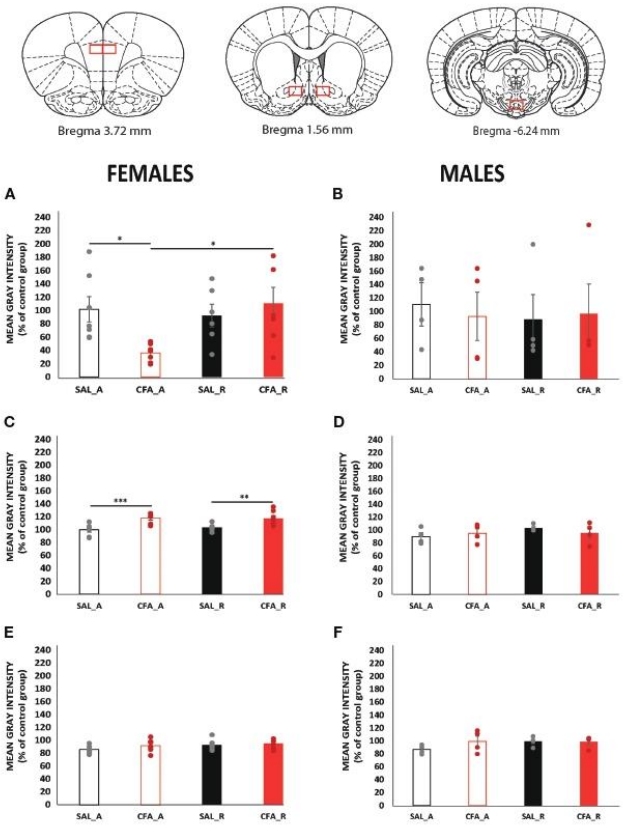

FIGURE 3 | Alterations of microglial activation measured as IBA1 staining during the abstinence and the alcohol reintroduction periods in the presence or absence of inflammatory pain in female and male rats. Data are expressed as mean ± SEM of IBA1 levels in % relative to control (n = 4–7/group). Black and red bars represent saline and CFA-females, respectively, during the abstinence (empty bars) and after abstinence (filled bars) periods. Points represent the individual data from each animal from the group. On the bottom, brain schematics representing the areas where the pictures were taken (Paxinos and Watson 2006). Graphs **(A, C, E)** gather the protein analysis of female rats, and graphs **(B, D, F)** those from male rats; PFC, NAc, and VTA protein analyses are represented in the following order, PFC: **(A, B)**; NAc: **(C, D)**; VTA: **(E, F)** Asterisks mark statistically significant differences in the Bonferroni multiple-comparison test applied when the two-way ANOVA detected significant differences in the main effects or in the interaction (*p < 0.05, **p < 0.01, ***p < 0.005). CFA, complete Freund adjuvant; SAL, saline; A, abstinence period; R, reintroduction period; PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; IBA1, ionized calcium-binding adapter molecule 1.

higher, about 20% higher, for CFA-females than for SAL-females in both abstinence ($p = 0.001$ and reintroduction ($p = 0.007$).

Very interestingly, no significant changes were observed when comparing groups in male rats for neither PFC (**Figure 3B**), NAc (**Figure 3D**), nor VTA (**Figure 3F**).

3.2.3 Downregulation and Upregulation of IL1β and IL10 in the NAc but Not in the PFC of CFA-Treated Female Rats Follow a Different Pattern Than That Observed in Saline-Treated Female Rats

Pro-inflammatory and anti-inflammatory cytokines mainly regulate the activity of several cells, above all immune cells, promoting cell communication. Since we observed alterations in microglial activation in PFC and NAc from female rats, we analyzed the levels of the pro-inflammatory cytokine IL1β and the anti-inflammatory one IL10 in those areas from females.

On the one hand, regarding PFC, the levels of IL1β were significantly lower during abstinence than after reintroduction regardless of the presence of inflammatory pain (**Figure 4A**. Bonferroni multiple comparison: SAL_A *vs.* SAL_R, $p = 0.001$; CFA_A *vs.* CFA_R, $p = 0.0001$). Interestingly, we observed the opposite changes in IL10, indicating a different regulation of pro- and anti-inflammatory events. The levels of IL10 suffered a decrease after reintroduction of the alcohol beverages

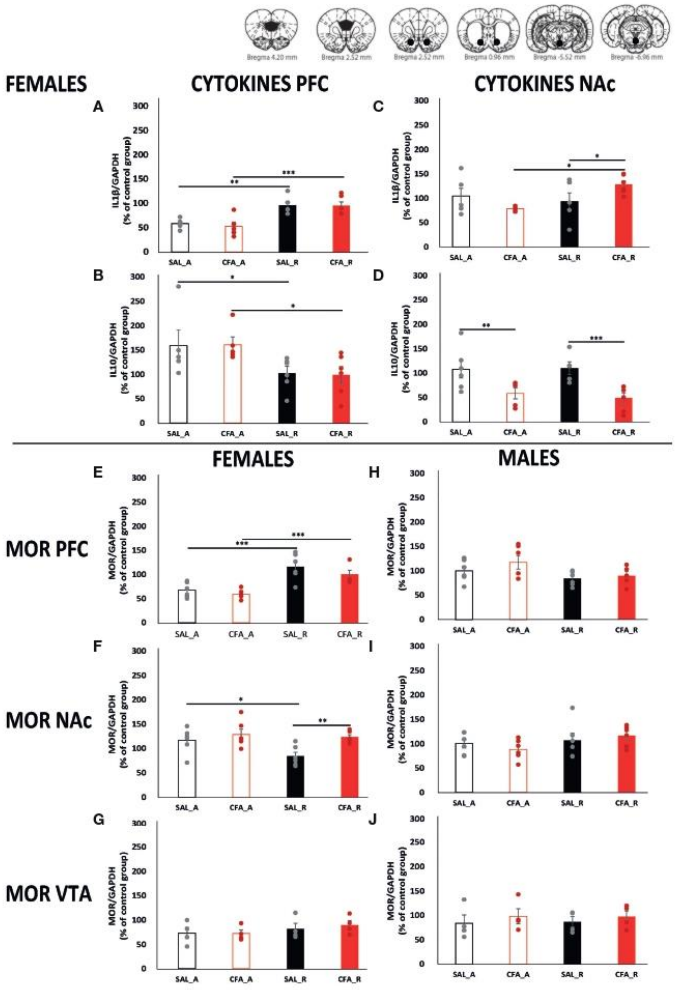

FIGURE 4 | Alterations of inflammatory mediators during the abstinence and the alcohol reintroduction periods in the presence or absence of inflammatory pain in female and male rats. Data are expressed as mean ± SEM of protein levels in % relative to control (n = 4–6/group). Black and red bars represent saline and CFA-females, respectively, during abstinence (empty bars) and after abstinence (filled bars) periods. Points represent the individual data from each animal from the group. On top, schematic representations of the punched brain areas harvested to perform the western blots. Graphs **(A–G)** gather the protein analysis of female rats and graphs **(H–J)** those from male rats; PFC, NAc, and VTA protein analyses are represented in the following order, PFC: **(A, B, E, H)**; NAc: **(C, D, F, I)**; VTA: **(G, J)**. The proteins analyzed are IL1β **(A, C)**, IL10 **(B, D)**, and MOR **(E–J)**. Asterisks mark statistically significant differences in the Bonferroni multiple-comparison test applied when the two-way ANOVA detected significant differences in the main effects or in the interaction (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$). CFA, complete Freund adjuvant; SAL, saline; A, abstinence period; R, reintroduction period; PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; IL1b, interleukin 1b; IL10, interleukin 10; MOR, mu-opioid receptor.

regardless of the presence of inflammatory pain (**Figure 4B**. Bonferroni multiple comparisons: SAL_A *vs.* SAL_R, $p = 0.037$; CFA_A *vs.* CFA_R, $p = 0.025$). On the other hand, in NAc of CFA-treated female rats, the levels of IL1β significantly increased after reintroduction ($p = 0.006$) and also when compared to SAL-animals ($p = 0.045$). Furthermore, when analyzing the levels of the anti-inflammatory cytokine IL10, the development of inflammatory pain significantly decreased the levels of IL10 during abstinence (0.011) and after reintroduction ($p = 0.002$) when compared to SAL-females (**Figure 4D**).

3.2.4 Alcohol and Inflammatory Pain Impacts MOR Expression Patterns in PFC and NAc During Abstinence and Reintroduction Phases in Female Rats: Specific Effect of the Presence of Pain in the NAc

MOR activation during reintroduction phases in areas of the MSCL has shown to play a crucial role in alcohol-relapse-like behavior (41). To investigate dynamic changes of MOR expression in the MSCL areas of interest in the abstinence and reintroduction phases of male and female rats in our model, we measured its relative expression levels by Western blot. **Figure 4** shows MOR expression in saline and CFA-treated female rats during abstinence and after alcohol reintroduction, and in control females in the same conditions. The two-way ANOVA tests performed confirmed that MOR levels were altered depending on the alcohol drinking period, the brain area studied, and the sex.

No changes were observed in the MOR expression of male rats in PFC (**Figure 4H**), NAc (**Figure 4I**), and VTA (**Figure 4J**). Moreover, the expression of MOR in VTA from female rats did not show any significant alterations between groups (**Figure 4G**).

Interestingly, MOR levels were significantly lower during abstinence than after reintroduction regardless of the presence of inflammatory pain in PFC from female rats (**Figure 4E**, Bonferroni multiple comparisons: SAL_A *vs.* SAL_R, $p = 0.0001$; CFA_A *vs.* CFA_R, $p = 0.001$). Finally, very interesting data show alterations in the pattern of MOR expression depending on the presence of pain in the NAc (**Figure 4F**). In the case of saline-treated female rats, MOR expression was reduced after the reintroduction of the alcohol beverages when compared to the abstinence period ($p = 0.018$). In addition, inflammatory pain significantly increases the levels of MORs by 50% in female rats after reintroduction when compared to saline-treated female rats ($p = 0.006$).

4 DISCUSSION

Our present results show dynamic alterations of microglial activation and neuroinflammatory mediator, cytokine, and MOR expression through the abstinence and reintroduction phases of a sex-dependent inflammatory pain-induced alcohol relapse rat model. Some of these alterations demonstrate to be dependent on the sex, abstinence, or reintroduction to alcohol drinking, the MCLS areas studied or their interaction providing new insights into neuroinflammatory properties of alcohol and its interaction with pain-induced alterations in these areas. Firstly, our drinking behavior results in female rats confirm that CFA-treated female rats show ADE, but the saline-treated ones do not as we have previously described in (25). Moreover, this group exhibited alterations in pNFκB and COX2, microglial activation (measured as IBA1 expression), and expression of IL1β and IL10 together with MOR in the PFC, NAc, and/or VTA, with those unique changes in NAc being of a special relevance. Indeed, these results showed a decrease of microglial activation in the PFC only during abstinence, and an augmentation of the microglial activation in the NAc of CFA-female rats in both abstinence and reintroduction phases. Additionally, the expression of pNFκB and IL1β also evidenced these dynamic changes through these two phases following similar expression patterns in both areas. As mentioned, these changes in NAc were observed in the presence of inflammatory pain only in female rats, which was the condition that triggered alcohol-relapse-like behavior in our animal model. Additionally, the expression of cytokine IL10 showed a different profile than the IL1β one, indicating anti-inflammatory processes occurring only during abstinence in the PFC of CFA-female rats, but not during the reintroduction phase in PFC or in the NAc. All in all, these data might indicate a downregulation of microglial activation and pro-inflammatory processes during abstinence in the PFC regardless of the presence of pain, whereas an upregulation can be observed in the NAc during abstinence that is maintained during the reintroduction phase only in CFA-treated females. Furthermore, we also investigated the expression of MORs in the same areas of the MCLS in the abstinence and reintroduction phases of our animal model. Notably, the same dynamics were also observed in the case of the MOR expression, suggesting that the already described interaction between MORs and neuroinflammation might also be underlying the adaptations developed during the abstinence and reintroduction phases.

To our knowledge, this is the first study analyzing neuroinflammatory mediators, microglial activation, and cytokines in the abstinence and alcohol relapse phases of a model that allows to compare sex-dependent behavior. Several reports have shown that chronic alcohol induces neuro inflammation, probably through TLR4, to produce release of diverse pro-inflammatory cytokines (21) and cause neural damage (22). These effects of alcohol exposure seem to be more intense in females than in males (42, 43), and, as shown in **Figure 2**, although the development of pain itself during abstinence altered some of these neuroinflammatory mediators in both male and female rats, our data failed to show increases in neuroinflammatory mediators in male rats. One plausible explanation might be related with the experimental protocol which allows free drinking, instead of forced alcohol intake shown in other studies (20, 44, 45), or the fact that sacrifices were carried out 24 h after the last alcohol consumption, allowing increased neuroinflammatory markers to return to normal levels. Very interestingly, the effect of inflammatory pain in neuroinflammation and microglial activation (see **Figures 2C, M, P**, **3C**) has recently captured the attention of researchers. Recent publications have shown that systemic inflammation leads also to the elevated presence of inflammatory mediators in the brain, which correlates with depressive-like behaviors in patients but also with negative affective state and alterations in neuronal excitability of neurons of NAc in mice (46–48). In line with these data, adaptations derived from the presence of pain might also account for the neuroinflammatory effects produced by alcohol, as is shown in our case for some studied proteins in CFA-treated female rats during abstinence and/or reintroduction phases.

Increasing evidence involves the innate immune system in alcohol drinking (49) and alcohol relapse behavior in female rats. Thus, in a very interesting set of studies, Ezquer and colleagues showed that administration of the secretome or exosomes from human mesenchymal cells prevented the increase in alcohol drinking after a period of abstinence in their female rat model (24, 50). In accordance with these results, our present data showed an increase of IBA1 staining in NAc of CFA-treated female rats during abstinence. Interestingly, in this same experimental group, IL10 expression in the NAc was downregulated, suggesting a pro-inflammatory state that might play a role in promoting ADE since we did not observe these changes in any of the non-relapsing groups. In addition to that, when alcohol was reintroduced, and presumably due to the presence of alcohol, the proinflammatory state was maintained as data of IBA1, pNFκB, and IL1β expression evidenced. Contrary to that, in PFC, CFA-treated female rats showed an anti-inflammatory state during abstinence that was reverted to pro-inflammatory state after alcohol reintroduction most likely as a consequence of alcohol intake and regardless of the presence of pain. In this case, IBA1 staining and the expression of pNFκB and IL1β were significantly lower, together with significantly higher levels of IL10 during abstinence. In line with these results, it has recently been shown that chronic intermittent access to ethanol and lipopolysaccharide exposure differentially alters PFC and NAc microglia soma volume 10 h after the end of the alcohol IA protocol, with microglia from PFC being more affected than that from NAc (51). These brain-region-dependent alterations might be a consequence of the presence of different subtypes of microglia populating in each area (52). It is also very interesting to observe that these alterations in the expression of IBA1 and IL1β in NAc of saline-treated females are different from those observed in CFA-treated females. Altogether, these significantly different alterations for CFA-treated female rats might be taken into consideration since they could potentially explain the inflammatory pain-induced alcohol relapse phenomenon that we observe in our model with only female rats.

Finally, abstinence and alcohol reintroduction did not increase microglial activation in VTA which is in accordance with previous results showing no effect of alcohol on microglial activation in VTA from postmortem human brains (53). From all the proteins studied, only pNFκB and iNOS after reintroduction of alcohol showed significant alterations in the VTA, but because of the involvement of this transcription factor in different physiological events, it is difficult to interpret its significance in the observed behavior. Moreover, we have not measured the levels of the non-phosphorylated form of NFκB, which is also a limitation of the study that makes even more difficult to interpret these results. Further studies should address the consequences of this increase in the pNFκB observed after alcohol reintroduction and its differences in saline versus CFA-treated females.

Interestingly, our results connect microglial activation and the expression of IL1β with MOR levels in relevant brain areas of MCLS. This correlation has already been described in a neonatal alcohol intake model in rats (12). In this report, the authors suggested that microglial MOR activation enhances the neuroinflammatory response, as other papers have also previously reported (11, 12, 54). In addition, the presence of neuroinflammation, and, more specifically, the increase of IL-1β, might influence MOR expression (13–16). In general, our results support these previous reports showing a bidirectional relationship between MORs and IL-1β. Nonetheless, it is interesting to mention that in the NAc, MOR expression remain unaltered during abstinence and after reintroduction to alcohol in CFA-female rats, whereas IL1β significantly increases only after the reintroduction of alcohol. Anyway, IL1β and MOR expressions are both significantly increased in CFA-treated females in the reintroduction phase (**Figures 4C, F**). This lack of correlation with IL1β in the abstinence period might be a consequence of different timelines in the expression patterns. In addition to this, MOR activation can also reduce the levels of anti-inflammatory cytokines such as IL10 (55, 56), and, as our results evidence, in NAc and PFC of females, MORs and IL10 expression levels are opposed along the different phases. The presence of neuroinflammation, and alterations of MCLS MOR expression, are suggested to underlie alcohol neurobiological effects and relapse-like behavior (23, 24, 57–60). All in all, these results point to neuroinflammation-MOR cross talk as a relevant piece in the abstinence and relapse neurobiological substrate puzzle.

Collectively, our results suggest that microglial activation and the resulting neuroinflammation, together with MOR level alterations in PFC and NAc, are likely to participate in an inflammatory pain-induced relapse-like behavior in female rats. Nonetheless, further research to clarify the role of the glial-neuron cross talk in alcohol relapse is warranted.

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 Ethics Committee in Experimentation and Animal Welfare of the University of Valencia and the local Goverment of Valencia (Conselleria d'Agricultura, Desenvolupament Rural, Emergéncia Climática i Transició Ecológica).

AUTHOR CONTRIBUTIONS

Conceptualization, LH and JC. Methodology, JC, JDL, YC-J, and LH. Formal analysis, JC and LH. Investigation, JC, JDL, and YC-J. Writing—original draft, JC. Resources, LH. Supervision, AP and LH. Writing—review and editing, JC, JDL, YC-J, AP, and LH. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.689453/full#supplementary-material

Supplementary Figure 1 | Representative images of DAPI and IBA1 staining from each group in females taken with 20x objective. White scale bars represent 100 µm. Brain schematics represent the areas where the pictures were taken (Paxinos and Watson 2006). CFA, complete Freund Adjuvant; SAL, saline; C, control; A, abstinence period; R, re-introduction period; PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; IBA1, ionized calcium-binding adapter molecule 1; DAPI, 4′,6-diamidino-2-phenylindole.

Supplementary Figure 2 | Representative images of DAPI and IBA1staining from each group in males taken with 20x objective. White scale bars represent 100 µm. Brain schematics represent the areas where the pictures were taken (Paxinos and Watson 2006). CFA, Complete Freund Adjuvant; SAL, saline; C, control; A, abstinence period; R, re-introduction period; PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; IBA1, ionized calcium-binding adapter molecule 1; DAPI, 4′,6-diamidino-2-phenylindole.

Supplementary Figure 3 | Representative measured bands obtained in the western blot from each group and each analysed protein. CFA, Complete Freund Adjuvant; C, control; SAL, saline; A, abstinence period; R, reintroductionperiod; PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; pNF-kB, phosphorylated Nuclear Factor kB; iNOS, inducible Nitric Oxide Synthase; COX2, Cyclooxygenase 2; IL1b, Interleukin 1b; IL10, Interleukin 10; MOR, Mu-Opioid Receptor.

Supplementary Table 1 | Statistical analysis for **Figures 2–4** (Two-Way ANOVA, SPSS 26) Partial Eta2: proportion of explained variance, prefrontal cortex (PFC), nucleus accumbens (NAc), ventral tegmental area (VTA), phosphorylated Nuclear Factor κB (pNF-κB), inducible Nitric Oxide Synthase (iNOS), Cyclooxygenase 2 (COX2), Interleukin 1β (IL1β), Interleukin 10 (IL10), Mu Opioid Receptor (MOR) and ionized calcium-binding adapter molecule 1 (IBA1).

Supplementary Table 2 | Summary of the significant changes observed in the immunofluorescence and western blot analysis. In yellow we present significant effects in main variable abstinence and re-introduction periods, in blue significant effects in main effect saline-treated and CFA-treated rats, in green the significant effects for both and in red no differences. + and - simbols are used to indicate if the group presents higher (+) or lower (-) levels of the protein of analysis when compared to another group with the Bonferroni multiple comparisons. In green cell two simbols are provided, the first one to indicate differences between abstinence and re-introduction and the second one to indicate differences between saline- and CFA-treated rats. CFA, Complete Freund Adjuvant; SAL, saline; A, abstinence period; R, re-introduction period; PFC, prefrontal cortex; NAc, nucleus accumbens.

REFERENCES

1. World Health Organization. World Health Organization. Ageing, Life Course Unit. In: *WHO Global Report on Falls Prevention in Older Age*. Geneva: World Health Organization (2008).
2. Organization WH. *Global Status Report on Alcohol and Health 2018*. Geneva: World Health Organization (2019).
3. Nutt DJ, King LA, Phillips LD. Drug Harms in the UK: A Multicriteria Decision Analysis. *The Lancet* (2010) 376:1558–65. doi: 10.1016/S0140-6736(10)61462-6
4. Gilpin NW, Koob GF. Neurobiology of Alcohol Dependence: Focus on Motivational Mechanisms. *Alcohol Res Health* (2008) 31:185–95.
5. Drobes DJ, Anton RF, Thomas SE, Voronin K. A Clinical Laboratory Paradigm for Evaluating Medication Effects on Alcohol Consumption: Naltrexone and Nalmefene. *Neuropsychopharmacology* (2003) 28:755–64. doi: 10.1038/sj.npp.1300101
6. Fitzgerald N, Angus K, Elders A, Andrade M, Raistrick D, Heather N, et al. Weak Evidence on Nalmefene Creates Dilemmas for Clinicians and Poses Questions for Regulators and Researchers. *Addict (Abingdon England)* (2016) 111:1477–87. doi: 10.1111/add.13438
7. Palpacuer C, Laviolle B, Boussageon R, Reymann JM, Bellissant E, Naudet F. Risks and Benefits of Nalmefene in the Treatment of Adult Alcohol Dependence: A Systematic Literature Review and Meta-Analysis of Published and Unpublished Double-Blind Randomized Controlled Trials. *PloS Med* (2015) 12:e1001924. doi: 10.1371/journal.pmed.1001924
8. Niciu M, Arias A. Targeted Opioid Receptor Antagonists in the Treatment of Alcohol Use Disorders. *CNS Drugs* (2013) 27:777–87. doi: 10.1007/s40263-013-0096-4
9. Gianoulakis C. Alcohol-Seeking Behavior: The Roles of the Hypothalamic-Pituitary-Adrenal Axis and the Endogenous Opioid System. *Alcohol Health Res World* (1998) 22:202–10.
10. Peana AT, Sánchez-Catalán MJ, Hipólito L, Rosas M, Porru S, Bennardini F, et al. Mystic Acetaldehyde: The Never-Ending Story on Alcoholism. *Front Behav Neurosci* (2017) 11:81. doi: 10.3389/fnbeh.2017.00081
11. Merighi S, Gessi S, Varani K, Fazzi D, Stefanelli A, Borea PA. Morphine Mediates a Proinflammatory Phenotype *via* µ-Opioid Receptor–PKCε–Akt–ERK1/2 Signaling Pathway in Activated Microglial Cells. *Biochem Pharmacol* (2013) 86:487–96. doi: 10.1016/j.bcp.2013.05.027
12. Shrivastava P, Cabrera MA, Chastain LG, Boyadjieva NI, Jabbar S, Franklin T, et al. Mu-Opioid Receptor and Delta-Opioid Receptor Differentially Regulate Microglial Inflammatory Response to Control Proopiomelanocortin Neuronal Apoptosis in the Hypothalamus: Effects of Neonatal Alcohol. *J Neuroinflamm* (2017) 14:83. doi: 10.1186/s12974-017-0844-3
13. Kraus J, Börner C, Giannini E, Höllt V. The Role of Nuclear Factor κb in Tumor Necrosis Factor-Regulated Transcription of the Human µ-Opioid Receptor Gene. *Mol Pharmacol* (2003) 64:876–84. doi: 10.1124/mol.64.4.876
14. Börner C, Kraus J, Schröder H, Ammer H, Höllt V. Transcriptional Regulation of the Human µ-Opioid Receptor Gene by Interleukin-6. *Mol Pharmacol* (2004) 66:1719–26. doi: 10.1124/mol.104.003806
15. Langsdorf EF, Mao X, Chang SL. A Role for Reactive Oxygen Species in Endotoxin-Induced Elevation of MOR Expression in the Nervous and

Immune Systems. *J Neuroimmunol* (2011) 236:57–64. doi: 10.1016/j.jneuroim.2011.05.009

16. Byrne LS, Peng J, Sarkar S, Chang SL. Interleukin-1 Beta-Induced Up-Regulation of Opioid Receptors in the Untreated and Morphine-Desensitized U87 MG Human Astrocytoma Cells. *J Neuroinflamm* (2012) 9:252. doi: 10.1186/1742-2094-9-252
17. Eisenstein TK. The Role of Opioid Receptors in Immune System Function. *Front Immunol* (2019) 10:2904. doi: 10.3389/fimmu.2019.02904
18. Zhang P, Yang M, Chen C, Liu L, Wei X, Zeng S. Toll-Like Receptor 4 (TLR4)/Opioid Receptor Pathway Crosstalk and Impact on Opioid Analgesia, Immune Function, and Gastrointestinal Motility. *Front Immunol* (2020) 11:1455. doi: 10.3389/fimmu.2020.01455
19. Alfonso-Loeches S, Ureña-Peralta JR, Morillo-Bargues MJ, Oliver-De La Cruz J, Guerri C. Role of Mitochondria ROS Generation in Ethanol-Induced NLRP3 Inflammasome Activation and Cell Death in Astroglial Cells. *Front Cell Neurosci* (2014) 8:216. doi: 10.3389/fncel.2014.00216
20. Montesinos J, Pascual M, Pla A, Maldonado C, Rodriguez-Arias M, Miñarro J, et al. TLR4 Elimination Prevents Synaptic and Myelin Alterations and Long-Term Cognitive Dysfunctions in Adolescent Mice With Intermittent Ethanol Treatment. *Brain Behav Immun* (2014) 45:233–44. doi: 10.1016/j.bbi.2014.11.015
21. Pascual M, Baliño P, Aragón CMG, Guerri C. Cytokines and Chemokines as Biomarkers of Ethanol-Induced Neuroinflammation and Anxiety-Related Behavior: Role of TLR4 and TLR2. *Neuropharmacology* (2015) 89:352–9. doi: 10.1016/j.neuropharm.2014.10.014
22. Alfonso-Loeches S, Alfonso-Loeches S, Ureña-Peralta J, Ureña-Peralta J, Morillo-Bargues M, Morillo-Bargues M, et al. Ethanol-Induced TLR4/NLRP3 Neuroinflammatory Response in Microglial Cells Promotes Leukocyte Infiltration Across the BBB. *Neurochem Res* (2016) 41:193–209. doi: 10.1007/s11064-015-1760-5
23. Cantacorps L, Alfonso-Loeches S, Moscoso-Castro M, Cuitavi J, Gracia-Rubio I, López-Arnau R, et al. Maternal Alcohol Binge Drinking Induces Persistent Neuroinflammation Associated With Myelin Damage and Behavioural Dysfunctions in Offspring Mice. *Neuropharmacology* (2017) 123:368–84. doi: 10.1016/j.neuropharm.2017.05.034
24. Ezquer F, Quintanilla ME, Morales P, Santapau D, Ezquer M, Kogan MJ, et al. Intranasal Delivery of Mesenchymal Stem Cell-Derived Exosomes Reduces Oxidative Stress and Markedly Inhibits Ethanol Consumption and Post-Deprivation Relapse Drinking. *Addict Biol* (2019) 24:994–1007. doi: 10.1111/adb.12675
25. Lorente JD, Cuitavi J, Campos-Jurado Y, Montón-Molina R, González-Romero JL, Hipólito L. Kappa Opioid Receptor Blockade in NAc Shell Prevents Sex-Dependent Alcohol Deprivation Effect Induced by Inflammatory Pain. *Pain (Amsterdam)* (2021). doi: 10.1097/j.pain.0000000000002332
26. Carnicella S, Ron D, Barak S. Intermittent Ethanol Access Schedule in Rats as a Preclinical Model of Alcohol Abuse. *Alcohol (Fayetteville NY)* (2014) 48:243–52. doi: 10.1016/j.alcohol.2014.01.006
27. Parent AJ, Beaudet N, Beaudry H, Bergeron J, Bérubé P, Drolet G, et al. Increased Anxiety-Like Behaviors in Rats Experiencing Chronic Inflammatory Pain. *Behav Brain Res* (2012) 229:160–7. doi: 10.1016/j.bbr.2012.01.001
28. Hipólito L, Wilson-Poe A, Campos-Jurado Y, Zhong E, Gonzalez-Romero J, Virag L, et al. Inflammatory Pain Promotes Increased Opioid Self-Administration: Role of Dysregulated Ventral Tegmental Area μ Opioid Receptors. *J Neurosci* (2015) 35:12217–31. doi: 10.1523/JNEUROSCI.1053-15.2015
29. Campos-Jurado Y, Lorente JD, González-Romero JL, Granero L, Polache A, Hipólito L. Impaired Alcohol-Induced Dopamine Release in the Nucleus Accumbens in an Inflammatory Pain Model: Behavioural Implications in Male Rats. *Pain (Amsterdam)* (2020) 161:2203–11. doi: 10.1097/j.pain.0000000000001915
30. Sánchez-Catalán MJ, Hipólito, Guerri C, Granero, Polache A. Distribution and Differential Induction of CYP2E1 by Ethanol and Acetone in the Mesocorticolimbic System of Rat. *Alcalc* (2008) 43:401–7. doi: 10.1093/alcalc/agn012
31. Campos-Jurado Y, Martí-Prats L, Morón JA, Polache A, Granero L, Hipólito L. Dose-Dependent Induction of CPP or CPA by intra-pVTA Ethanol: Role of Mu Opioid Receptors and Effects on NMDA Receptors. *Prog Neuropsychopharmacol Biol Psychiatry* (2020) 100:109875. doi: 10.1016/j.pnpbp.2020.109875
32. Lupp A, Richter N, Doll C, Nagel F, Schulz S. UMB-3, a Novel Rabbit Monoclonal Antibody, for Assessing μ-Opioid Receptor Expression in Mouse, Rat and Human Formalin-Fixed and Paraffin-Embedded Tissues. *Regul Peptides* (2011) 167:9–13. doi: 10.1016/j.regpep.2010.09.004
33. Tang Y, Wang C, Wang Y, Zhang J, Wang F, Li L, et al. Isoliquiritigenin Attenuates LPS-Induced AKI by Suppression of Inflammation Involving NF-κb Pathway. *Am J Trans Res* (2018) 10:4141–51.
34. Yang B, Deng G, Zhao R, Dai C, Jiang C, Wang X, et al. Porous Se@SiO2 Nanosphere-Coated Catheter Accelerates Prostatic Urethra Wound Healing by Modulating Macrophage Polarization Through Reactive Oxygen Species-NF-κb Pathway Inhibition. *Acta Biomater* (2019) 88:392–405. doi: 10.1016/j.actbio.2019.02.006
35. Groenendyk J, Paskevicius T, Urra H, Viricel C, Wang K, Barakat K, et al. Cyclosporine A Binding to COX-2 Reveals a Novel Signaling Pathway That Activates the IRE1α Unfolded Protein Response Sensor. *Sci Rep* (2018) 8:16678–15. doi: 10.1038/s41598-018-34891-w
36. Zhao Y, Tian X, Liu G, Wang K, Xie Y, Qiu Y. Berberine Protects Myocardial Cells Against Anoxia-Reoxygenation Injury *via* P38 MAPK-Mediated NF-κb Signaling Pathways. *Exp Ther Med* (2019) 17:230–6. doi: 10.3892/etm.2018.6949
37. Bradley T, Peppa D, Pedroza-Pacheco I, Li D, Cain DW, Henao R, et al. RAB11FIP5 Expression and Altered Natural Killer Cell Function Are Associated With Induction of HIV Broadly Neutralizing Antibody Responses. *Cell (Cambridge)* (2018) 175:387–99.e17. doi: 10.1016/j.cell.2018.08.064
38. Hoogland ICM, Houbolt C, van Westerloo DJ, van Gool WA, van de Beek D. Systemic Inflammation and Microglial Activation: Systematic Review of Animal Experiments. *J Neuroinflamm* (2015) 12:114. doi: 10.1186/s12974-015-0332-6
39. Agustín-Pavón C, Mielcarek M, Garriga-Canut M, Isalan M. Deimmunization for Gene Therapy: Host Matching of Synthetic Zinc Finger Constructs Enables Long-Term Mutant Huntingtin Repression in Mice. *Mol Neurodegeneration* (2016) 11:64. doi: 10.1186/s13024-016-0128-x
40. Fernandez-Lizarbe S, Pascual M, Gascon MS, Blanco A, Guerri C. Lipid Rafts Regulate Ethanol-Induced Activation of TLR4 Signaling in Murine Macrophages. *Mol Immunol* (2008) 45:2007–16. doi: 10.1016/j.molimm.2007.10.025
41. Cuitavi J, Hipólito L, Canals M. The Life Cycle of the Mu-Opioid Receptor. *Trends Biochem Sci (Amsterdam Regular ed)* (2021) 46:315–28. doi: 10.1016/j.tibs.2020.10.002
42. Alfonso-Loeches S, Pascual M, Guerri C. Gender Differences in Alcohol-Induced Neurotoxicity and Brain Damage. *Toxicol (Amsterdam)* (2013) 311:27–34. doi: 10.1016/j.tox.2013.03.001
43. Barton EA, Baker C, Leasure JL. Investigation of Sex Differences in the Microglial Response to Binge Ethanol and Exercise. *Brain Sci* (2017) 7:139. doi: 10.3390/brainsci7100139
44. Pascual M, Blanco AM, Cauli O, Miñarro J, Guerri C. Intermittent Ethanol Exposure Induces Inflammatory Brain Damage and Causes Long-Term Behavioural Alterations in Adolescent Rats. *Eur J Neurosci* (2007) 25:541–50. doi: 10.1111/j.1460-9568.2006.05298.x
45. Montesinos J, Pascual M, Millán-Esteban D, Guerri C. Binge-Like Ethanol Treatment in Adolescence Impairs Autophagy and Hinders Synaptic Maturation: Role of TLR4. *Neurosci Lett* (2018) 682:85–91. doi: 10.1016/j.neulet.2018.05.049
46. Menard C, Pfau M, Hodes G, Kana V, Wang V, Bouchard S, et al. Social Stress Induces Neurovascular Pathology Promoting Depression. *Nat Neurosci* (2017) 20:1752–60. doi: 10.1038/s41593-017-0010-3
47. Dudek KA, Dion-Albert L, Lebel M, LeClair K, Labrecque S, Tuck E, et al. Molecular Adaptations of the Blood-Brain Barrier Promote Stress Resilience vs. Depression. *Proc Natl Acad Sci PNAS* (2020) 117:3326–36. doi: 10.1073/pnas.1914655117
48. Klawonn AM, Fritz M, Castany S, Pignatelli M, Canal C, Similä F, et al. Microglial Activation Elicits a Negative Affective State Through Prostaglandin-Mediated Modulation of Striatal Neurons. *Immun (Cambridge Mass)* (2021) 54:225–34.e6. doi: 10.1016/j.immuni.2020.12.016

49. Mayfield J, Ferguson L, Harris RA. Neuroimmune Signaling: A Key Component of Alcohol Abuse. *Curr Opin Neurobiol* (2013) 23:513–20. doi: 10.1016/j.conb.2013.01.024
50. Ezquer F, Morales P, Quintanilla ME, Santapau D, Lespay-Rebolledo C, Ezquer M, et al. Intravenous Administration of Anti-Inflammatory Mesenchymal Stem Cell Spheroids Reduces Chronic Alcohol Intake and Abolishes Binge-Drinking. *Sci Rep* (2018) 8:4325–15. doi: 10.1038/s41598-018-22750-7
51. Siemsen BM, Landin JD, McFaddin JA, Hooker KN, Chandler LJ, Scofield MD. Chronic Intermittent Ethanol and Lipopolysaccharide Exposure Differentially Alter Iba1-Derived Microglia Morphology in the Prelimbic Cortex and Nucleus Accumbens Core of Male Long-Evans Rats. *J Neurosci Res* (2020) 99:1922–39. doi: 10.1002/jnr.24683
52. Tan Y, Yuan Y, Tian L. Microglial Regional Heterogeneity and its Role in the Brain. *Mol Psychiatry* (2020) 25:351–67. doi: 10.1038/s41380-019-0609-8
53. He J, Crews FT. Increased MCP-1 and Microglia in Various Regions of the Human Alcoholic Brain. *Exp Neurol* (2008) 210:349–58. doi: 10.1016/j.expneurol.2007.11.017
54. Gessi S, Borea PA, Bencivenni S, Fazzi D, Varani K, Merighi S. The Activation of μ-Opioid Receptor Potentiates LPS-Induced NF-kB Promoting an Inflammatory Phenotype in Microglia. *FEBS Lett* (2016) 590:2813–26. doi: 10.1002/1873-3468.12313
55. Azuma Y, Ohura K. Endomorphins 1 and 2 Inhibit IL-10 and IL-12 Production and Innate Immune Functions, and Potentiate NF-kappaB DNA Binding in THP-1 Differentiated to Macrophage-Like Cells. *Scandinavian J Immunol* (2002) 56:260–9. doi: 10.1046/j.1365-3083.2002.01128.x
56. Duncker SC, Philippe D, Martin-Paschoud C, Moser M, Mercenier A, Nutten S. Nigella Sativa (Black Cumin) Seed Extract Alleviates Symptoms of Allergic Diarrhea in Mice, Involving Opioid Receptors. *PloS One* (2012) 7:e39841. doi: 10.1371/journal.pone.0039841
57. Mitchell JM, O'Neil JP, Janabi M, Marks SM, Jagust WJ, Fields HL. Alcohol Consumption Induces Endogenous Opioid Release in the Human Orbitofrontal Cortex and Nucleus Accumbens. *Sci Trans Med* (2012) 4:116ra6. doi: 10.1126/scitranslmed.3002902
58. Perry CJ, McNally GP. μ-Opioid Receptors in the Nucleus Accumbens Shell Mediate Context-Induced Reinstatement (Renewal) But Not Primed Reinstatement of Extinguished Alcohol Seeking. *Behav Neurosci* (2013) 127:535–43. doi: 10.1037/a0032981
59. Castro DC, Berridge KC. Advances in the Neurobiological Bases for Food 'Liking'versus 'Wanting'. *Physiol Behav* (2014) 136:22–30. doi: 10.1016/j.physbeh.2014.05.022
60. Richard JM, Fields HL. Mu-Opioid Receptor Activation in the Medial Shell of Nucleus Accumbens Promotes Alcohol Consumption, Self-Administration and Cue-Induced Reinstatement. *Neuropharmacology* (2016) 108:14–23. doi: 10.1016/j.neuropharm.2016.04.010

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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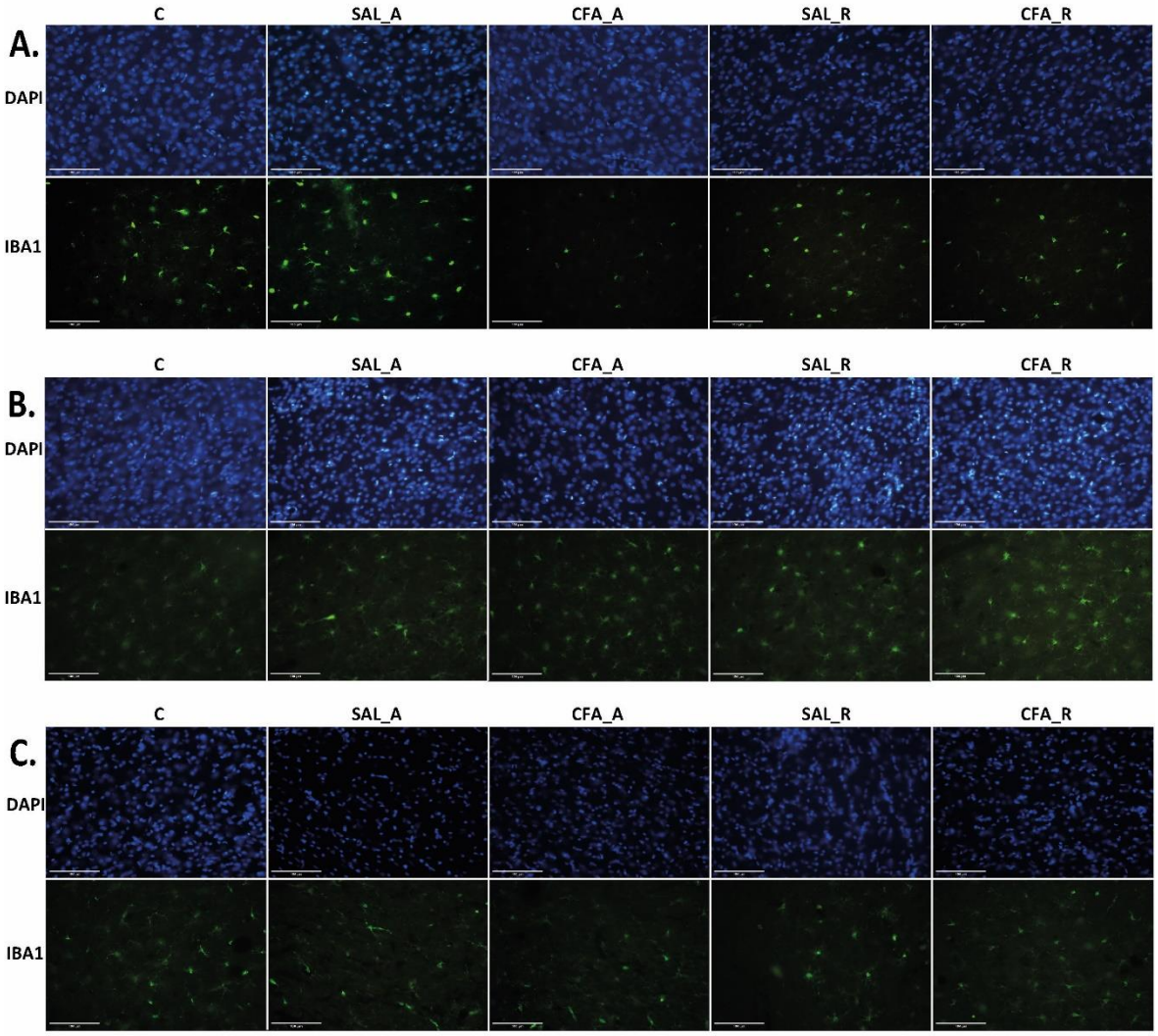
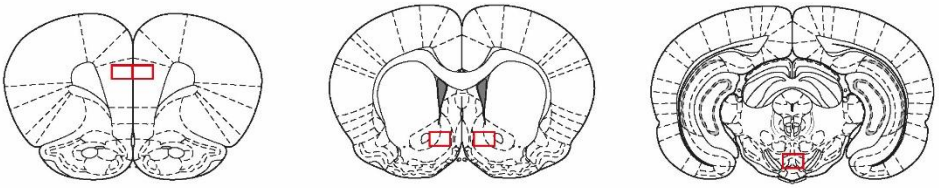
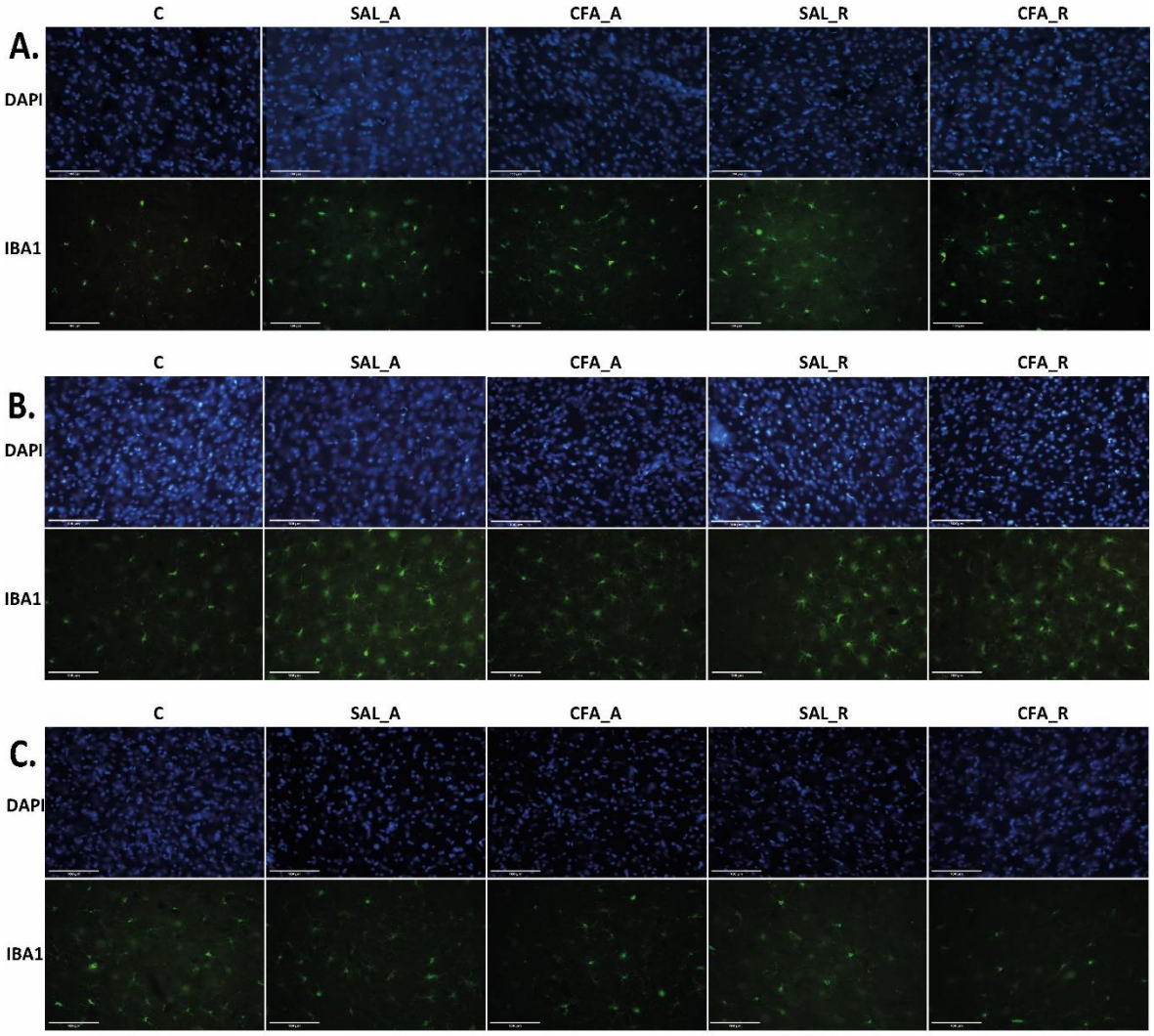
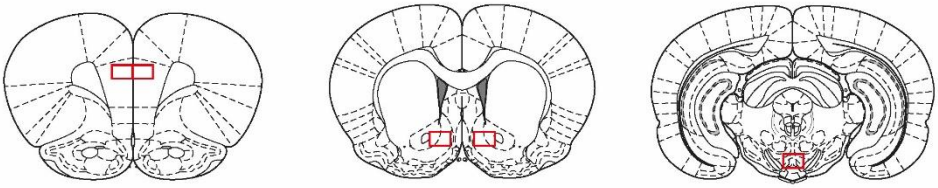
SUPPLEMENTARY FIGURE 1. IBA1 STAINING IN FEMALES





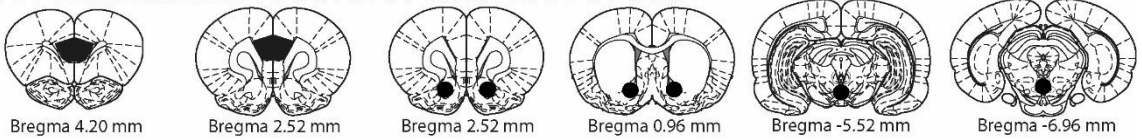
SUPPLEMENTARY FIGURE 2. IBA1 STAINING IN MALES



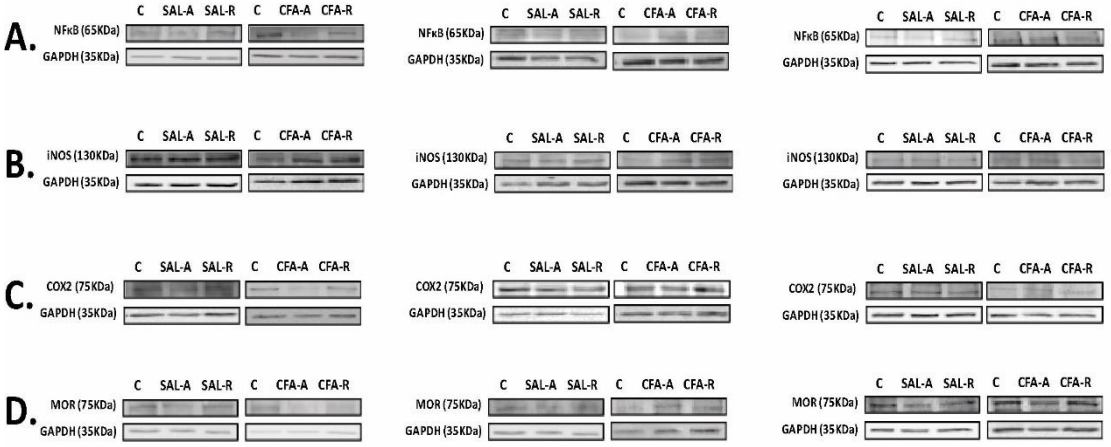


Bregma 3.72 mm | Bregma 1.56 mm | Bregma -6.24 mm

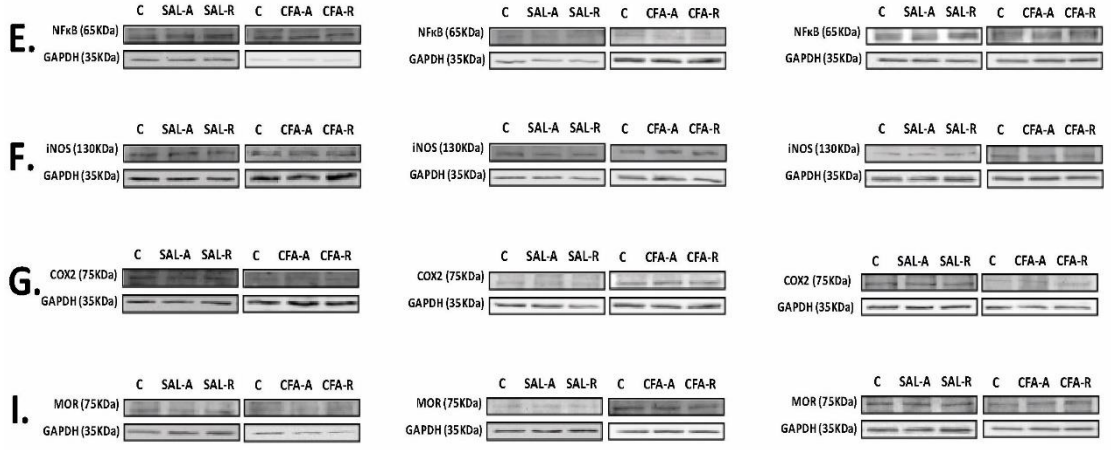
SUPPLEMENTARY FIGURE 3. WESTERN BLOTS



FEMALES



MALES



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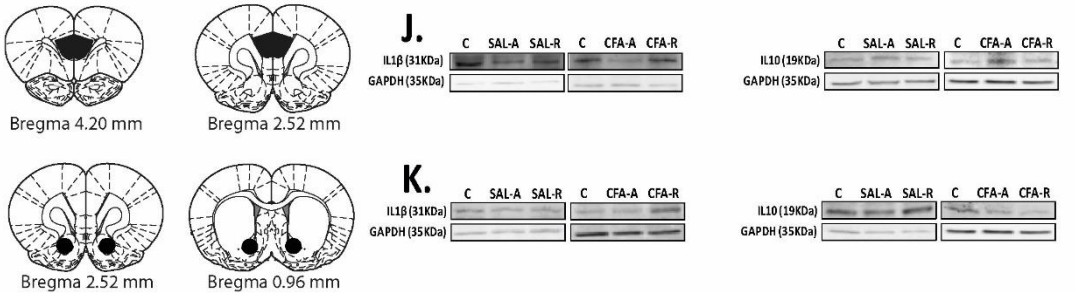

SUPPLEMENTARY TABLE 1.

Figure number	Statistical Test	Factor name	F-value	p-value	Partial Eta^2
Figure 2A		**Abstinence**	F(1,24) = 19.189	<0.001*	0.49
pNFκB PFC	Two-Way ANOVA	**Pain**	F(1,24) = 0.120	0.732	0.006
females		**Intersection**	F(1,24) = 1.165	0.293	0.055
Figure 2B		**Abstinence**	F(1,24) = 2.650	0.119	0.117
pNFκB NAc	Two-Way ANOVA	**Pain**	F(1,24) = 2.476	0.131	0.110
females		**Intersection**	F(1,24) = 0.222	0.643	0.011
Figure 2C		**Abstinence**	F(1,16) = 2.573	0.135	0.177
pNFκB VTA	Two-Way ANOVA	**Pain**	F(1,16) = 13.311	0.003*	0.526
females		**Intersection**	F(1,16) = 38.055	<0.001*	0.760
Figure 2D		**Abstinence**	F(1,24) = 12.187	0.002*	0.379
iNOS PFC	Two-Way ANOVA	**Pain**	F(1,24) = 1.200	0.286	0.057
females		**Intersection**	F(1,24) = 0.099	0.756	0.005
Figure 2E		**Abstinence**	F(1,24) = 0.108	0.745	0.005
iNOS NAc	Two-Way ANOVA	**Pain**	F(1,24) = 2.043	0.168	0.093
females		**Intersection**	F(1,24) = 0.663	0.425	0.032
Figure 2F		**Abstinence**	F(1,16) = 0.743	0.406	0.058
iNOS VTA	Two-Way ANOVA	**Pain**	F(1,16) = 7.612	0.017*	0.388
females		**Intersection**	F(1,16) = 1.489	0.246	0.110
Figure 2G		**Abstinence**	F(1,24) = 15.714	<0.001*	0.440
COX2 PFC	Two-Way ANOVA	**Pain**	F(1,24) = 0.100	0.755	0.005
females		**Intersection**	F(1,24) = 5.614	0.028*	0.219
Figure 2H		**Abstinence**	F(1,24) = 1.003	0.319	0.048
COX2 NAc	Two-Way ANOVA	**Pain**	F(1,24) = 2.467	0.132	0.110
females		**Intersection**	F(1,24) = 1.748	0.201	0.080
Figure 2I		**Abstinence**	F(1,16) = 0.909	0.359	0.070
COX2 VTA	Two-Way ANOVA	**Pain**	F(1,16) = 0.857	0.373	0.067
females		**Intersection**	F(1,16) = 0.743	0.405	0.058
Figure 2J		**Abstinence**	F(1,24) = 3.751	0.067	0.158
pNFκB PFC	Two-Way ANOVA	**Pain**	F(1,24) = 3.033	0.097	0.132
males		**Intersection**	F(1,24) = 2.722	0.115	0.120
Figure 2K		**Abstinence**	F(1,24) = 0.494	0.490	0.024
pNFκB NAc	Two-Way ANOVA	**Pain**	F(1,24) = 0.016	0.902	0.001
males		**Intersection**	F(1,24) = 0.104	0.751	0.005
Figure 2L		**Abstinence**	F(1,16) = 2.932	0.113	0.196
pNFκB VTA	Two-Way ANOVA	**Pain**	F(1,16) = 0.515	0.487	0.041
males		**Intersection**	F(1,16) = 0.021	0.888	0.002
Figure 2M		**Abstinence**	F(1,24) = 12.758	0.002*	0.389
iNOS PFC	Two-Way ANOVA	**Pain**	F(1,24) = 16.799	0.001*	0.457
males		**Intersection**	F(1,24) = 10.083	0.005*	0.335
Figure 2N		**Abstinence**	F(1,24) = 0.112	0.742	0.006
iNOS NAc	Two-Way ANOVA	**Pain**	F(1,24) = 2.967	0.100	0.129
males		**Intersection**	F(1,24) = 0.839	0.371	0.040
Figure 2O		**Abstinence**	F(1,16) = 1.457	0.251	0.108
iNOS VTA	Two-Way ANOVA	**Pain**	F(1,16) = 0.219	0.648	0.018
males		**Intersection**	F(1,16) = 0.471	0.505	0.038
Figure 2P		**Abstinence**	F(1,24) = 4.426	0.048*	0.181
COX2 PFC	Two-Way ANOVA	**Pain**	F(1,24) = 6.663	0.018*	0.250
males		**Intersection**	F(1,24) = 3.523	0.075	0.150
Figure 2Q		**Abstinence**	F(1,24) = 1.003	0.329	0.048
COX2 NAc	Two-Way ANOVA	**Pain**	F(1,24) = 2.467	0.132	0.110
males		**Intersection**	F(1,24) = 1.748	0.201	0.080
Figure 2R		**Abstinence**	F(1,16) = 0.918	0.357	0.071
COX2 VTA	Two-Way ANOVA	**Pain**	F(1,16) = 0.538	0.477	0.043
males		**Intersection**	F(1,16) = 0.150	0.709	0.012
Figure 3A		**Abstinence**	F(1,24) = 3.948	0.058	0.141
IBA1 PFC	Two-Way ANOVA	**Pain**	F(1,24) = 2.058	0.165	0.079
females		**Intersection**	F(1,24) = 6.550	0.017*	0.214
Figure 3B		**Abstinence**	F(1,24) = 0.067	0.800	0.006
IBA1 PFC	Two-Way ANOVA	**Pain**	F(1,24) = 0.014	0.908	0.001
males		**Intersection**	F(1,24) = 0.134	0.721	0.011
Figure 3C		**Abstinence**	F(1,24) = 0.091	0.765	0.004
IBA1 NAc	Two-Way ANOVA	**Pain**	F(1,24) = 24.114	<0.001*	0.501
females		**Intersection**	F(1,24) = 0.537	0.471	0.0022
Figure 3D		**Abstinence**	F(1,24) = 0.039	0.847	0.003
IBA1 NAc	Two-Way ANOVA	**Pain**	F(1,24) = 1.202	0.294	0.091
males		**Intersection**	F(1,24) = 1.043	0.327	0.080
Figure 3E		**Abstinence**	F(1,24) = 2.019	0.168	0.078

IBA1 VTA	Two-Way ANOVA	**Pain**	F(1,24) = 3.168	0.088	0.117
females		**Intersection**	F(1,24) = 0.555	0.463	0.023
Figure 3F		**Abstinence**	F(1,24) = 1.297	0.277	0.098
IBA1 VTA	Two-Way ANOVA	**Pain**	F(1,24) = 1.208	0.293	0.091
males		**Intersection**	F(1,24) = 1.382	0.263	0.103
Figure 4A		**Abstinence**	F(1,24) = 37.202	<0.001*	0.650
IL1β PFC	Two-Way ANOVA	**Pain**	F(1,24) = 0.219	0.645	0.011
females		**Intersection**	F(1,24) = 0.142	0.710	0.007
Figure 4B		**Abstinence**	F(1,24) = 10.896	0.004*	0.353
IL10 PFC	Two-Way ANOVA	**Pain**	F(1,24) = 0.001	0.977	0.001
females		**Intersection**	F(1,24) = 0.017	0.896	0.001
Figure 4C		**Abstinence**	F(1,24) = 3.004	0.098	0.131
IL1β NAc	Two-Way ANOVA	**Pain**	F(1,24) = 0.142	0.710	0.007
females		**Intersection**	F(1,24) = 7.044	0.015*	0.260
Figure 4D		**Abstinence**	F(1,24) = 0.072	0.792	0.007
IL10 NAc	Two-Way ANOVA	**Pain**	F(1,24) = 19.719	<0.001*	0.496
females		**Intersection**	F(1,24) = 0.231	0.636	0.011
Figure 4E		**Abstinence**	F(1,24) = 32.690	<0.001*	0.620
MOR PFC	Two-Way ANOVA	**Pain**	F(1,24) = 2.223	0.152	0.100
females		**Intersection**	F(1,24) = 0.210	0.651	0.010
Figure 4F		**Abstinence**	F(1,24) = 4.492	0.047*	0.183
MOR Nac	Two-Way ANOVA	**Pain**	F(1,24) = 7.823	0.011*	0.281
females		**Intersection**	F(1,24) = 2.314	0.144	0.104
Figure 4G		**Abstinence**	F(1,16) = 1.668	0.221	0.122
MOR VTA	Two-Way ANOVA	**Pain**	F(1,16) = 0.133	0.721	0.011
females		**Intersection**	F(1,16) = 0.209	0.656	0.017
Figure 4H		**Abstinence**	F(1,24) = 5.345	0.032	0.211
MOR PFC	Two-Way ANOVA	**Pain**	F(1,24) = 1.493	0.236	0.069
males		**Intersection**	F(1,24) = 0.363	0.554	0.018
Figure 4I		**Abstinence**	F(1,24) = 2.596	0.123	0.115
MOR Nac	Two-Way ANOVA	**Pain**	F(1,24) = 0.008	0.930	0.001
males		**Intersection**	F(1,24) = 1.042	0.320	0.050
Figure 4J		**Abstinence**	F(1,16) = 0.005	0.944	0.001
MOR VTA	Two-Way ANOVA	**Pain**	F(1,16) = 0.807	0.387	0.063
males		**Intersection**	F(1,16) = 0.017	0.898	0.001

SUPPLEMENTARY TABLE 2.

Brain area	Protein	Sex	Figure	SAL_A	CFA_A	SAL_R	CFA_R
PFC	pNFκB	females	Figure 2A	-	-	+	+
		males	Figure 2J				
	iNOS	females	Figure 2D	+	+	-	-
		males	Figure 2M	-	+/+		-
	COX2	females	Figure 2G		-		+
		males	Figure 2P	-	+/+		-
	IBA1	females	Figure 3A	+	-/-		+
		males	Figure 3B				
	IL1β	females	Figure 4A	-	-	+	+
	IL10	females	Figure 4B	+	+	-	-
	MOR	females	Figure 4E	-	-	+	+
		males	Figure 4H				
NAc	pNFκB	females	Figure 2B				
		males	Figure 2K				
	iNOS	females	Figure 2E				
		males	Figure 2N				
	COX2	females	Figure 2H				
		males	Figure 2Q				
	IBA1	females	Figure 3C	-	+	-	+
		males	Figure 3D				
	IL1β	females	Figure 4C		-	-	+/+
	IL10	females	Figure 4D	+	-	+	-
	MOR	females	Figure 4F	+		-/-	+
		males	Figure 4I				
VTA	pNFκB	females	Figure 2C	-/-	+/+	+	-
		males	Figure 2L				
	iNOS	females	Figure 2F			-	+
		males	Figure 2O				
	COX2	females	Figure 2I				
		males	Figure 2R				
	IBA1	females	Figure 3E				
		males	Figure 3F				
	MOR	females	Figure 4G				
		males	Figure 4J				

CHAPTER 8

Discussion

In general, the results obtained in the present doctoral thesis following the set objectives bring to light a deep connection between the immune system and the opioidergic system, specifically Mu-Opioid receptors (MORs) and their role in pain-induced alcohol relapse. On the one hand, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), a selective MOR agonist, elicits proinflammatory cytokines release in the nucleus accumbens (NAc) and microgliosis in the ventral tegmental area (VTA). Moreover, intra-VTA DAMGO alters microglial proliferation in areas of projection. Interestingly, inflammatory pain alters these microgliosis pattern and induces microglial proliferation in some brain areas in the absence of DAMGO. On the other hand, microglial activation plays a role in neuronal MORs expression and signalling. It is important to notice that MOR activation-induced neuroinflammation also increases neuronal MOR expression and activation. Altogether, these results seem to be key to understand the pain-induced alcohol relapse phenomenon, only given in females, since microglial activation, proinflammatory mediators release, and MOR levels seem to correlate in areas of the mesocorticolimbic system (MCLS) in the relapsing animals.

This section will try to answer several questions that arise given the results obtained in the present doctoral thesis: (1) How tight is the relationship between MORs, microglia, and pain? (2) What are the implications of microglial control over neuronal MORs? (3) How does the MORs-neuroinflammation relationship influence sex-dependent pain-induced alcohol relapse?

8.1. How tight is the relationship between Mu-Opioid receptors, microglia, and pain?

MORs have traditionally been thought to be located only on neurons, in which they exert their main role in reinforcement and pain management (Cuitavi et al., 2021). However, there is evidence that MORs can also be found in glial cells, and more concretely in microglia, which opens a great deal of new possibilities in the study of pain and substance use disorders. The results of the present doctoral thesis also hint that microglia express MORs, since we can observe how they are activated in the presence of a MOR agonist. However, despite our, and others', contribution to the field, whether MORs can be found in microglia or not is still controversial.

Until very recently, the expression or function of MORs in microglia had mainly been tested *in vitro* (Chao et al., 1997; Calvo et al., 2000; Turchan-Cholewo et al., 2008; Horvath and DeLeo, 2009; Merighi et al., 2012; 2013; Mika et al., 2014; Gessi et al., 2016; Popiolek-Barczyk et al., 2017). However, Maduna et al. (2019) found, while using microglia datasets, that microglial cells from cerebral cortex, hippocampus, striatum, and spinal cord express the *OPRM1* gene. Nonetheless, there is controversy regarding this matter. In fact, Kao et al. (2012) failed to find a colocalisation between the microglial marker ionized calcium-binding adapter molecule 1 (IBA1) and MOR mRNA in the spinal cord.

Surprisingly, there are not many research papers dealing with the protein expression of MOR in microglia. Maduna and collaborators indicate that 35.4-51.6% of microglial cells in different brain areas (frontal cortex, central amygdala, ventral tegmental area, and periaqueductal gray), and 36.8-42.4% of microglia in the spinal cord express protein MOR (Maduna et al., 2019). Another research carried out by Horvath et al. (2010) also shows MOR protein expression in spinal microglia.

Recently, Reiss et al. (2022) developed a conditional knockout mouse line in which microglial cells do not express MORs. Interestingly, this mouse line showed none or reduced opioid-induced hyperalgesia and dependence. This piece of research indicates, although indirectly, that microglia express MOR. Moreover, it is one of the first papers wherein a possible role for microglial MORs is suggested. Nonetheless, another piece of research indicates that the loss of microglial MORs does not play a role in opioid-induced hyperalgesia and tolerance (Corder et al., 2017). It is clear that there is a lack of consensus when addressing this matter and we hope that our results will help to clarify this matter, although further research will be needed to clarify if microglial MORs are a plausible target to approach chronic pain and analgesia.

Microglial MOR activation accounts for neuroinflammation (Merighi et al., 2013; Gessi et al., 2016), which very possibly plays a role in pain processing. In fact, microglia contribute to pain pathogenesis through its control over synaptic plasticity in the spinal cord (Chen et al., 2018). Moreover, it is well-stablished that pain induces microgliosis in the spinal cord (Gilmore, 1975; Glimore and Skinner, 1979). However, whether other parts of the central nervous system experience this pain-induced microgliosis remains a barely explored subject. In this sense, the MCLS is compromised in the presence of a pain condition, which leads to a variety of comorbidities (Seminowicz et al., 2009; Taylor, 2016). Nonetheless, although there is evidence that neuropathic pain produces microgliosis within this system (Taylor et al., 2017), to the best of our knowledge, the results of this doctoral thesis are the first ones to present this phenomenon in an inflammatory pain model.

All in all, the results obtained in the present doctoral thesis not only corroborate the findings exposed and discussed in this section but also go a step further. We provide evidence of a complex regulatory system that involves MOR signalling in the MCLS, microgliosis, and a clear influence of pain in controlling the

system. This discovery is important when addressing pain-induced comorbidities, especially drug use disorders. In this sense, MORs within the MCLS play a critical role in the development of opioid use disorders derived from opioid prescription to treat pain. By knowing more about how microglial MORs interact with opioids or other MOR agonists in the presence of pain, more accurate treatment will be developed.

8.2. What are the implications of microglial control over neuronal Mu-Opioid receptors?

Microglia are immune cells in charge of protecting the central nervous system. However, there is evidence that they can also participate in the disruption of the homeostasis of this very system under pathological conditions (Marinelli et al., 2019). In fact, they contribute to substance use disorders and chronic pain by releasing cytokines, chemokines, and reactive oxygen species (Kawasaki et al., 2008; Gu et al., 2016; Bachtell et al., 2017; Henriques et al., 2018; Inoue and Tsuda, 2018; Catale et al., 2019; Locke et al., 2020; Kane and Drew, 2021). Nonetheless, further research is needed to deepen the knowledge about how microglia disrupt homeostasis in substance use disorders and chronic pain. The results of the present doctoral thesis shed light on this matter and propose a new way of communication between microglia and neurons by which microglia release proinflammatory cytokines which affect neuronal MOR expression and signalling. Briefly, MOR agonism induces the release of proinflammatory cytokines and microgliosis in a dose-dependent fashion, which is altered by the presence of an inflammatory pain condition. Generally, the presence of pain impairs the agonist-elicited effect. Furthermore, the resulting inflammation modifies neuronal MOR signalling induced by the presence of an agonist and expression.

The organism relies on microglia to control pain transmission and analgesia (Tsuda et al., 2013; Tsuda, 2019) by inducing microgliosis in the spinal cord (Gu et al., 2016; Locke et al., 2020). Our results suggest that this microgliosis would be increasing MORs expression and activation which would have a direct impact on the mechanism of endogenous analgesia. However, we can only suggest this mechanism for acute pain since our experiments were set at 24 h. It would be interesting to test

this microglia-neuronal MOR connection for longer periods of time since this information would be very useful to improve chronic pain current treatments. Moreover, *in vivo* studies are needed to further confirm our results and observe possible sex-dependent factors that may alter the results obtained.

In this doctoral thesis we also provide evidence that acute inflammatory pain induces microglial proliferation in some areas of the MCLS, which has previously been described for neuropathic pain (Taylor et al., 2017). It is very likely that this microgliosis contributes to alterations in mesocorticolimbical MORs. It is extremely complicated to predict how these changes would affect pain-induced comorbidities. On the one hand, MORs are widespread in the system and their function greatly depends on the neuron they are expressed in and in which part of it they are localised (Cuitavi et al., 2021). On the other hand, we have shown in this doctoral thesis that the presence of a MOR agonist modifies both microglial and neuronal MOR responses. However, based on the existing literature, we can hypothesise that, in the presence of pain and an opioid-like drug, when neuroinflammation and the opioid-like drug are widespread in the brain stem, the microglia-MOR relationship would be promoting substance use disorders. In this sense, insightful *in vivo* experiments focused on how this relationship acts in specific neurons within concrete brain areas would be very useful to further clarify the role it plays in pain-induced substance use disorders.

8.3. How does the Mu-Opioid receptors-neuroinflammation relationship influence sex-dependent pain-induced alcohol relapse?

There is a lot of information regarding the sex-dependency inherent to substance abuse biology and epidemiology, specifically about alcohol use disorders (AUDs; Hommer, 2003; Anker and Carroll, 2010; Sanchis-Segura and Becker, 2016; Grant et al., 2017). Moreover, pain also affects men and women in different ways (Kurita et al., 2012; Mogil, 2012; Ruau et al., 2012; Landmark et al., 2013; Steingrímsdóttir et al., 2017). In this sense, the results of the present thesis alongside previous published work from our research group (Lorente et al., 2022) show how female rats with inflammatory pain are more prone to suffer from alcohol deprivation effect than females without inflammatory pain and males in general. Furthermore, in this doctoral thesis we show how only relapsing females present with dynamic changes in different areas of the MCLS in microglia proliferation, proinflammatory cytokines release, and MOR levels during abstinence and after relapsing. Moreover, after the reintroduction of ethanol, the aforementioned changes correlated. These alterations suggest that the bidirectional relationship between MOR and microglia, studied in the present doctoral thesis, could be playing a role in the pain-induced alcohol relapse phenomenon in a sex-dependent way. Additionally, it is important to pinpoint that we did not observe sex differences when we measured cytokine release derived from the treatment with a MOR agonist. This suggests that pain is most probably the factor that shapes the sex differences in microglial activation in our model.

As mentioned in the previous sections, sex is a factor that we need to further study when observing the connection between MORs and microglia since our work

has been mainly carried out in males and *in vitro*. However, we can hypothesise that these sex-dependent changes observed in this doctoral thesis are probably linked to differences in MORs expression and microglial reaction. In fact, there is evidence that MOR density in the brain, including areas of the MCLS, varies between males and females (Zubieta et al., 1999; Wang et al., 2012; Zamfir et al., 2022). Additionally, the immune system is widely affected by sex hormones (Klein and Flanagan, 2016), including microglial cells, which also work in a sex-dependent manner (Lenz and McCarthy, 2015; Han et al., 2021). Bearing in mind everything that has been discussed in this section, the need to test both sexes in preclinical and clinical studies is highlighted once again. In this sense, it would be interesting to carry out further clinical research in AUDs to observe in detail risk factors such as pain and how they behave when dividing the population by sex. This would be extremely useful to approach treatments for AUDs in a more personalised manner, which would increase the rates of success.

8.4. References

Anker, J. J., & Carroll, M. E. (2010). Females are more vulnerable to drug abuse than males: evidence from preclinical studies and the role of ovarian hormones. Biological basis of sex differences in psychopharmacology, 73-96.

Bachtell, R. K., Jones, J. D., Heinzerling, K. G., Beardsley, P. M., & Comer, S. D. (2017). Glial and neuroinflammatory targets for treating substance use disorders. *Drug and alcohol dependence*, *180*, 156-170.Schroder, K., & Tschopp, J. (2010). The inflammasomes. *cell*, *140*(6), 821-832.

Calvo, C. F., Cesselin, F., Gelman, M., & Glowinski, J. (2000). Identification of an opioid peptide secreted by rat embryonic mixed brain cells as a promoter of macrophage migration. *European Journal of Neuroscience*, *12*(8), 2676-2684.

Catale, C., Bussone, S., Iacono, L. L., & Carola, V. (2019). Microglial alterations induced by psychoactive drugs: A possible mechanism in substance use disorder?. In *Seminars in Cell & Developmental Biology* (Vol. 94, pp. 164-175). Academic Press.

Chao, C. C., Hu, S., Shark, K. B., Sheng, W. S., Gekker, G., & Peterson, P. K. (1997). Activation of mu opioid receptors inhibits microglial cell chemotaxis. *Journal of pharmacology and experimental therapeutics*, *281*(2), 998-1004.

Chen, G., Zhang, Y. Q., Qadri, Y. J., Serhan, C. N., & Ji, R. R. (2018). Microglia in pain: detrimental and protective roles in pathogenesis and resolution of pain. *Neuron*, *100*(6), 1292-1311.

Corder, G., Tawfik, V. L., Wang, D., Sypek, E. I., Low, S. A., Dickinson, J. R., ... & Scherrer, G. (2017). Loss of μ opioid receptor signaling in nociceptors, but not

microglia, abrogates morphine tolerance without disrupting analgesia. *Nature medicine*, *23*(2), 164-173.

Cuitavi, J., Hipólito, L., & Canals, M. (2021). The life cycle of the mu-opioid receptor. *Trends in Biochemical Sciences*, *46*(4), 315-328.

Gessi, S., Borea, P. A., Bencivenni, S., Fazzi, D., Varani, K., & Merighi, S. (2016). The activation of μ-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia. *FEBS letters*, *590*(17), 2813-2826.

Gilmore, S. A. (1975). Proliferation of non-neuronal cells in spinal cords of irradiated, immature rats following transection of the sciatic nerve. *The Anatomical Record*, *181*(4), 799-811.

Gilmore, S. A., & Skinner, R. D. (1979). Intraspinal non-neuronal cellular responses to peripheral nerve injury. *The Anatomical Record*, *194*(3), 369-387.

Grant, B. F., Chou, S. P., Saha, T. D., Pickering, R. P., Kerridge, B. T., Ruan, W. J., ... & Hasin, D. S. (2017). Prevalence of 12-month alcohol use, high-risk drinking, and DSM-IV alcohol use disorder in the United States, 2001-2002 to 2012-2013: results from the National Epidemiologic Survey on Alcohol and Related Conditions. JAMA psychiatry, 74(9), 911-923.

Gu, N., Peng, J., Murugan, M., Wang, X., Eyo, U. B., Sun, D., ... & Wu, L. J. (2016). Spinal microgliosis due to resident microglial proliferation is required for pain hypersensitivity after peripheral nerve injury. *Cell reports*, *16*(3), 605-614.

Han, J., Fan, Y., Zhou, K., Blomgren, K., & Harris, R. A. (2021). Uncovering sex differences of rodent microglia. *Journal of neuroinflammation*, *18*(1), 1-11.

Henriques, J. F., Portugal, C. C., Canedo, T., Relvas, J. B., Summavielle, T., & Socodato, R. (2018). Microglia and alcohol meet at the crossroads: Microglia as critical modulators of alcohol neurotoxicity. *Toxicology letters*, *283*, 21-31.

Hommer, D. W. (2003). Male and female sensitivity to alcohol-induced brain damage. Alcohol Research & Health, 27(2), 181.

Horvath, R. J., & DeLeo, J. A. (2009). Morphine enhances microglial migration through modulation of P2X4 receptor signaling. *Journal of Neuroscience*, *29*(4), 998-1005.

Horvath, R. J., Romero-Sandoval, E. A., & De Leo, J. A. (2010). Inhibition of microglial P2X4 receptors attenuates morphine tolerance, Iba1, GFAP and μ opioid receptor protein expression while enhancing perivascular microglial ED2. *Pain*, *150*(3), 401-413.

Inoue, K., & Tsuda, M. (2018). Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. *Nature Reviews Neuroscience*, *19*(3), 138-152.

Kane, C. J., & Drew, P. D. (2021). Neuroinflammatory contribution of microglia and astrocytes in fetal alcohol spectrum disorders. *Journal of neuroscience research*, *99*(8), 1973-1985.

Kao, S. C., Zhao, X., Lee, C. Y., Atianjoh, F. E., Gauda, E. B., Yaster, M., & Tao, Y. X. (2012). Absence of mu opioid receptor mRNA expression in astrocytes and microglia of rat spinal cord. *Neuroreport*, *23*(6), 378.

Kawasaki, Y., Zhang, L., Cheng, J. K., & Ji, R. R. (2008). Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1β, interleukin-6, and

tumor necrosis factor-α in regulating synaptic and neuronal activity in the superficial spinal cord. *Journal of neuroscience*, *28*(20), 5189-5194.

Klein, S. L., & Flanagan, K. L. (2016). Sex differences in immune responses. Nature Reviews Immunology, 16(10), 626-638.

Kurita, G. P., Sjøgren, P., Juel, K., Højsted, J., & Ekholm, O. (2012). The burden of chronic pain: a cross-sectional survey focussing on diseases, immigration, and opioid use. *PAIN®*, *153*(12), 2332-2338.

Landmark, T., Romundstad, P., Dale, O., Borchgrevink, P. C., Vatten, L., & Kaasa, S. (2013). Chronic pain: one year prevalence and associated characteristics (the HUNT pain study). *Scandinavian journal of pain*, *4*(4), 182-187.

Lenz, K. M., & McCarthy, M. M. (2015). A starring role for microglia in brain sex differences. *The Neuroscientist*, *21*(3), 306-321.

Locke, S., Yousefpour, N., Mannarino, M., Xing, S., Yashmin, F., Bourassa, V., & Ribeiro-da-Silva, A. (2020). Peripheral and central nervous system alterations in a rat model of inflammatory arthritis. *Pain*, *161*(7), 1483-1496.

Lorente, J. D., Cuitavi, J., Campos-Jurado, Y., Montón-Molina, R., González-Romero, J. L., & Hipólito, L. (2022). Kappa opioid receptor blockade in the nucleus accumbens shell prevents sex-dependent alcohol deprivation effect induced by inflammatory pain. *Pain*, *163*(1), e137-e147.

Maduna, T., Audouard, E., Dembélé, D., Mouzaoui, N., Reiss, D., Massotte, D., & Gaveriaux-Ruff, C. (2019). Microglia express mu opioid receptor: insights from transcriptomics and fluorescent reporter mice. *Frontiers in psychiatry*, *9*, 726.

Marinelli, S., Basilico, B., Marrone, M. C., & Ragozzino, D. (2019). Microglia-neuron crosstalk: Signaling mechanism and control of synaptic transmission. In *Seminars in cell & developmental biology* (Vol. 94, pp. 138-151). Academic Press.

Merighi, S., Gessi, S., Varani, K., Fazzi, D., Mirandola, P., & Borea, P. A. (2012). Cannabinoid CB2 receptor attenuates morphine-induced inflammatory responses in activated microglial cells. *British journal of pharmacology*, *166*(8), 2371-2385.

Merighi, S., Gessi, S., Varani, K., Fazzi, D., Stefanelli, A., & Borea, P. A. (2013). Morphine mediates a proinflammatory phenotype via μ-opioid receptor–PKCε–Akt–ERK1/2 signaling pathway in activated microglial cells. *Biochemical pharmacology*, *86*(4), 487-496.

Mika, J., Popiolek-Barczyk, K., Rojewska, E., Makuch, W., Starowicz, K., & Przewlocka, B. (2014). Delta-opioid receptor analgesia is independent of microglial activation in a rat model of neuropathic pain. *PLoS One*, *9*(8), e104420.

Mogil, J. S. (2012). Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nature Reviews Neuroscience*, *13*(12), 859-866.

Popiolek-Barczyk, K., Piotrowska, A., Makuch, W., & Mika, J. (2017). Biphalin, a dimeric enkephalin, alleviates LPS-induced activation in rat primary microglial cultures in opioid receptor-dependent and receptor-independent manners. *Neural plasticity*, *2017*.

Reiss, D., Maduna, T., Maurin, H., Audouard, E., & Gaveriaux-Ruff, C. (2022). Mu opioid receptor in microglia contributes to morphine analgesic tolerance, hyperalgesia, and withdrawal in mice. *Journal of Neuroscience Research*, *100*(1), 203-219.

Ruau, D., Liu, L. Y., Clark, J. D., Angst, M. S., & Butte, A. J. (2012). Sex differences in reported pain across 11,000 patients captured in electronic medical records. *The Journal of Pain*, *13*(3), 228-234.

Sanchis-Segura, C., & Becker, J. B. (2016). Why we should consider sex (and study sex differences) in addiction research. Addiction biology, 21(5), 995-1006.

Seminowicz, D. A., Laferriere, A. L., Millecamps, M., Jon, S. C., Coderre, T. J., & Bushnell, M. C. (2009). MRI structural brain changes associated with sensory and emotional function in a rat model of long-term neuropathic pain. *Neuroimage*, *47*(3), 1007-1014.

Steingrímsdóttir, Ó. A., Landmark, T., Macfarlane, G. J., & Nielsen, C. S. (2017). Defining chronic pain in epidemiological studies: a systematic review and meta-analysis. *Pain*, *158*(11), 2092-2107.

Taylor, A. M., Becker, S., Schweinhardt, P., & Cahill, C. (2016). Mesolimbic dopamine signaling in acute and chronic pain: implications for motivation, analgesia, and addiction. *Pain*, *157*(6), 1194.

Taylor, A. M., Mehrabani, S., Liu, S., Taylor, A. J., & Cahill, C. M. (2017). Topography of microglial activation in sensory-and affect-related brain regions in chronic pain. *Journal of neuroscience research*, *95*(6), 1330-1335.

Tsuda, M. (2019). Microglia-mediated regulation of neuropathic pain: molecular and cellular mechanisms. *Biological and Pharmaceutical Bulletin*, *42*(12), 1959-1968.

Tsuda, M., Beggs, S., Salter, M. W., & Inoue, K. (2013). Microglia and intractable chronic pain. *Glia*, *61*(1), 55-61.

Turchan-Cholewo, J., Dimayuga, F. O., Ding, Q., Keller, J. N., Hauser, K. F., Knapp, P. E., & Bruce-Keller, A. J. (2008). Cell-specific actions of HIV-Tat and morphine on opioid receptor expression in glia. *Journal of neuroscience research*, *86*(9), 2100-2110.

Wang, Y. J., Huang, P., Ung, A., Blendy, J. A., & Liu-Chen, L. Y. (2012). Reduced expression of the mu opioid receptor in some, but not all, brain regions in mice with OPRM1 A112G. *Neuroscience*, *205*, 178-184.

Zamfir, M., Sharif, B., Locke, S., Ehrlich, A. T., Ochandarena, N. E., Scherrer, G., ... & Séguéla, P. (2022). Distinct and sex-specific expression of mu opioid receptors in anterior cingulate and somatosensory S1 cortical areas. *Pain*, 10-1097.

Zubieta, J. K., Dannals, R. F., & Frost, J. J. (1999). Gender and age influences on human brain mu-opioid receptor binding measured by PET. *American Journal of Psychiatry*, *156*(6), 842-848.

CHAPTER 9

Conclusions

The most relevant conclusions derived from the data obtained in the present doctoral thesis are:

Conclusion 1. Mu-Opioid receptors (MORs) activation in the mesocorticolimbic system (MCLS) induces proinflammatory cytokines release and microgliosis within the system.

Conclusion 2. Inflammatory pain alters microglial proliferation in areas of the MCLS.

Conclusion 3. Inflammatory pain irrupts in the neuroinflammation triggered by MOR activation within the MCLS.

Conclusion 4. Microglial activation induces neuronal MOR activation and expression through proinflammatory cytokines signalling.

Conclusion 5. MOR activation-induced neuroinflammation leads to an increase in neuronal MOR expression and signalling.

Conclusion 6. Inflammatory pain induces alcohol relapse only in female rats.

Conclusion 7. The levels of microglial cells, IL1β, and MORs correlate in females suffering from inflammatory pain in an alcohol deprivation effect model suggesting that their all implicated in pain-induced alcohol relapse in female rats.

CHAPTER 10

Resum

10.1. Objecte i objectius de la tesi

Els trastorns per consum d'alcohol (AUD) són una condició mèdica caracteritzada per l'impediment a l'hora de controlar l'ús de begudes alcohòliques malgrat les conseqüències adverses a nivell social, ocupacional, o de salut. És important remarcar que, actualment, existeix una tendència a l'alça en el nombre d'afectats per AUD a nivell global, i la magnitud d'aquesta problemàtica esdevé major si tenim en compte la complexitat del diagnòstic i l'estigma social que representa el AUD. Una altra cosa que es precís considerar és que el sexe juga un paper fonamental en l'epidemiologia del AUD. De fet, en les últimes dècades la prevalença de AUD en dones s'ha incrementat en un 84%, mentre que en els homes només un 35%. És cert que, fins fa poc, les dones estaven infrarepresentades en els estudis clínics, i que per convencions socials tenien menys accés a begudes alcohòliques, però estudis previs han demostrat que les dones son més susceptibles a desenvolupar comportaments relacionats amb el consum de substàncies i intoxicacions etíliques. A més a més, hi ha diversos factors que influeixen en el desenvolupament i manteniment del AUD. En este sentit, recentment, s'ha relacionat la presència de condicions doloroses amb les recaigudes en el consum d'alcohol. No obstant això, degut a la incipiència d'aquesta troballa, les evidències científiques que indaguen en el substrat bioquímic subjacent són escasses.

El dolor es defineix com una experiència sensorial i emocional desagradable que va associada o que pareix que va associada a un dany tissular existent o potencial. D'acord amb la duració de la condició dolorosa podem classificar-lo en dolor agut o crònic. Per un costat, el dolor agut té una duració de menys de 3 mesos i va associat a una hiperactivitat del sistema nerviós vegetatiu,

taquicàrdia, sudoració, i pal·lidesa. D'altra banda, el dolor crònic apareix quan persisteix durant més temps del necessari per a la guarició de l'organisme. És important remarcar que, en països desenvolupats, al voltant d'un 18% d'adults sofreixen de dolor crònic. Al igual que en el AUD, el dolor crònic està molt condicionat pel sexe. En efecte, aquesta condició és un 20% més prevalent en dones que en homes.

Tant el AUD com el component emocional del dolor comparteixen connexions neuronals dins del sistema cerebral de reforç i aversió, conegut com sistema mesocorticolimbic (MCLS). Dins d'aquest sistema, les neurones que el connecten estan estretament regulades pel sistema opioidèrgic endogen, composat principalment pels receptors opioides. Entre ells, el receptor Mu-opioide (MORs) destaca en la regulació de processos d'analgèsia i recompensa. Els MORs estan codificats al gen *OPRM1*. Durant la traducció del seu RNA missatger esdevé el fenomen de "splicing" alternatiu el qual dóna lloc a diverses isoformes que es caracteritzen per la quantitat de dominis transmembrana que posseeixen. Una vegada a la membrana, els MORs actuen com receptors acoblats a proteïna G amb diferents rutes de senyalització que regulen l'entrada i eixida d'ions, així com la producció d'AMPc. Després d'acomplir la seua tasca els receptors s'internalitzen mitjançant l'acció de quinases com les GRK i de proteïnes inductores de la internalització com les arrestines.

Així mateix, tant el dolor com el AUD són patologies que tenen una vessant neuroinflamatòria important. De fet, les citocines proinflamatòries, les espècies reactives d'oxigen, i altres mediadors de la inflamació són una de les causes principals de cronificació del dolor, i també es postulen com una de les causes del desenvolupament i cronificació del AUD. Des dels anys 90, es ve estudiant de manera intermitent la relació entre els MORs i els mecanismes neuroinflamatoris,

així com el seu paper en la modulació del dolor en la medul·la espinal. En aquest sentit, estudis previs indiquen que certes citocines proinflamatòries incrementen l'expressió del RNA missatger i proteica del MOR en diferents tipus de cèl·lules tant immunològiques com neuronals. A més a més, s'ha observat que diversos agonistes dels MORs com, per exemple, alguns opioides indueixen un ambient inflamatori, encara que la majoria de assajos s'han fet *in vitro.* Tanmateix, hi ha una mancança de bibliografia d'aquesta temàtica i les investigacions d'aquesta relació MOR-neuroinflamació en AUD són nul·les.

Tenint en compte la informació exposada en aquesta secció, l'objectiu principal d'aquesta tesi doctoral és l'estudi de la relació existent entre els MORs i el sistema immune, i la investigació de com aquesta relació juga un paper principal en AUD induïts per dolor que, a més a més, tenen un component dependent del sexe que també es proposa analitzar. Per tal de desenvolupar aquest objectiu principal, es plantegen els següents objectius específics:

Objectiu 1. Validar el paper dels MORs com inductors de neuroinflamació en el MCLS a través de l'activació de cèl·lules de micròglia i observar quin és l'efecte del dolor en aquesta relació.

Objectiu 2. Observar si la neuroinflamació modula l'activitat, la internalització, i l'expressió dels MORs neuronals.

Objectiu 3. Estudiar el fenomen dependent del sexe de la recaiguda l'alcohol induïda per dolor i el paper que hi juga la relació MOR-neuroinflamació.

10.2. Plantejament i metodologia emprats

Els objectius es desenvolupen al llarg de tres capítols de la tesi. El capítol 5 "Mu-Opioid Receptors: Neuroinflammation Drivers" (Receptors Mu-Opioides: Inductors de neuroinflamació) correspon a l'objectiu 1. En ell, es revisen i discuteixen resultats de diversos experiments en els que l'activació focal dels MORs indueix processos immunes que varen ser mesurats amb tècniques de biologia molecular, i com la presència d'una condició dolorosa altera aquests processos.

Per un costat, el primer experiment està basat en l'ús de la microdiàlisi com a mètode per a l'obtenció de citocines proinflamatòries. En aquest experiment vàrem implantar quirúrgicament sondes de microdiálisis en els nuclis accumbens (NAc), un àrea fonamental per a entendre el reforç dins del MCLS, de rates mascle i femella. 48 h després de la implantació vàrem recollir 5 dialitzats, un cada 100 min. D'aquests dialitzats, el primer va ser el basal i la resta els vàrem recollir després d'un tractament per retrodiàlisis de 1 µM [D-Ala2, N-MePhe4, Gly-ol]-encefalina (DAMGO), un pèptid agonista dels MOR, durant 20 min a un flux de 2,2 µL/min. Seguidament, la presència i quantitat de citocines proinflamatòries als dialitzats es varen mesurar en un citòmetre de flux amb l'ajuda d'un kit comercial personalitzat. És interessant remarcar que les concentracions de les citocines IL1α, IL1β, i IL6 varen experimentar un increment significatiu després del tractament amb DAMGO.

Per altre costat, en el segon experiment vàrem microinjectar DAMGO (7 ng i 14 ng) o líquid cefalorraquidi artificial (aCSF) en el àrea ventral tegmental (VTA) en rates mascle. A més a més, vàrem triar el model de dolor inflamatori de l'adjuvant complet de Freund (CFA), el qual va ser induït 48 h abans de les microinjeccions, de manera que la meitat d'animals per grup varen ser injectats en la pota amb salí

com a control i l'altra meitat amb CFA. És important dir que vàrem observar que les microinjeccions en el VTA no varen afectar el llindar nociceptiu mecànic en el test de Von Frey. Els resultats varen revelar que 7 ng DAMGO, però no 14 ng, va induir microgliosi en l'àrea d'injecció. Addicionalment, les diferents dosis de DAMGO en VTA van produir canvis en la proliferació microglial en àrees de projecció. Per últim, la presència de dolor inflamatori va alterar els resultats obtinguts.

Comptat i debatut, l'activació farmacològica del MOR en diferent àrees del MCLS indueix mecanismes neuroinflamatoris com la proliferació de la micròglia i l'alliberament de citocines proinflamatòries que, a més a més, tenen un efecte més enllà de l'àrea de tractament. Així mateix, el dolor inflamatori altera aquests esdeveniments neuroinflamatoris, indicant que la micròglia del MCLS és fonamental per entendre el desenvolupament de les comorbiditats del dolor en les que un agonista dels MOR com els opiacis està present.

El capítol 6 amb títol "Microglial control over Mu-opioid receptors signalling and activation" (Control microglial sobre la senyalització i l'activació dels receptors Mu-opioides) desenvolupa l'objectiu 2. En aquest capítol s'utilitzen tècniques *in vitro* de cultiu cel·lular i de biologia molecular que mostren com la micròglia és capaç d'alterar la senyalització del MOR neuronal. Este capítol conta amb dos experiments principals.

En primer lloc, en un dels experiments vàrem realitzar cultius primaris de micròglia, els quals vàrem tractar amb 10 ng/mL lipopolisacàrid (LPS) o vehicle durant 24 h. Després, vàrem caracteritzar el perfil d'alliberament de citocines proinflamatòries al medi extracel·lular amb reaccions en cadena de la polimerasa (PCRs) i un *array* de proteïnes. Això ens va permetre observar increments significatius en la producció d'IL1β, TNFα, i IL6. A més a més, vàrem extraure la part

proteica de la meitat de les mostres de medi obtingudes. A continuació, vàrem procedir a la realització d'assajos de "bioluminescence resonance energy transfer" (BRET) en cèl·lules SH-SY5Y. Aquestes cèl·lules tenen un origen neuronal i, per tant, els resultats obtinguts en elles són traslladables a les alteracions que esdevenen al sistema nerviós central en organismes complexes. Es varen emprar dos assajos BRET diferents: (1) observació de l'alliberament de la subunitat βγ de la proteïna G com a mesura d'activació dels MORs en presència de 10 µM DAMGO, i (2) estudi del reclutament de la β-Arrestina 2 per part dels MOR com a mesura d'internalització dels mateixos front a 10 µM DAMGO. En aquests assajos vàrem tractar les cèl·lules SH-SY5Y durant 24 h amb el medi de la micròglia per un costat, i amb la part proteica d'eixe medi per l'altre. Addicionalment, vàrem emprar les citocines L1β, TNFα, i IL6 a concentracions detectades en el medi després del tractament amb LPS (60 pg/µL, 500 pg/µL, i 1800 pg/µL respectivament) com a control positiu per investigar si són elles, almenys parcialment, les responsables dels possibles efectes observats amb el medi i la seua fracció soluble. És interessant remarcar que l'activació dels MORs en presència de DAMGO es va veure incrementada tant quan les cèl·lules SH-SY5Y varen ser tractades amb el medi o la seua fracció soluble com amb les citocines proinflamatòries. Malgrat això, no aconseguirem veure canvis en la internalització del receptor en les condicions del nostre experiment, probablement degut a limitacions de sensibilitat de la tècnica. Per últim, vàrem observar si el secretoma de micròglia activada, la seua part soluble, i les citocines emprades podien alterar l'expressió del receptor mitjançant una tècnica de biolumiscència en cèl·lules SH-SY5Y. Com és en el cas de l'activació del receptor, tots els tractaments incrementaren significativament l'expressió dels MORs en comparació als controls adients.

En el segon experiment, vàrem utilitzar els dialitzats obtinguts de l'experiment de microdiálisis del capítol 5, concretament el basal i el segon dialitzat després del tractament amb DAMGO on ja veiem un increment significatiu en l'alliberament de citocines proinflamatòries. Vàrem emprar aquests dialitzats per a tractar durant 24 h les cèl·lules SH-SY5Y i fer els mateixos assajos que a l'experiment anterior. Els resultats d'aquest experiment mostren com els dialitzats després del tractament amb DAMGO incrementen significativament l'activació, la internalització, i l'expressió dels MORs neuronals quan comparem amb els dialitzats basals.

És interessant remarcar que els resultats d'aquest capítol mostren una clara regulació dels MOR neuronals per part de la micròglia a través de processos neuroinflamatoris, cosa que té una alta rellevància en processos de trastorn per consum de drogues i en la regulació del dolor i el seu tractament amb medicaments que contenen opiacis. A més a més, veiem com aquests mateixos efectes ocorren conjuntament amb mecanismes neuroinflamatoris derivats de l'activació de MORs glials, la qual cosa té una gran importància a nivell de tractaments amb opiacis.

Per últim, l'objectiu 3 s'enquadra en el capítol 7 titulat "Pain-Induced Alcohol Relapse: Role of Mu-Opioid Receptors and Neuroinflammation" (Recaiguda al alcohol induïda per dolor: Paper dels receptors Mu-Opioides i de la neuroinflamació). En aquest capítol s'explica el desenvolupament i replicació d'un nou model animal de recaiguda induïda pel desenvolupament d'una condició dolorosa, fet observat a estudis en pacients en abstinència en el context clínic. A més a més, s'utilitzen tècniques de biologia molecular per detectar i quantificar variacions en proteïnes claus durant les diferents fases del model animal.

En primer lloc vàrem establir el nou model en rata combinant dos models preexistents, el model de CFA de dolor inflamatori i el model d'accés intermitent a alcohol en un paradigma de dues botelles. En este sentit, rates mascle i femella varen estar exposades a una botella d'aigua i una d'alcohol 20% en aigua tres vegades a la setmana (dilluns, dimecres i divendres) durant 8 setmanes, de manera que la botella d'alcohol es posava al començament del seu període d'activitat i es retirava 24 h després. En acabar aquest període d'adquisició, vàrem retirar les botelles d'alcohol forçant l'abstinència que va tindre una duració de 3 setmanes. El primer dia de la tercera setmana d'abstinència forçada, vàrem injectar els animals amb CFA o solució salina estèril. En acabar les 3 setmanes d'abstinència, les botelles d'alcohol es varen reintroduir durant 5 noves sessions d'accés a alcohol. Els animals es varen sacrificar en dos punts del procés, al final de les 3 setmanes d'abstinència forçada i després de les 5 sessions de reintroducció. En ambdós sacrificis s'extragueren els cervells i es processaren per fer immunoelectrotransferència i immunohistoquímica. Els resultats mostren com només les rates femella varen incrementar significativament el consum d'alcohol quan comparem el consum de les 5 sessions prèvies a l'abstinència i les 5 sessions post abstinència. És interessant remarcar que aquests resultats sexe dependents mostren una realitat que no s'havia observat prèviament a la literatura. Addicionalment, les tècniques de biologia molecular emprades ens varen proveir de dades d'expressió de proteïnes de la cascada de senyalització de inflamació (pNFκB), de mediadors neuroinflamatoris (iNOS, COX2 i IL1β) i antiinflamatoris (Il10), IBA1, i MOR al còrtex prefrontal (PFC), al NAc, i a l'Àrea Ventral Tegmental (VTA). Els resultats mostren alteracions dinàmiques en les tres àrees estudiades al llarg de les diferents fases del model. A més a més, els mascles quasi no van experimentar canvis d'expressió en les proteïnes analitzades, cosa que correlaciona

amb les dades de comportament del model. Per altra banda, les alteracions observades en relació al marcador de micròglia IBA1 i als MORs correlacionen al PFC i al NAc en femelles amb dolor inflamatori en el període de post abstinència, suggerint que les interaccions MOR-micròglia estudiades als capítols 5 i 6 poden estar jugant un paper clau en les recaigudes al alcoholisme induïdes per dolor. És també important subratllar que l'expressió de l'IL1β sofreix les mateixes alteracions que els MORs, sent indicatiu de la possible regulació del MOR per part d'aquesta citocina en el model.

Els resultats obtinguts en el capítol 7 esclareixen el paper fonamental del factor sexe i de la presència de dolor inflamatori en les recaigudes en el consum d'alcohol. A més a més, suggereixen que la relació descrita en capítols anteriors entre els MORs i mecanismes neuroinflamatoris pot estar intervenint en el procés de recaiguda. No obstant això, precisem de més recerca per poder dictaminar la profunditat del paper d'aquesta interrelació.

10.3. Aportacions originals

Les principals aportacions de la present tesi doctoral es poden resumir en tres: (1) L'aprofundiment de l'estudi de la interrelació entre els MORs, la micròglia, i la neuroinflamació, (2) el paper que el dolor inflamatori perifèric juga en el control del sistema immunològic en àrees concretes del sistema nerviós central pertanyents al sistema de reforç, i (3) la rellevància del factor sexe, dolor i interrelació MOR-micròglia en les recaigudes en el consum d'alcohol.

En relació al primer punt, aquesta tesi doctoral ha suposat un impuls vertaderament important dins del camp d'investigació en el qual s'enquadra. Això és així per dues raons principals. En primer lloc, hem vist com l'activació farmacològica dels MOR indueix l'alliberament de molècules amb un caràcter inflamatori a més a més d'incrementar el número de cèl·lules IBA1 positives. Els estudis anteriors basen les seues troballes en experiments majoritàriament *in vitro* i en evidències indirectes. El nostre estudi aporta un punt de vista *in vivo* i *ex vivo* que complementa i correlaciona amb resultats extrets de la literatura. Addicionalment, els nostres experiments s'han realitzat en àrees cerebrals del sistema de reforç i no en zones típicament involucrades en el processament del dolor com la medul·la espinal com en experiments prèviament publicats, cosa que els fa traslladables a una innumerable llista de malalties neuropsiquiàtriques basades en alteracions mesocorticolímbiques com la depressió, l'ansietat, o els trastorns per consum de drogues. Per altra banda, també hem aprofundit en com mecanismes neuroinflamatoris modifiquen l'expressió dels MORs neuronals mitjançant la senyalització de citocines proinflamatòries. Açò ja es coneixia des dels anys 90 del segle passat, però va quedar sense resoldre als començament dels 2000. La present tesi doctoral indica que l'increment en la expressió dels MORs

neuronals esdevé per mecanismes de control del RNA missatger. També presentem una nova relació que ningú ha descrit amb anterioritat en la que la micròglia regula l'activitat dels MORs també a través de citocines proinflamatòries. Finalment, aportem evidència de com l'activació farmacològica dels MORs, probablement microglials, també modifiquen l'expressió i la senyalització dels MORs neuronals, cosa que suggereix la presència d'un mecanisme regulador de tipus retroalimentació positiva. A més a més, esta troballa suposa un gran avanç a l'hora d'entendre com actuen fàrmacs de tipus opioide i molècules similars que tenen afinitat pels MORs.

El segon punt es recolza en literatura prèvia en la qual s'ha observat com la presència d'una condició dolorosa produeix microgliosi en medul·la espinal. Tanmateix, els nostres resultats aporten una nova visió en la qual una condició de dolor inflamatori agut incrementa la proliferació microglial en diferents àrees del sistema de reforç. Açò vol dir que el dolor inflamatori no només altera la resposta immunològica en zones del sistema nerviós central dedicades al processament de la sensació dolorosa, sinó que a més a més modifica l'activitat de la micròglia en àrees mesocorticolímbiques, cosa que és molt reveladora i podria suposar un avanç en l'estudi de com el dolor indueix el desenvolupament de comorbiditats neuropsiquiatriques. Addicionalment, en la present tesi doctoral es posa de manifest el paper que el dolor inflamatori té en el control dels mecanismes neuroinflamatoris derivats de l'activació dels MOR en el sistema de reforç. Aquestes dades impacten directament al camp de les comorbiditats del dolor, en concret el trastorns per consum de drogues, i aporten un nou punt de vista en relació als tractaments del dolor.

Per últim, al tercer punt, tractant de traslladar la recerca bàsica a situacions clíniques, desenvolupem i validem un model en rata de recaiguda en el consum

d'alcohol desenvolupada per dolor i estudiem el factor sexe. En primer lloc, aquesta tesi doctoral descriu com les femelles tenen una major vulnerabilitat a les recaigudes en el consum d'alcohol quan sofreixen una condició dolorosa. El nostre grup és el primer en observar aquest fenomen, encara que un treball anterior va observar com els mascles eren més propensos a iniciar el consum d'alcohol que les femelles quan sofrien de dolor inflamatori. Tant els nostres resultats com els aportats prèviament pel grup del Dr. Kash son fonamentals per poder tractar d'una manera molt més personalitzada i adient als pacients amb un AUD. Aquest punt és essencial, ja que en moltes investigacions bàsiques modelant condicions patològiques humanes només empren mascles als seus experiments, cosa que ignora un factor biològic tan important com és el sexe. Addicionalment, els nostres resultats suggereixen que la interrelació MORs-micròglia regulada per la presència de dolor inflamatori explicada en els dos punts anteriors pot estar jugant un paper essencial en el procés de recaiguda en el consum d'alcohol induïda per dolor en femelles. En el moment d'escriptura de la present tesi doctoral som l'únic grup de recerca que presenta aquesta evidència amb un interès molt important en els AUD degut al seu component inflamatori.

10.4. Conclusions

Les principals conclusions derivades de les dades compilades en la present tesi doctoral són les següents:

Conclusió 1. L'activació dels MORs en el sistema de reforç indueix l'alliberament de citocines proinflamatòries i microgliosi en aquest sistema.

Conclusió 2. El dolor inflamatori altera la proliferació microglial en àrees del sistema de reforç.

Conclusió 3. El dolor inflamatori irromp en la neuroinflamació desencadenada per l'activació dels MORs en el sistema de reforç.

Conclusió 4. L'activació microglial indueix l'activació i l'expressió dels MORs neuronals a través de la senyalització mitjançant citocines proinflamatòries.

Conclusió 5. La neuroinflamació induïda per l'activació dels MORs porta a un increment en l'expressió i la senyalització dels MORs neuronals.

Conclusió 6. El dolor inflamatori indueix la recaiguda en el consum d'alcohol només en rates femella.

Conclusió 7. Els nivells de cèl·lules microglials, IL1β, i MORs correlaciona en femelles que sofreixen de dolor inflamatori en un model de privació d'alcohol, cosa que suggereix que estan tots implicats en les recaigudes en el consum d'alcohol induïdes per dolor en rates femella.

10.5. Futures línies d'investigació

Al llarg d'aquest resum s'han mencionat diverses mancances i limitacions de la nostra investigació que, per raons obvies de la temporalitat de la tesi doctoral, no han pogut ser abordades. En aquesta secció no només aprofundirem en nous enfocaments i experiments per cobrir aquestes mancances sinó que, a més a més, n'afegirem de noves.

En primer lloc, malgrat el gran paper que juga la perspectiva de gènere en la present tesi doctoral, els resultats obtinguts al capítol 5 es deriven de, principalment, rates mascle. Seria, per tant, interessant repetir els experiments realitzats en rates femella per veure si es repliquen els resultats. Aquest punt és essencial si tenim en compte que les diferències observades en el model de recaiguda en el consum d'alcohol induïda per dolor inflamatori tenen un component de gènere molt important. Addicionalment, encara que hem emprat ratolins mascle i femella de manera conjunta en el capítol 6 per fer els cultius primaris de micròglia, és possible que al fer una distinció de sexe a l'hora de la seua realització observem uns resultats una mica diferents.

Altre punt a considerar és la possibilitat de que els mecanismes neuroinflamatoris i la seua relació amb els MOR també esdevinga amb altres receptors de la mateixa família com els Kappa (KOR) o els delta (DOR). De fet hi ha evidència de que aquesta relació existeix, encara que el mecanisme pel qual ocorre és del tot desconegut. Si es descobrira que els KOR i els DOR interactuen de manera estreta amb cèl·lules glials, s'obriria un nou camp de recerca que utilitzaren aquesta relació com a diana per millorar les vides de pacients amb dolor crònic i trastorns per consum de substancies.

Per altra banda, emprem el CFA com model de dolor inflamatori i no hem fet ús de cap model de dolor neuropàtic, el qual en ocasions es comporte de manera diferent als models de dolor inflamatori. Tenint en compte que el sistema immunològic és fonamental en el processament del dolor inflamatori i nosaltres ens focalitzem en canvis neuroimmunològics, és de suposar que ambdós tipus de dolor actuaran de manera distinta. A més a més, seria interessant observar les alteracions que es produeixen en la relació entre els MORs i la micròglia amb altres insults perifèrics de caràcter inflamatori.

Una continuació clara de la tesi doctoral seria també iniciar-se en l'estudi d'un possible tractament farmacològic que tinguera com diana terapèutica la relació entre els MORs i la neuroinflamació per tal d'evitar les recaigudes en el consum d'alcohol induïdes per dolor o altres comorbiditats relacionades. Una possibilitat seria la inhibició de l'activitat de les cèl·lules microglials amb agents com l'ibudilast. A més a més, s'hauria d'estudiar el mecanisme d'administració del fàrmac per tal d'evitar que afecte a la perifèria de manera no intencionada. Per aconseguir-ho, podríem utilitzar diferents tipus de formes farmacèutiques innovadores que vehiculen el fàrmac principalment a la diana terapèutica. Aquesta recerca és inherent a l'àmbit de la tecnologia farmacèutica.

A més a més, en el nostre model de recaiguda en el consum d'alcohol induïda per dolor examinem les alteracions dinàmiques al MCLS produïdes durant els períodes d'abstinència i recaiguda. Tanmateix, desconeguem si aquestes diferències provenen de canvis que ja es produeixen durant l'etapa d'adquisició. A més a més, també desconeixem de quina manera el dolor inflamatori modifica els mecanisme immunològics al MCLS, ja que no hi ha massa bibliografia al respecte i la que hi ha es centra fonamentalment a la medul·la espinal.

També seria lògic aprofundir en altres trastorns per consum de substàncies com el opiacis o la cocaïna, per tal de veure si la relació entre els MORs i la neuroinflamació derivada de l'activació de les cèl·lules microglials juga un paper en aquests. Sobretot, caldria focalitzar la recerca en les diferents parts del procés d'addició a cadascuna d'aquestes drogues d'abús.

Finalment, el nostre laboratori té la intenció de continuar amb les línies de recerca propostes com a part de futures tesis doctorals.