Received: 14 August 2022 | Revised: 14 March 2023 | Accepted: 24 March 2023

DOI: 10.1002/glia.24374

GLIA

WILEY

RESEARCH ARTICLE

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

Javier Cuitavi[1,2] | Paula Andrés-Herrera[1,2] | David Meseguer[1] | Yolanda Campos-Jurado[1] | Jesús D. Lorente[1] | Hannah Caruana[1] | Lucía Hipólito[1,2]

[1]Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Avda. Vicent Andrés Estellés s/n, Burjassot 46100, Spain

[2]Instituto de Biotecnología y Biomedicina (BIOTECMED), Universidad de Valencia, Valencia 46100, Spain

Correspondence
Lucía Hipólito, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Avenida Vicent Andrés Estellés s/n, 46100 Burjassot, Spain.
Email: lucia.hipolito@uv.es

Funding information
Ministerio de Ciencia e Innovación, Grant/Award Number: PID2019-109823RB-I00; Ministerio de Sanidad, Delegación del Gobierno para el Plan Nacional sobre Drogas, Grant/Award Number: PNSD2019I038; Universitat de València, Grant/Award Number: UV-INV-PREDOC-1327981

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 ($42 \times 27 \times 18$ 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,

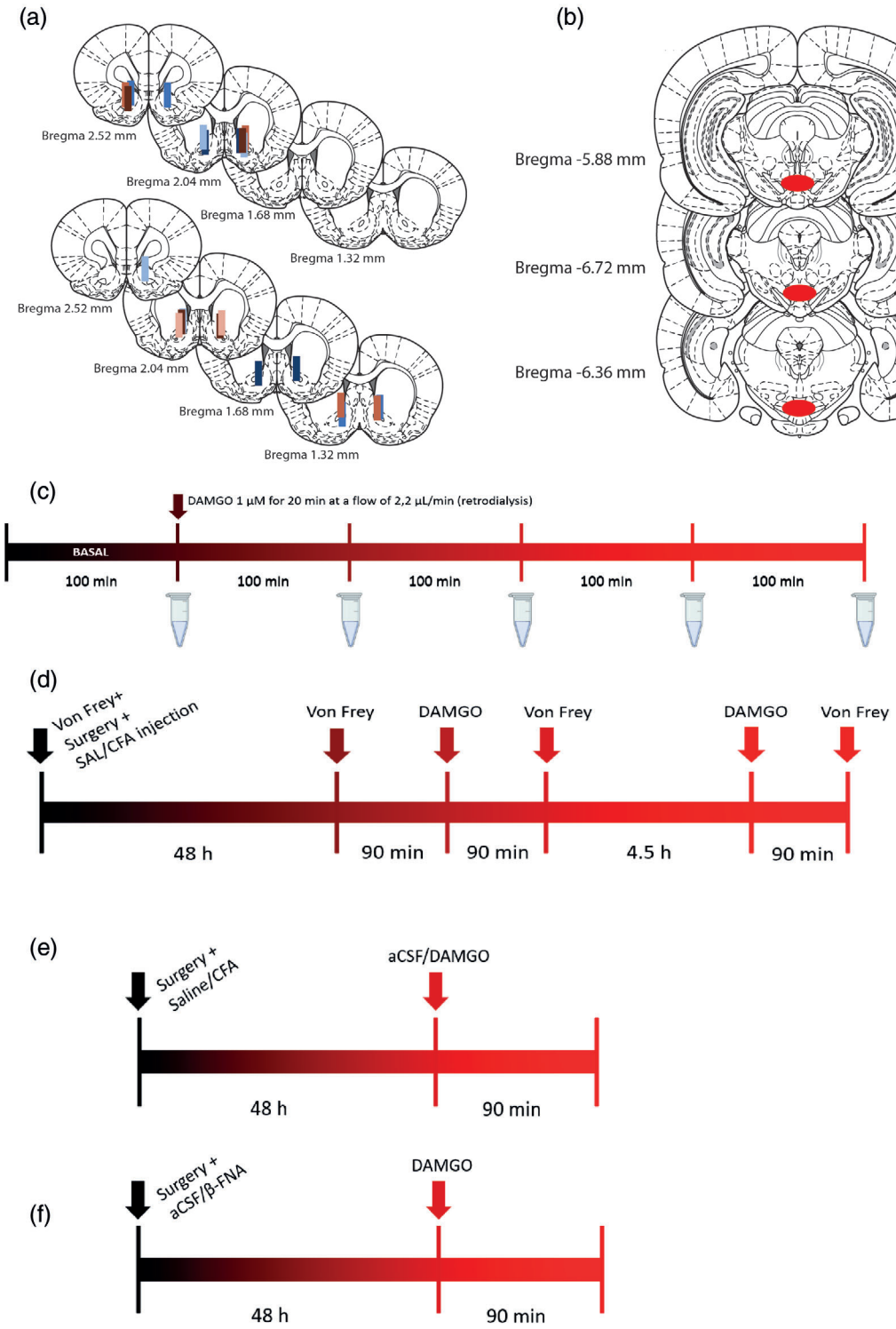

FIGURE 1 Cannulae placements and experimental designs. (a) Diagram of coronal sections indicating the placement of microdialysis probes. (b) Diagram of coronal sections indicating the placement of the tip of the injectors at the moment of the treatment. All the injectors were included in the red circle 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

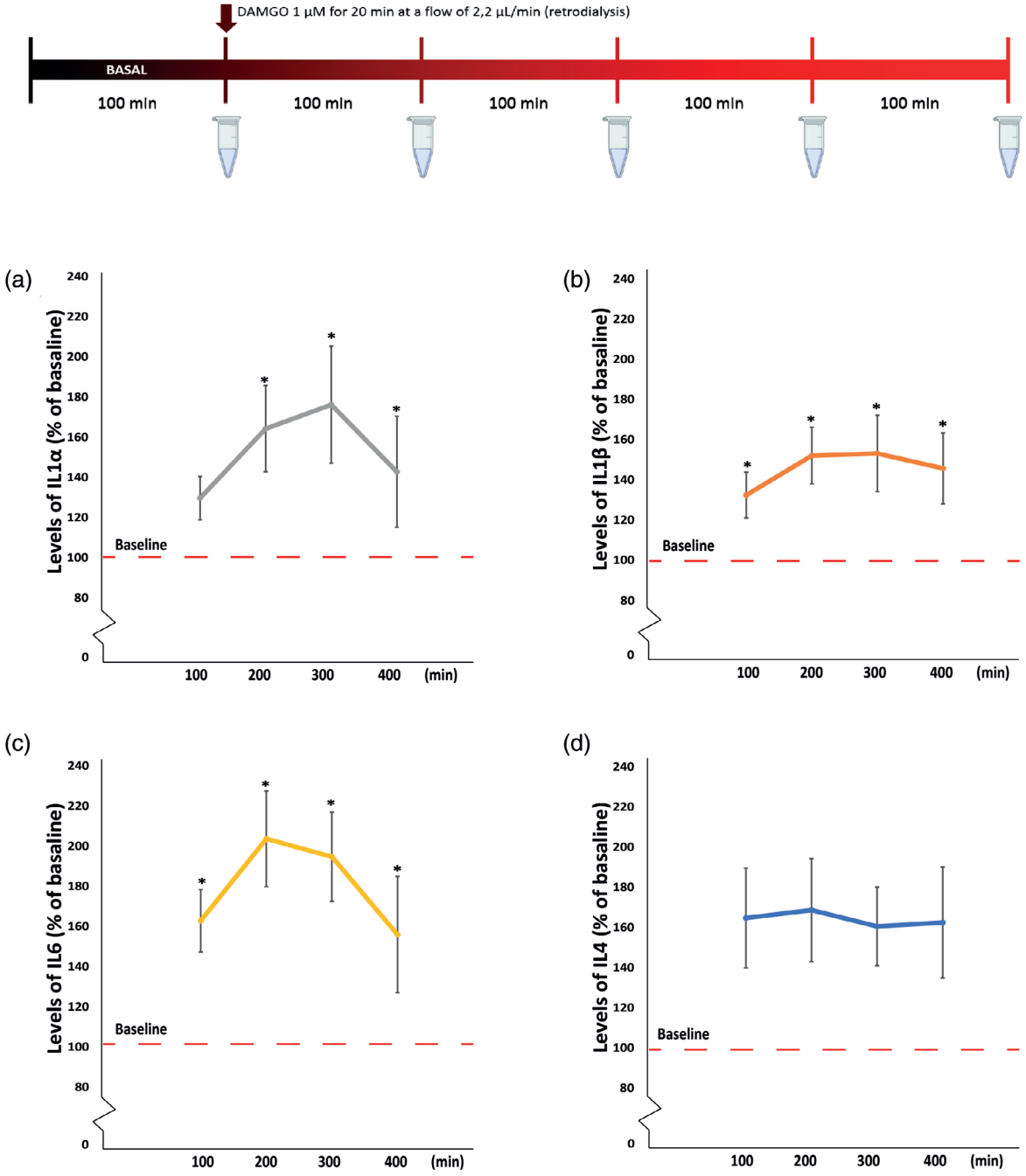

FIGURE 2 DAMGO intra-NAc increases cytokines local extracellular levels. Levels of the measured cytokines (Expressed in % of baseline) along time post DAMGO administration by retrodialysis. Data are expressed as mean ± SEM. *$p < .05$ (ANOVA repeated measures followed by Dunnett). (a) IL1α; (b) IL1β; (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

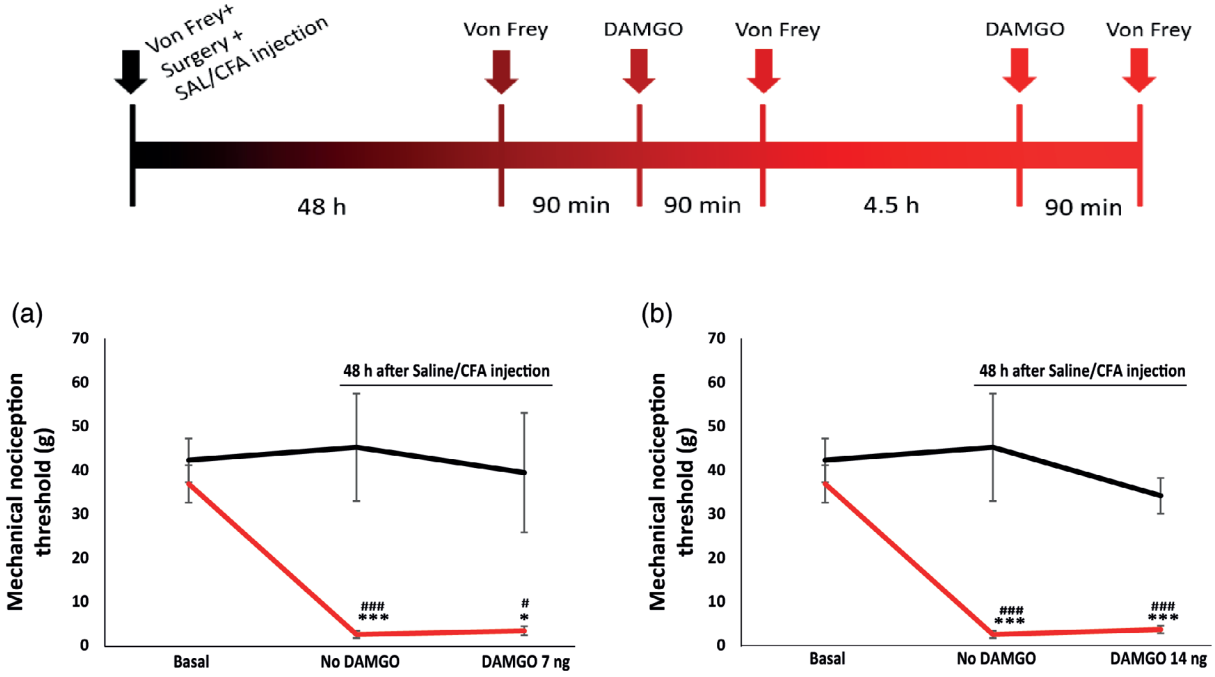

FIGURE 3 DAMGO administered into the VTA does not alter mechanical nociception. Data are expressed as mean ± SEM of g ($n = 5$–6/group). Data were collected at three different time points: basal (prior to the SAL/CFA injections), No DAMGO (48 h after the SAL/CFA injections), DAMGO (90 min after DAMGO 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.

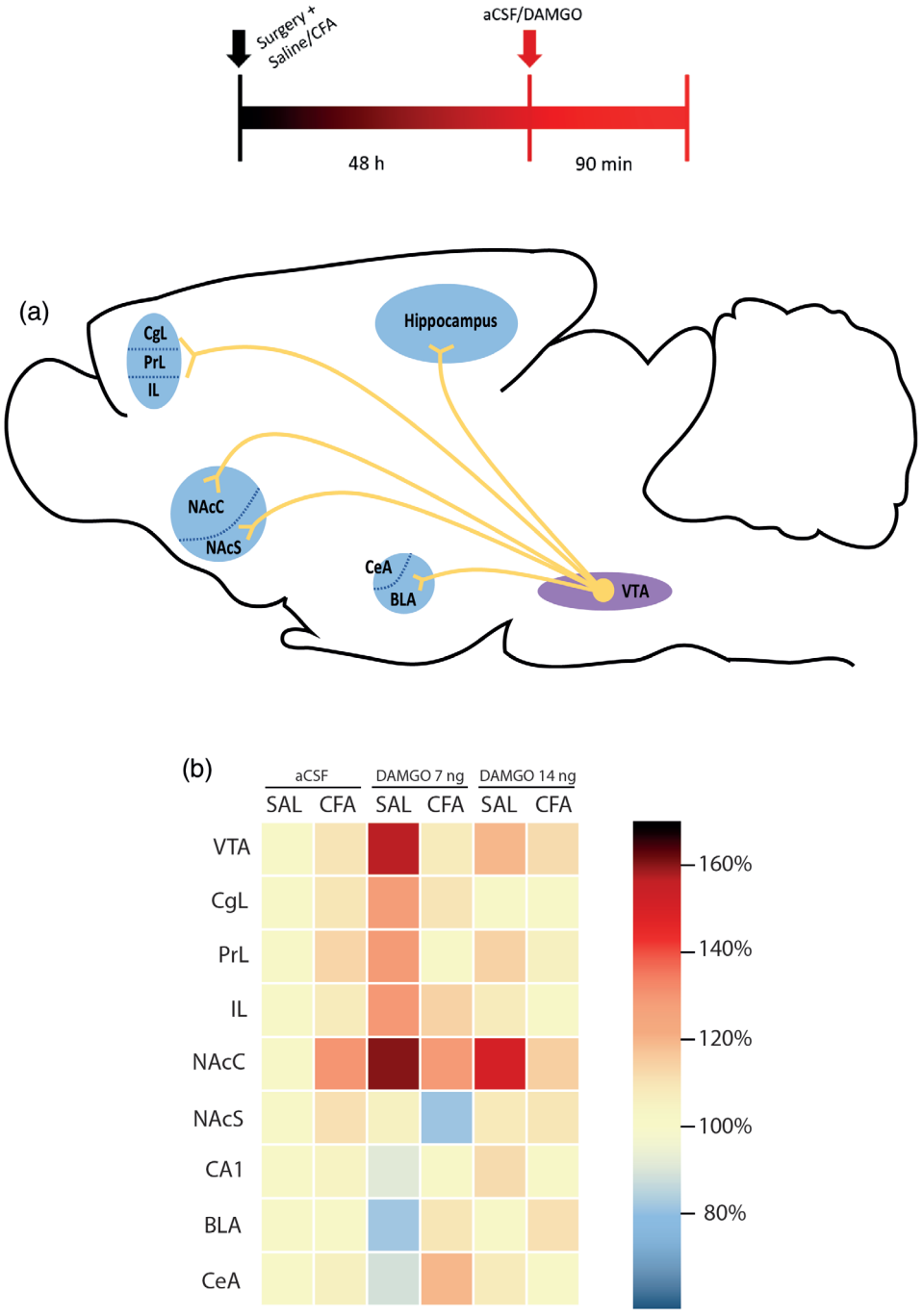

FIGURE 4 DAMGO administered into the VTA produces dose-dependent and area-dependent alterations in the number of IBA1-positive cells. (a) Schematic of the VTA projection areas in the MCLS analyzed in this study. (b) Heatmap indicating the levels of IBA1-positive cells (Expressed in % of the SAL-aCSF 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

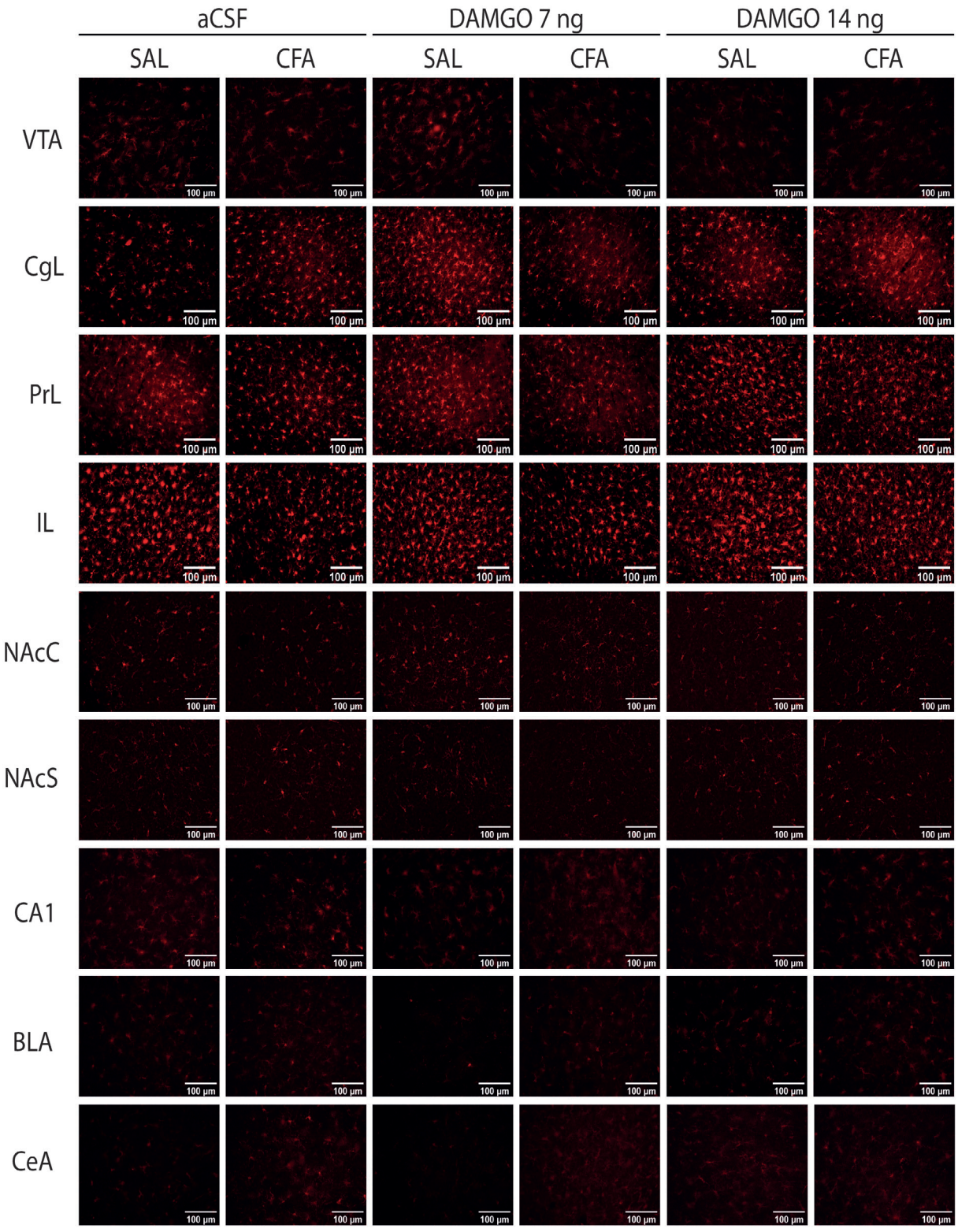

FIGURE 5 Representative images of the alterations of IBA1-positive cells in each brain area after the microinjections and the SAL/CFA injection. White scale bars represent 100 µm. aCSF, artificial cerebrospinal fluid; BLA, basolateral amygdala; CeA, central amygdala; CFA, Complete Freund Adjuvant; CgL, 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

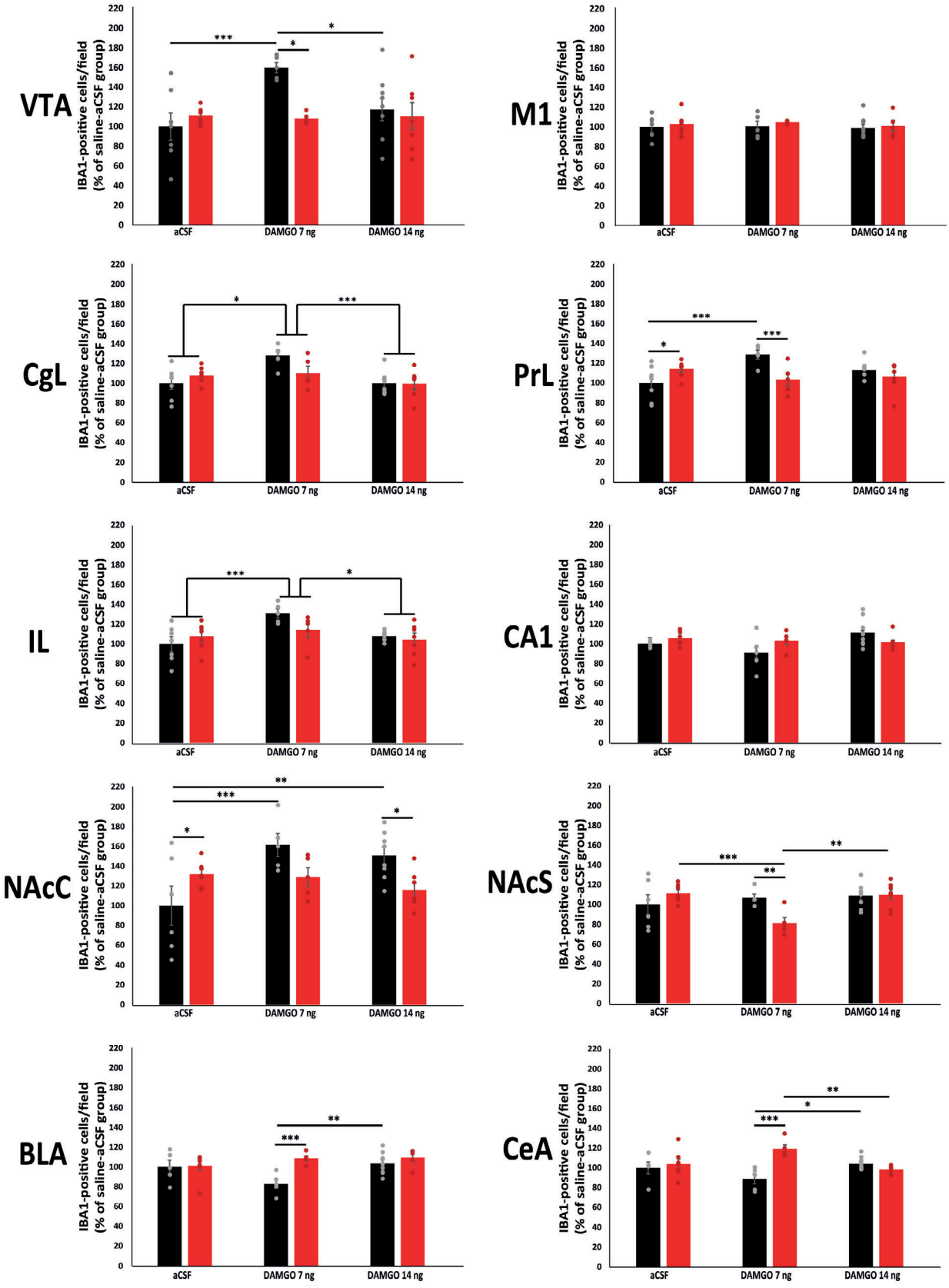

FIGURE 6 Legend on next page.

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.

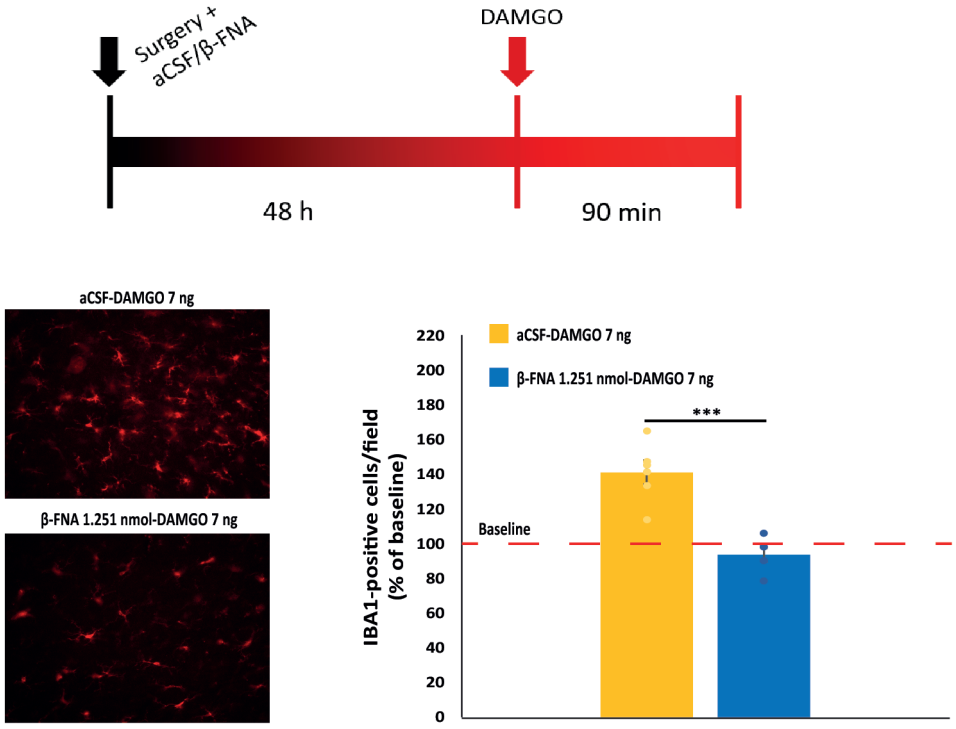

FIGURE 7 Alterations of IBA1-positive cells (Expressed in % of the saline-aCSF group) in the VTA after the microinjections aCSF/β-FNA–DAMGO. Data are expressed as mean ± SEM of IBA1-positive cells ($n = 6$/group). The red line corresponds to the baseline data average (saline-aCSF group). The yellow bar 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.

ACKNOWLEDGMENTS

We would like to thank Ms. Pilar Laso for grant management. Moreover, we thank Dr. Inmaculada Noguera, Chief Veterinarian Officer, and the personnel of the Animal facilities (SCSIE) at the University of Valencia for their help and effort in assuring animal welfare. We also thank Dr. Sandra Norte, Flow Cytometry Technician from SCSIE at the University of Valencia for her help and support.

FUNDING INFORMATION

This study has been supported by Spanish Ministerio de Ciencia e Innovación PID2019-109823RB-I00 (Lucía Hipólito) and by Spanish Ministerio de Sanidad, Delegación del Gobierno para el Plan Nacional sobre Drogas PNSD2019I038 (Lucía Hipólito). Javier Cuitavi is supported by an Atracció de Talent PhD Fellowship from the University of Valencia (UV-INV-PREDOC-1327981).

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.

ORCID

Javier Cuitavi https://orcid.org/0000-0003-3418-3135
Lucía Hipólito https://orcid.org/0000-0002-6834-5748

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

How to cite this article: 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*, 1–15. https://doi.org/10.1002/glia.24374