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RESEARCH ARTICLE

Dopamine D_3 receptor blockade accelerates the extinction of opioid withdrawal-induced drug-seeking behaviours and alters microglia in dopaminoceptive nuclei

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Background and Purpose: Among all drugs of abuse, opioids cause most of the deaths and treatment seeking. Despite abundant research in multifaceted therapeutic strategies, a high rate of relapse still characterises this condition. Dopamine D_3 receptor antagonists combined with cue-exposure therapies have been proposed to ameliorate the abused drugs-induced cognitive deficits, which consequently would aid to prevent the maladaptive behaviours responsible for drug use.

Experimental Approach: We used the morphine withdrawal-induced conditioned place aversion (CPA) paradigm to assess, in male rats, the efficacy of D_3 receptor blockade to improve the extinction of drug-seeking behaviours associated with the aversive contextual stimuli of its withdrawal. Then, using immunohistochemical methods, we evaluated the participation of neuroimmune mechanisms in the striatum and infralimbic cortex in D_3 receptor modulation of CPA extinction.

Key Results: Whereas the selective D_3 antagonist PG01037 accelerated the extinction of the morphine withdrawal-induced CPA, our findings indicate that decreased motivation might be involved in this action. Increased D_3 receptor expression in glial cells and the modulation of microglia activation state in specific dopaminoceptive areas could intervene in the behavioural outcomes of D_3 receptor blockade.

Conclusions and Implications: Our findings reveal a facilitatory role of D_3 antagonists in the inhibition of morphine-seeking behaviours triggered by contextual stimuli associated with its withdrawal. Nonetheless, their potential ability to reduce motivation might influence their therapeutic use. Future investigations elucidating the precise function of D_3 receptors will facilitate the identification of this receptor as a valuable therapeutic target for mitigating the recurrence of opioid withdrawal-induced drug-seeking behaviour.

KEYWORDS
conditioned place aversion, extinction, GFAP, Iba1, PG01037, substance use disorder

Abbreviations: Λ, lacunarity; CPA, conditioned place aversion; DS, dorsal striatum; GFAP, glial fibrillar acidic protein; Iba1, ionised calcium-binding adapter molecule 1; IL, infralimbic cortex; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; SUD, substance use disorders.

1 | INTRODUCTION

Substance use disorders (SUD) are a global health burden, being opioids the most lethal drugs and the ones that cause most people to seek treatment (UNODC, 2023). The rewarding effects of abused substances or the aversive emotional symptoms of their withdrawal are associated with cues and contextual stimuli that, afterwards, even after many years of abstinence, can trigger drug-seeking and intake (Kaplan et al., 2011). Amid the behavioural approaches most used to treat SUD are the exposure-based therapies, in which cues priorly paired with the abused compound are presented to the person in a controlled and drug-free atmosphere, so that the association with the substance extinguishes (Perry & Lawrence, 2017). Nonetheless, the cognitive deficits that define SUD are in brain circuits involved in the therapeutic behavioural change required for recovery (Perry & Lawrence, 2017). Thus, it is believed that the combination of extinction-based therapies with cognitive enhancers might facilitate the extinction of the abused drugs' associations with cues and, consequently, diminish relapses in drug-seeking behaviours (Ma et al., 2019).

The **dopamine** system integrated by neurons of the ventral tegmental area that project to the nucleus accumbens (NAc), hippocampus, amygdala and medial prefrontal cortex (mPFC) has been thoroughly studied in the context of SUD as it modulates reward, cognition and motivation, which are key factors for the onset and maintenance of addictive behaviours (Klein et al., 2019; Solinas et al., 2019). Enhanced activity of this system is associated with reward and reward-motivated behaviour (Baik, 2013), while its hypofunction has been related with drug withdrawal and negative affect (Solinas et al., 2019). Moreover, dopaminergic projections to the striatum and mPFC modulate aversion-based learning (Verharen et al., 2020) and the extinction of aversive memories (Hamati et al., 2024; Peters et al., 2009).

Recent investigations have bestowed the **D_3 receptor** a pivotal role in SUD because it is the only **DA receptor** expressed exclusively in the limbic system and, consequently, appears to be particularly engaged in reward, motivation and cognition (Banks & Sprague, 2024; Sokoloff & Le Foll, 2017). Furthermore, D_3 receptor ligands have been postulated as potential therapeutic tools to treat SUD and other psychiatric and neurodegenerative diseases (Banks & Sprague, 2024; Prieto, 2017). Although conventionally considered as a neurotransmitter, dopamine exerts also as an immune modulator, therefore many immune cells express proteins involved in dopamine production, metabolism and D receptors, including D_3 (Channer et al., 2023). Relevantly, some of the therapeutic effects of D_3 ligands in several psychiatric and neurological conditions have been attributed to their actions on glial cells (Elgueta et al., 2017; Montoya et al., 2019; J. Wang et al., 2022; Yang et al., 2020).

In this work, we used the conditioned place aversion (CPA) paradigm to evaluate the efficacy of D_3 antagonism to improve the extinction of drug-seeking behaviours triggered by **morphine** abstinence-associated contextual stimuli in male rats. So far, D_3 receptor function in SUD has been investigated in neurons. The ability of D_3 antagonists to regulate the altered D_3 receptor expression observed during the behavioural responses to abused drugs have been reported before (R. Guerrero-Bautista et al., 2020, 2021). However, D_3 receptor has been found in microglia and/or astrocytes in animal models of some neurodegenerative and psychiatric disorders, such as **Parkinson Disease** and depression, and D_3 antagonism or genetic deletion have been published to modulate the activity of these cells, the production of cytokines as well as the behavioural symptoms of these conditions (Elgueta et al., 2017; Montoya et al., 2019; J. Wang et al., 2018, 2020; J. Wang et al., 2022). Because glial cells have been reported to be essential players in the behavioural responses to opioids and other drugs of abuse (Lacagnina et al., 2017; Linker et al., 2019; H. Zhang et al., 2020), we aimed to examine, in brain areas known to mediate in the extinction of fear and drug-related memories, the impact of D_3 blockade on: (i) the activation of astrocytes and microglia; (ii) total and cell-specific D_3 receptor expression in glial and neuronal cells; and, because D_3 antagonism affected microglial D_3 receptor expression, (iii) the morphology of these cells as an indicator of their activation state (Reddaway et al., 2023). Remarkably, we found that D_3 blockade accelerated the extinction of the morphine withdrawal-induced CPA and that this effect could be at least partially associated with diminished motivation. Moreover, our data shows, for the first time, that

What is already known?

- Opioids withdrawal-associated contextual stimuli can trigger drug-seeking and intake even after years of abstinence.
- D_3 antagonists might be useful pharmacological tools to prevent abused drug-seeking behaviours.

What does this study add?

- D_3 receptor blockade accelerates the extinction of opioid-seeking behaviours prompted by cues associated with their withdrawal.
- Microglial cells of specific dopaminoceptive areas might mediate in the effects of D_3 receptor antagonism.

What is the clinical significance?

- D_3 antagonists may be useful pharmacological tools to facilitate the extinction of opioid-seeking behaviours.
- D_3 blockers could induce a diminished motivational state that would limit their therapeutical use.

in the therapeutic effects of D_3 antagonists for SUD treatment might involve their modulation of neuroinflammatory mechanisms in limbic areas.

2 | METHODS

2.1 | Animals

Wistar male rats were cared for in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/UE). All surgical and experimental procedures were approved by the local Committee for animal research (Comité de Ética y Experimentación Animal; RD 53/2013; REGA ES300305440012) and performed during the light phase. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Thus, this study was designed to obtain equally sized experimental groups, with a subject number similar to previous publications (Myers & Carlezon, 2010). Rats were housed (four per methacrylate cage; length: 45 cm; width: 24 cm; height: 20 cm) on a standard light cycle (light on at 8 AM) provided *ad libitum* food (standard rodent chow, Tecklad rodent diet, Inotiv, West Lafayette, IN, USA) and water and handled daily during the first week after arrival to minimise stress. Because chronic exposure to opioids is known to decrease the body weight gain because of a lesser food intake (Ferenczi et al., 2010; Núñez et al., 2008; Pinter-Kuebler et al., 2013), the body weight of placebo- and morphine-treated animals was measured daily to corroborate that morphine was being adequately released from the pellets.

2.2 | Drugs

Based on previous studies (García-Pérez et al., 2016; Pinter-Kuebler et al., 2013), rats were rendered dependent on morphine by subcutaneous implantation of two constant release morphine base pellets (75 mg) under isoflurane (Isoflo®, Ecuphar, Barcelona, Spain) anaesthesia. Placebo pellets contained lactose instead of morphine. **Naloxone** hydrochloride dissolved in sterile saline (0.9% NaCl) and injected subcutaneously (s. c.) at a dose of 15 μg kg^{-1} in volumes of 1 ml kg^{-1} of body weight. This dose was selected as it is known to evoke aversive emotional symptoms of opioid withdrawal able to induce CPA but reduced physical ones (Frenois et al., 2002; Myers et al., 2012; Schulteis et al., 1994). Finally, the highly selective (Grundt et al., 2005; Higley et al., 2011) D_3 antagonist **PG01037** was prepared in 0.5% Tween 80 in sterile distilled water and injected intraperitoneally (i.p.) at doses of 3, 10 and 30 mg kg^{-1} in volumes of 1 ml kg^{-1} of body weight. These doses were based on previous investigations (Higley et al., 2011; Moreno et al., 2022). Despite the route of administration used, peripheral actions of PG01037 are highly unlikely because of, on the one hand, its high affinity for the D_3 receptor (Ki = 0.7 ± 0.1 nM) and 133-fold selectivity over **D_2 receptors** (Higley et al., 2011) and, on the other hand, the restricted D_3 receptor expression to the limbic system (Banks & Sprague, 2024; Sokoloff & Le Foll, 2017). Moreover, PG01037 has been shown to preferentially bind to D_3 receptors over many other receptors in the central nervous system (Higley et al., 2011). Therefore, this D_3 antagonist has been used in several studies in the last two decades (Collins et al., 2005; Higley et al., 2011; Riddle et al., 2011).

2.3 | Behavioural procedures

The three chamber conditioning apparatus and the unbiased morphine withdrawal-induced CPA protocol were as previously described (Franco-Garcia et al., 2022, 2023) (Figure 1a, a'). Exclusion criteria and scores were estimated as in Myers and Carlezon (2010). Briefly, for each test and animal, a score was calculated as the time in seconds spent in the naloxone-paired chamber minus that in the saline-paired chamber. Then, the change scores for the post-conditioning or extinction tests were determined as their score in that test minus the score of the pre-conditioning test. Because animals that did not acquire a CPA cannot undergo CPA extinction, morphine-dependent rats not showing a strong aversion (≤ − 300 s in the post-conditioning test) were excluded from both Experiments 1 and 2 (25 out of a total of 116 morphine-dependent animals).

In Experiment 1, 1 day after the CPA test, one set of placebo- and morphine-treated animals was subjected to an extinction training (ET) that lasted three consecutive days and consisted of two 30-min confinements in the previously saline- and naloxone-paired compartments separated 3 h. The order of exposure was counterbalanced across rats and reversed relative to the preceding session. Saline was administered immediately prior to each confinement. The following day after the last extinction training session, animals were tested for extinction (Figure 1a).

In Experiment 2, 1 day after the CPA test, another group of placebo- and morphine-treated rats was subjected to an ET that lasted 1 day and 30 min before saline administration animals received an i.p. injection of PG01037 or vehicle. Animals were tested for extinction the next day (Figure 1a').

2.4 | Immunofluorescence

The immunolabelling assay complied with the *British Journal of Pharmacology* guidelines (Alexander et al., 2018). For this experiment, rats subjected to a 3-day extinction training and those that received vehicle or 30 mg kg^{-1} of PG01037 before the 1-day ET were anesthetised with a sublethal dose of **pentobarbital** (100 mg kg^{-1}, i. p.; Dolethal, Vetoquinol, Madrid, Spain) and later transcardially perfused with 250 ml of saline 0.9% followed by 500 ml of fixative solution (paraformaldehyde 4% in borate buffer 0.1 M pH 9.5). Then, fixed brains were extracted and kept first in fixative solution with sucrose

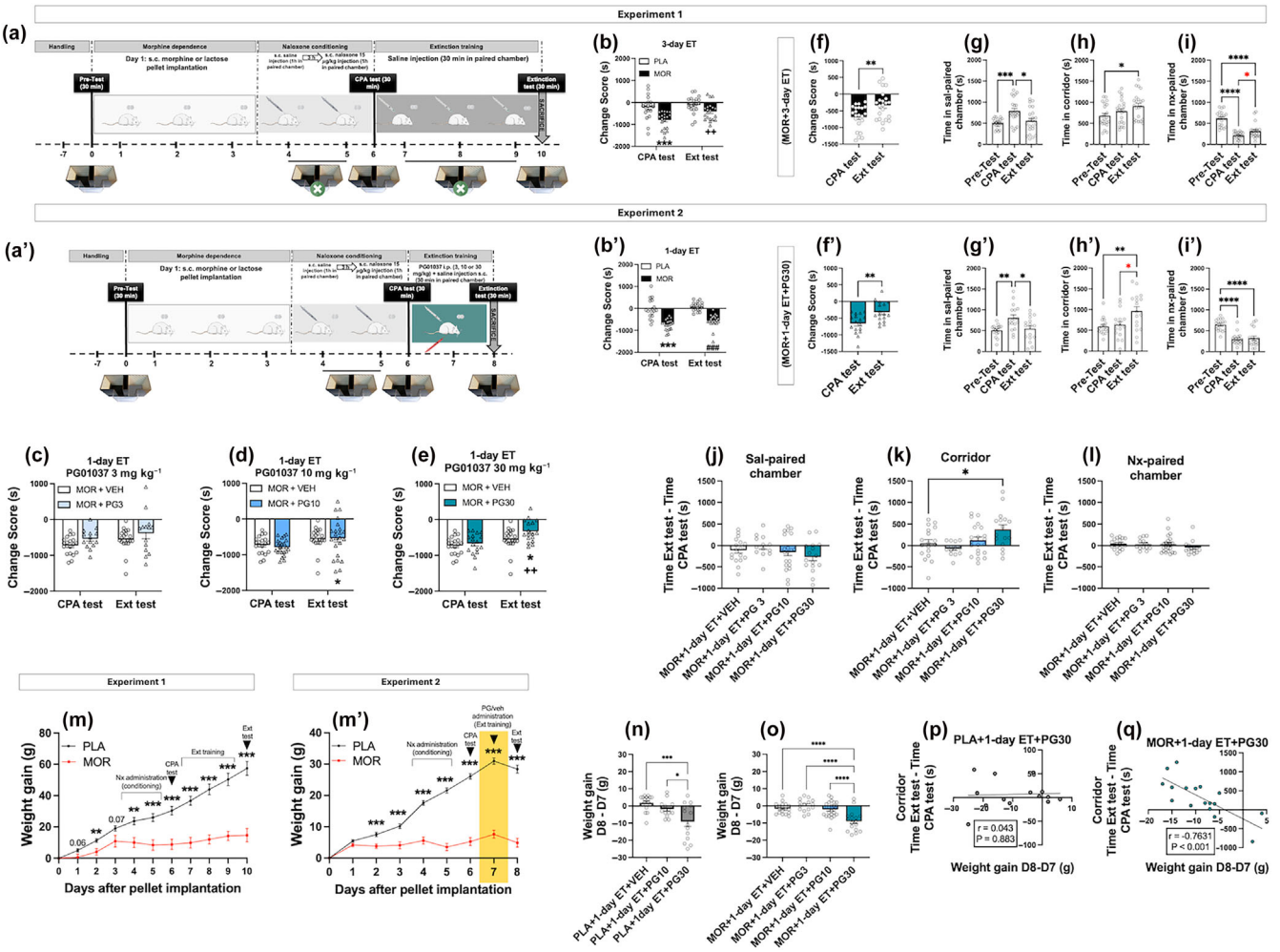

FIGURE 1 **D_3 antagonism accelerates morphine-withdrawal extinction training and alters weight gain**. (a, a') Schematic representation of behavioural and pharmacological procedures followed in both experiments. (b) Three-day extinction training protocol returned change score to control levels. (b′) One day extinction training did not return change score to basal levels. *** $P < 0.001$ versus placebo (PLA)-conditioned place aversion (CPA) test, $^{++}$ $P < 0.01$ versus morphine (MOR)-CPA test, $^{\#\#\#}$ $P < 0.001$ versus PLA- extinction training (Ext) test. (c) Three mg kg^{-1} dose of PG01037 did not improve extinction learning. (d) Ten mg kg^{-1} dose of the antagonist showed a slight effect on extinction training. (e) Full extinction was achieved when animals were injected with the highest dose of the antagonist (30 mg kg^{-1}). * $P < 0.05$ versus MOR+VEH + CPA test, $^{++}$ $P < 0.01$ versus MOR+VEH + Ext test. (f) Change score of morphine-dependent animals increased after 3-day extinction training (ET). (f′) Likewise, change score of morphine-dependent animals increased after PG01037 administration and 1-day ET. **P <0.01. After the 3-day ET protocol, regarding the CPA test, (g) animals spent less time in the saline-paired chamber, (h) same time in corridor and (i) more time in the naloxone-paired environment. (g') Animals injected with the antagonist at 30 mg kg^{-1} decreased the time spent in saline-paired chamber, (h′) increased the time in the corridor and (i′) time in naloxone-paired environment remained the same as during CPA test. * P <0.05, ** P <0.01, ***P <0.001, **** P <0.0001. (j) Time spent during Ext test minus time spent during CPA test after PG01037 administration was unchanged regarding saline- and (l) naloxone-paired compartments (k) but was increased towards the corridor. * P <0.05. (m) Morphine-administered animals gained less weight than control animals in the 3-day paradigm and (m') the 1-day extinction training. * P <0.05, ** P <0.01, *** P <0.001 versus MOR group within the same day. (n) Placebo animals injected with the antagonist exhibited less weight gain after PG01037 administration. (o) Morphine-dependent animals also exhibited less weight gain after PG01037 administration. * P <0.05, *** P <0.001, **** $P < 0.0001$. (p) Weight gain after PG01037 injection in placebo animals did not correlate with the difference of time spent across tests towards the corridor, but (q) it showed a high negative correlation in morphine-dependent animals.

30% to preserve the tissue for 3 h, and later in phosphate buffered saline (PBS) with sucrose 30%. Thirty micrometre rostro-caudally coronal sections were obtained using a freezing microtome (Leica Microsystems, Barcelona, Spain), collected in cryoprotectant and stored at −20°C until processing.

To identify the brain regions of interest, Paxinos and Watson Atlas (Paxinos & Watson, 2007) was used. Four to five animals of each group were used for immunostaining ($n = 4–5$). Sections were washed with PBS and, for antigens retrieval, exposed to citrate buffer (citric acid 10 mM in 0.05% of Tween-20, pH 6.0, 90 °C; 2 times for 10 min

each). After washing with PBS, unspecific bindings were blocked with normal donkey serum solution (10% in 0.3% of Triton-X-100 in PBS for 2 h, RT). Afterwards, sections were incubated for 72 h at 4°C with gentle agitation with rabbit polyclonal anti-D_3 receptor (1:200, Merck Millipore, MA, USA, Cat# AB1786P, RRID: AB_90963), mouse monoclonal anti-NeuN (1:1000, Abcam, Cambridge, UK, Cat# AB104224, RRID: AB_10711040), chicken polyclonal anti-glial fibrillar acidic protein (GFAP, 1:500, Merck Millipore, Cat# AB5541, RRID: AB_177521) and goat polyclonal anti-ionised calcium-binding adapter molecule 1 (Iba1, 1:200, Abcam, Cat# AB5076, RRID: AB_2224402) antibodies. Later, brain tissue samples were washed with PBS and incubated 4 h with the following secondary donkey antibodies: anti-rabbit conjugated with Alexa Fluor 488 (1:1000, Thermo Fisher Scientific, MA, USA, Cat# A-21206, RRID: AB_2535792), anti-mouse conjugated with Alexa 555 (1:1000, Thermo Fisher Scientific, Cat# A-31570, RRID: AB_253618), anti-chicken conjugated with Alexa 647 (1:1000, Thermo Fisher Scientific, Cat# A78952, RRID: AB_2921074) and anti-goat conjugated with Alexa 405 (1:250, Thermo Fisher Scientific, Cat# A48259, RRID: AB_2890272). After this, sections were washed with PBS and mounted with ProLong antifade (P36980, Invitrogen).

2.5 | Immunoreactivity quantification analysis

Images were captured by using a Leica epifluorescence microscope (Leica Microsystems, DM4 B) connected to a video camera (Leica Microsystems, DFC7000 T). Light emission from specific labelling was obtained through **DAPI** filter cube (Leica Microsystems; excitation range: 325–375; dichromatic mirror: 400; emission filter: 435–485), L5 filter cube (Leica Microsystems; excitation range: 460–500; dichromatic mirror: 505; emission filter: 512–542), RHO filter cube (Leica Microsystems; excitation range: 541–551 nm; dichromatic mirror: 560 nm; emission filter: 565–605 nm) and Y5 filter cube (Leica Microsystems; excitation range: 590–650 nm; dichromatic mirror: 660 nm; emission filter: 662–738 nm). Time exposure and settings for all nuclei were constant through experimental groups, and images were captured at 20X magnification. All sections were quantified by a blinded investigator. The mean grey value was measured for each channel separately by using FIJI software v. 2.1.0/1.53c (NIH ImageJ, Bethesda, MD, USA). A minimum of three sections of each animal were evaluated, and a mean value for each animal was then calculated.

2.6 | Confocal analysis and 3D rendering

Confocal images were obtained by using a confocal microscope Leica TCS SP8 (Leica Microsystems) and LAS X (Leica Microsystems) processing software. Images from the nuclei were captured from low magnification to high magnification (10X to 63X). Confocal images were obtained using 405-nm excitation for Alexa Fluor 405, 488-nm excitation for Alexa Fluor 488, 555-nm excitation for Alexa Fluor 555 and 647-nm excitation for Alexa Fluor 647. Emitted light was detected in the range of 409–457 nm for Alexa Fluor 405, 499–565 nm for Alexa Fluor 488, 565–632 nm for Alexa Fluor 555 and 696–750 nm for Alexa Fluor 647. Every channel was captured separately to avoid spectral crosstalk. The confocal microscope settings were established and maintained by local technicians for optimal resolution. Ten micrometre Z-stacks (step size: 0.3 μm) were obtained, and three-dimensional reconstructions of the z-stacks of confocal images were rendered in Imaris software (Bitplane Scientific Software, Zurich, Switzerland).

2.7 | Quantitative co-localisation

In order to detect modifications in the expression of D_3 receptors in neurons, microglia and/or astrocytes after CPA extinction achieved by a 3-day ET or a 1-day ET + PG01037, co-localisation in each nucleus was estimated by using Manders' overlap coefficient (MOC) (Dunn et al., 2011). Co-localisation analysis was performed in images in which Costes' thresholding method was applied for each channel through Imaris Software (Costes et al., 2004). Two to three images per animal were analysed.

Manders' overlap coefficient (MOC) represents the fraction of one protein that colocalises with a second protein and is a measure of the overlapped fraction of each signal. Its values are in the range 0–1.0. For two probes (*S1* and *S2*), MOC was calculated as

$$M1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i}$$

Where *M1* is the fraction of *S1* (D_3 receptor) in compartments containing *S2* (each one of the cellular markers).

2.8 | Microglial morphometric analysis

As microglia morphology is broadly used as an indicator of their functional state, fractal analysis was employed to quantitatively assess the morphology of microglia based on complexity (Karperien & Jelinek, 2015; Morrison et al., 2017). Confocal images were obtained under a 63X objective. According to the protocol followed (Young & Morrison, 2018), three to four randomly selected regions per animal containing microglia were selected and four microglial cells per section were analysed. For that, all images were processed using ImageJ exactly as previously described (Young & Morrison, 2018). Fractal analysis of individual microglial cells was carried out using the box-counting method provided with the FracLac plug-in for ImageJ. We determined lacunarity (Λ) that describes the degree of heterogeneity that exists in a cell's shape (Karperien & Jelinek, 2015); circularity, which is calculated as $4\pi \times (\text{area}/\text{perimeter}^2)$ and varies from 0 (linear polygon) to 1 (perfect circular object) (Zanier et al., 2015); and Ramification Index, measured as the ratio of the perimeter to the area, normalised by that same ratio calculated for a circle of the same area, as it has been described before (Madry et al., 2018). The regions of

interest (ROIs) used for analysis were identical for all cells. Four cells were analysed per image.

2.9 | Data analysis

Data were analysed using GraphPad Prism 10.0 (GraphPad Software; San Diego, CA, USA) and *P*-values <0.05 were considered statistically significant. All statistical groups were at least of five independent values. Within these datasets, no significant variance in homogeneity was found. All descriptive data were presented as means ± standard error of the mean (SEM). Results of behavioural tests, body weight and image analyses were analysed using Student's *t* test, one- or two-way analysis of variance (ANOVA) followed by Sidak's post hoc test. The post hoc tests were conducted only if *F* in ANOVA achieved $P < 0.05$. The absence of outliers was confirmed (ROUT Method; $Q = 1\%$). Pearson's correlation was used to explore whether there was a linear association between the extinction score towards the corridor and the body weight gained 24 h after the administration of 30 mg kg of PG01037, and its strength. Together, our data analysis followed the *British Journal of Pharmacology* guidelines (Curtis et al., 2025).

2.10 | Materials

Morphine base was obtained from Alcaliber Laboratories (Madrid, Spain). Naloxone hydrochloride was purchased from Sigma Chemical (St. Louis, MO, U.S.A.), while PG01037 was kindly supplied by Dr. Amy Newman (Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse – Intramural Research Program, National Institutes of Health, 333 Cassell Drive, Baltimore, Maryland, USA). The flowing compounds Tween 20, Tween 80, Triton-X-100, citric acid, donkey serum and sodium tetraborate were obtained from Merck KGaA (Darmstadt, Germany).

Details of other materials and suppliers are provided in the specific sections.

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023).

3 | RESULTS

The parameters of all the statistical analyses can be found in Tables S1 and S2.

3.1 | D_3 antagonism facilitates the extinction of morphine withdrawal-induced CPA

The extinction of drug memories is a potential strategy for preventing drug craving and relapses (Kaplan et al., 2011). This kind of behavioural approaches alone, however, are not consistently effective in reducing reactivity to drug cues in humans. This is probably due to the cognitive impairments induced by continued drug abuse and exposure that includes attention, learning and memory deficits, and result in suboptimal decision-making, poor executive function and inappropriate prioritisation of goals (Nic Dhonnchadha & Kantak, 2011; Perry & Lawrence, 2017). As D_3 receptor has been shown to modulate cognitive processes (Lapointe et al., 2023; Millan et al., 2020; Schneider et al., 2021), we used the morphine withdrawal-induced CPA paradigm to assess the ability of D_3 antagonism to improve the extinction of morphine-seeking behaviours associated with aversive contextual stimuli of its withdrawal. In Experiment 1, unlike controls ($n = 19$), rats with morphine pellets ($n = 20$) exhibited a strong aversion for the naloxone-paired compartment in the CPA test and, as in our previous work (Franco-Garcia et al., 2022; Franco-García et al., 2023), the 3-day ET effectively reverted this aversive behaviour in the extinction test (Figure 1b). In Experiment 2, morphine-treated animals receiving vehicle ($n = 20$) displayed aversion to the morphine withdrawal-associated environment in the CPA and also in the extinction tests, while placebo-treated and vehicle-injected rats ($n = 18$) did not (Figure 1b'). In Experiment 2, the lower dose of the D_3 antagonist did not affect the score of morphine-treated animals ($n = 14$) during the extinction test when compared with their CPA score and with the extinction score of the opioid-dependent vehicle-injected rats (Figure 1c). While the injection of 10 mg kg^{-1} of PG01037 to morphine-dependent animals ($n = 23$) significantly diminished their extinction score regarding their CPA score, it provoked no differences with the extinction score of morphine-treated rats receiving vehicle (Figure 1d). Nonetheless, the extinction score of the opioid-treated animals injected with the higher dose of the D_3 antagonist ($n = 17$) was significantly lower than their CPA score and also than the extinction score of the morphine rats treated with vehicle (Figure 1e). Administration of 10 ($n = 14$) and 30 mg kg^{-1} ($n = 14$) of PG01037 did not affect the extinction score of placebo-implanted rats (Figure S1A, B).

We then went on to study the process of extinction of aversive behaviours of contexts associated with morphine withdrawal achieved by behavioural approaches as opposed to the extinction accomplished through the combination of behavioural and pharmacological tools. First, we observed that the significantly increased extinction change score of morphine-treated animals subjected to a 3-day ET (Experiment 1) was similar to that of the morphine dependent rats administered with 30 mg kg^{-1} of PG01037 during the 1-day ET (Experiment 2) (Figure 1f, f'). Next, we analysed the time that these animals spent in each compartment of the conditioning apparatus during the successive tests. In both experiments, the time that rats spent in the saline chamber increased significantly during the CPA test regarding the pre-conditioning one and significantly decreased in the extinction test

in comparison with the CPA one (Figure 1g, g'). Dependent animals in Experiments 1 and 2 similarly spent significantly more time in the corridor during the extinction test than during the pre-conditioning test (Figure 1h, h'). Moreover, rats in both experiments spent significantly less time in the morphine withdrawal environment during the CPA and extinction tests than during the preconditioning one (Figure 1i, i'). Nevertheless, while animals subjected to the 3-day ET increased the time spent in the naloxone-associated compartment during the extinction test regarding the CPA one (Figure 1i) as a result from the new learning acquired during the 3-day ET, those injected with PG01037 did not spent more seconds in the withdrawal environment during the extinction test (Figure 1i') but significantly enhanced their time in the corridor (Figure 1h'). This corridor has transparent walls and floor in order to minimise the time spent in it and was the only compartment that did not become a conditioned contextual stimulus along the behavioural procedures. For a more exhaustive analysis, we evaluated the effect of D_3 blockade on the difference in time that morphine-treated rats spent in each chamber of this apparatus in the extinction test minus that in the CPA test and found no changes for the saline -(Figure 1j) and naloxone-associated compartments (Figure 1l) at any D_3 antagonist dose. Moreover, animals injected with 30 mg kg^{-1} of PG01037 enhanced this difference of time in the corridor when compared with those receiving vehicle (Figure 1k), thus suggesting that D_3 blockade could have affected their motivational state.

On the other hand, controls in both experiments gained significantly more weight than morphine-treated rats (Figure 1m, m'). However, in contrast with animals in Experiment 1 (Figure 1m), both placebo and morphine rats in Experiment 2 decreased their body weight the day after vehicle/PG01037 administration (day 8; Figure 1m'). Because the reward system regulates motivation for feeding (Baik, 2013, 2021), we then analysed the effect of D_3 receptor blockade on the body weight gain 24 h after PG01037 administration or its vehicle. Both placebo- and morphine-implanted rats receiving the higher dose of the D_3 antagonist gained significantly less weight than those injected with vehicle, 3 or 10 mg kg^{-1} of the drug (Figure 1n,o). Noteworthy was that we uncovered a high negative correlation between the increased time spent in the corridor in the extinction test regarding the CPA one and the body weight loss was likely caused by a decreased food intake 1 day after the administration of 30 mg kg^{-1} of PG01037 to morphine-treated rats (Figure 1q). This did not occur in placebo animals (Figure 1p), hence reinforcing that in the facilitation of the extinction of aversive behaviours related with morphine negative affect induced by D_3 antagonism could participate a decreased motivational state. Together, a summary of these behavioural outcomes can be found in Figure 5a.

3.2 | The blockade of D_3 receptor diminished Iba1-IR in the infralimbic cortex (IL), NAc (shell) and dorsal striatum (DS)

D_3 receptor has been reported to modulate the activity of distinct brain cell populations and thus participate in the aetiology of distinct neurodegenerative and psychiatric conditions (Elgueta et al., 2017; Simpson et al., 2014; J. Wang et al., 2022). Hence, we assessed the functional state of neurons, astrocytes and microglia as well as D_3 receptor expression through NeuN-, GFAP-, Iba1- and D_3 receptor-immunoreactivity (IR) quantification, respectively, in several dopaminoceptive areas involved in learning and motivation (Haber, 2016). First, we compared the data of morphine-treated animals subjected to a 3-day ET with their controls. Morphine-dependent rats displayed diminished NeuN-IR in the IL, NAc core and shell, and DS (Figure 2e,f,g,h). Surprisingly, GFAP-IR level also dropped in the IL and NAc (core) (Figure 2i,j), while no changes were detected in the shell and DS (Figure 2k,l). Morphine induced no alterations in Iba1-IR in any of the regions analysed (Figure 2m–p). Finally, D_3 receptor-IR in the IL and DS of opioid-treated rats was lower than in controls (Figure 2q,t), whereas no changes were detected in the NAc core and shell (Figure 2r,s). In contrast, NeuN-, GFAP-, Iba1- and D_3 receptor-IR did not vary in any of these areas of morphine-treated rats that underwent a 1-day ET and received vehicle when compared with placebo-treated rats subjected to the same procedures (Figures S2A-E, S3A-E, S4A-E and S5A-E, hence suggesting the influence of the duration of morphine exposure and/or the extinction training on these parameters. Lastly, we evaluated the effect of CPA extinction through D_3 receptor antagonism on NeuN-, GFAP-, Iba1- and D_3 receptor-IR. No main effects of morphine, PG01037 nor their interaction were detected in NeuN-, GFAP-and D_3 receptor -IR in the IL, NAc core and shell and DS (Figures S2F-I, S3F-I, S4F-I and S5F-I). There were no significant effects of the opioid, the D_3 antagonist nor their interaction on Iba1-IR in the NAc (core). Nonetheless, PG01037 exerted a significant effect on Iba1-IR in the IL, NAc (shell) and DS. Additionally, placebo animals injected with the D_3 antagonist showed reduced Iba1-IR in the DS when compared with those receiving vehicle (Figure 2x). Nevertheless, no significant differences between groups were detected for Iba1-IR in the IL (Figure 2u), NAc (core) (Figure 2v) and NAc (shell) (Figure 2w). Altogether, our results indicate that the D_3 antagonist attenuated Iba1-IR in the IL, the NAc (shell) and the DS. Representative bregma levels of the areas analysed are shown in Figure 2a–d.

3.3 | PG01037 administration increased Iba1/D_3 receptor colocalization in the NAc (shell) and DS of rats that extinguished the CPA

Because D_3 receptor has been found in neurons, astrocytes and microglia of distinct brain nuclei and different conditions modulate its expression in each cell population (Elgueta et al., 2017; Montoya et al., 2019), we subsequently studied D_3 receptor expression in each kind of cells. This was carried out through the measurement of NeuN/D_3 receptor, GFAP/D_3 receptor and Iba1/D_3 receptor colocalizations. NeuN/D_3 receptor colocalization was significantly lower in the IL (Figure 3a) and DS (Figure 3d) of morphine-treated animals subjected to a 3-day ET when compared to controls. Additionally, GFAP/D_3 receptor and Iba1/ D_3 receptor colocalizations decreased in the IL and

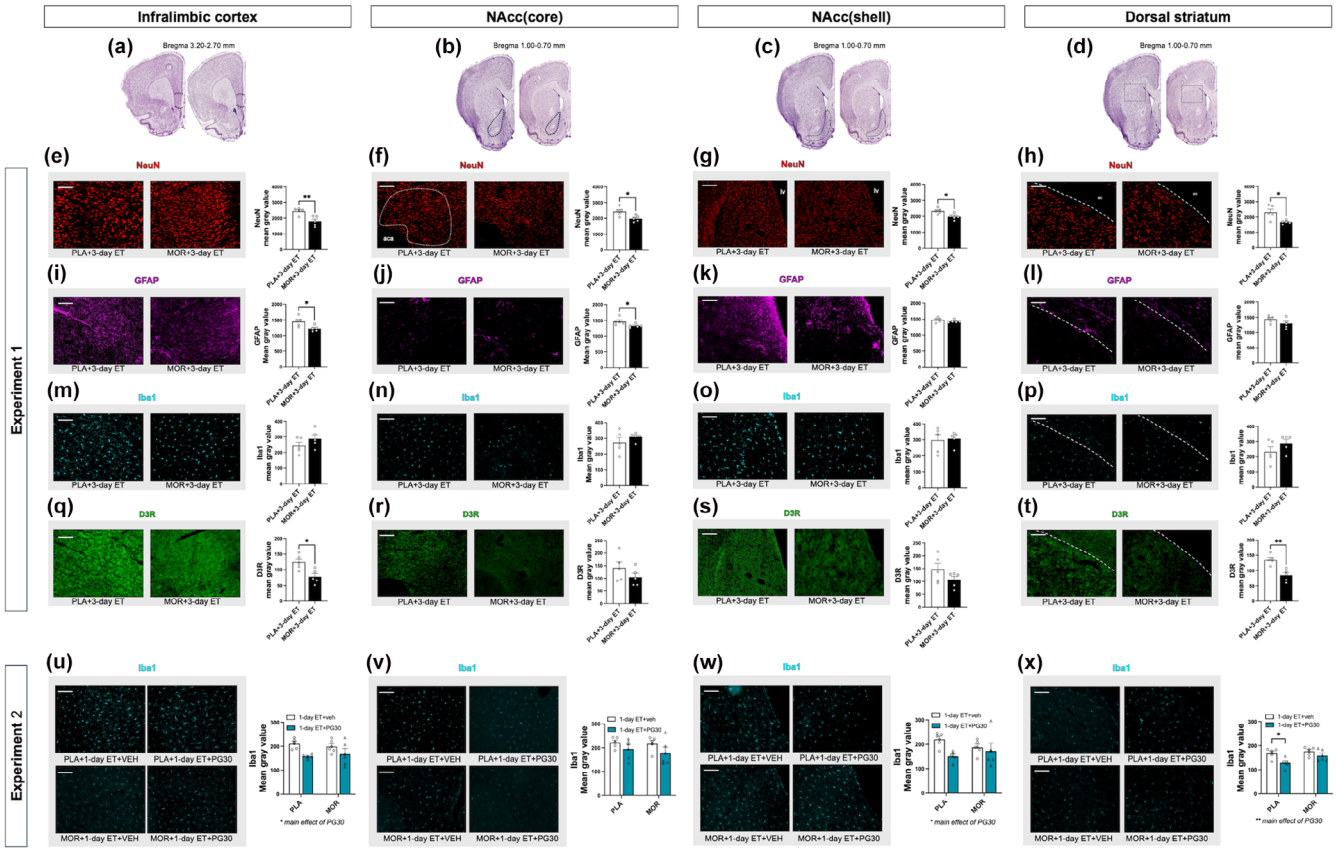

FIGURE 2 **Analysis of D_3 receptor (R), NeuN, glial fibrillar acidic protein (GFAP) and ionised calcium-binding adapter molecule (1Iba) markers after extinction training in the infralimbic cortex (IL), core and shell of the nucleus accumbens (NAc) and dorsal striatum (DS).** Anatomical localisation of the (a) IL, (b) core and (c) shell of the NAc, and (d) DS in the rat brain. Extracted from Paxinos and Watson Atlas (Paxinos & Watson, 2007). (e–h) NeuN-IR was decreased across regions after 3-day ET in morphine-dependent animals. GFAP-IR was decreased in the (i) IL and (j) core of the NAc after 3-day ET in morphine-dependent animals but not in the shell of the (k) NAc nor (l) DS. (m–p) Iba1-IR remained unchanged across brain nuclei. D3R-IR decreased in the (q) IL and (t) DS after 3-day ET in morphine-dependent animals but not in the (r, s) NAc. PG01037 at 30 mg kg^{-1} diminished Iba1-IR in the (u) IL, (x) shell of the NAc and (y) DS but not in the (w) core of the NAc. Scale bars: 10 μm. * P <0.05, ** P <0.01.

DS of morphine-dependent rats regarding the placebo group (Figure 3a,d). We detected no changes in the colocalization of NeuN/D_3 receptor, GFAP/D_3 receptor or Iba1 D_3 receptor in the NAc (Figure 3b,c). Distinctly, colocalizations of NeuN/D_3 receptor, GFAP/D_3 receptor or Iba1/D_3 receptor did not vary in any of these areas of morphine-treated rats that underwent a 1-day ET and received vehicle when compared with placebo-treated rats subjected to the same procedures (Figures S2J-L, S3J-L, S4J-L and S5J-L). This again suggests that the length of morphine treatment and/or the ET probably affected the expression of D_3 receptors in each cell population. When we studied the effect of CPA extinction through D_3 antagonism on D_3 receptor colocalization with NeuN, GFAP and Iba1, we found no main effects of morphine, PG01037 nor their interaction for NeuN/D_3 receptor in the IL and NAc (shell), GFAP/D_3 receptor in the IL, NAc shell and core, and Iba1 D_3 receptor in the IL. An effect of the opioid was uncovered for GFAP/D_3 receptor and Iba1/D_3 receptor in the DS, and an effect of PG01037 was observed on NeuN/D_3 receptor in the NAc (core) and DS, GFAP/D_3 receptor in the DS and Iba1/D_3 receptor in NAc core and shell and DS. Moreover, a significant enhancement of Iba1/D_3 receptor colocalization was detected in the NAc (shell) (Figure 3g) and the DS (Figure 3h) of morphine-treated animals that extinguished the CPA after PG01037 injection during the 1-day ET regarding those receiving vehicle, hence supporting that D_3 receptor blockade increase this receptor expression in microglial cells in the abovementioned brain nuclei. However, post hoc analyses did not reveal any significant changes between groups for NeuN/D_3 receptor in any of the areas analysed (Figure 3e–h). GFAP/D_3 receptor colocalization levels were increased in the DS of morphine-dependent animals injected with the D_3 antagonist (Figure 3h) and Iba1/ D_3 receptor colocalization augmented significantly in the NAc (shell) of morphine-dependent animals injected with PG01037 (Figure 3g). However, no significant alterations were detected for GFAP/D_3 receptor nor Iba1/D_3 receptor in the rest of brain regions studied (Figure 3e–h).

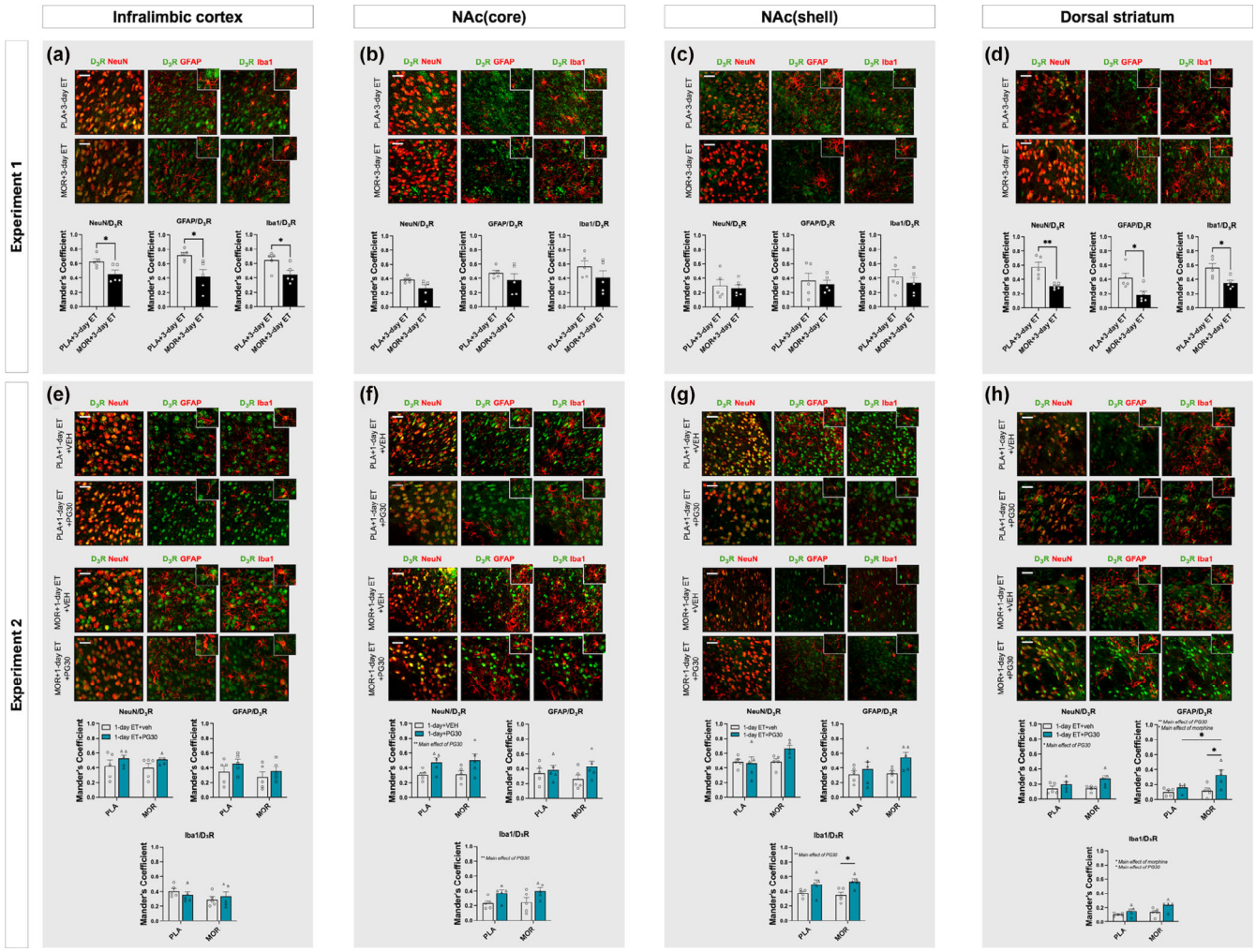

FIGURE 3 **Analysis of colocalization levels of D_3 receptor (R) and neuronal and glial markers after extinction training paradigms in the infralimbic cortex (IL), core and shell of the nucleus accumbens (NAc) and dorsal striatum (DS).** (a) Representative colocalization staining and analysis of D_3R and neurons, astrocytes and microglia after 3-day extinction training protocol in the IL. Scale bar: 5 μm. Three-day extinction training (ET) in morphine-dependent animals is related to decreased colocalization levels of D_3R in neurons, astrocytes and microglia in the IL. (e) Representative colocalization images and analysis of D_3R and NeuN-, GFAP- and ionised calcium-binding adapter molecule 1 (Iba1)-positive cells after 1-day ET protocol. PG01037 administration did not alter D_3R colocalization with neurons, astrocytes and microglia in the IL. (b) Representative colocalization staining and analysis of D_3R and neurons, astrocytes and microglia after 3-day extinction training protocol in the core of the NAc. Scale bar: 5 μm. Three-day ET in morphine-dependent animals did not alter colocalization levels in neurons, astrocytes and microglia in the core of the NAc. (f) Representative colocalization images and analysis of D3R and NeuN-, GFAP- and Iba1-positive cells after 1-day ET protocol. PG01037 administration enhanced D_3R colocalization with neurons and microglia but not with astrocytes in the core of the NAc. (c) Representative colocalization staining and analysis of D_3R and neurons, astrocytes and microglia after 3-day extinction training protocol in the shell of the NAc. Three-day ET in morphine-dependent animals did not alter colocalization levels in neurons, astrocytes or microglia. (g) Representative colocalization images and analysis of D_3R and NeuN-, GFAP- and Iba1-positive cells after 1-day ET protocol in the shell of the NAc. PG01037 administration enhanced D_3R colocalization with microglia but not with neurons or astrocytes in the shell of the NAc. (d) Representative colocalization staining and analysis of D_3R and neurons, astrocytes and microglia after 3-day extinction training protocol in the DS. Three-day ET in morphine-dependent animals decreased colocalization levels in neurons, astrocytes and microglia in the DS. (h) Representative colocalization images and analysis of D3R and NeuN-, GFAP- and Iba1-positive cells after 1-day ET protocol in the DS. PG01037 administration enhanced D_3R colocalization with neurons, astrocytes and microglia in the DS. * P <0.05, ** P <0.01. Scale bars: 5 μm.

3.4 | D_3 blockade modulate the activation state of microglia in the IL and the NAc (shell)

So far, our results demonstrated that PG01037 lessened Iba1-IR and augmented Iba1/D_3 receptor colocalization in specific dopaminoceptive nuclei of rats with facilitated CPA extinction after D_3 blockade. As the distinct activation states of microglia are associated with modifications in their shape (Reddaway et al., 2023), we then performed a morphometric study of these cells. We discovered that microglia of morphine-dependent animals that extinguished CPA

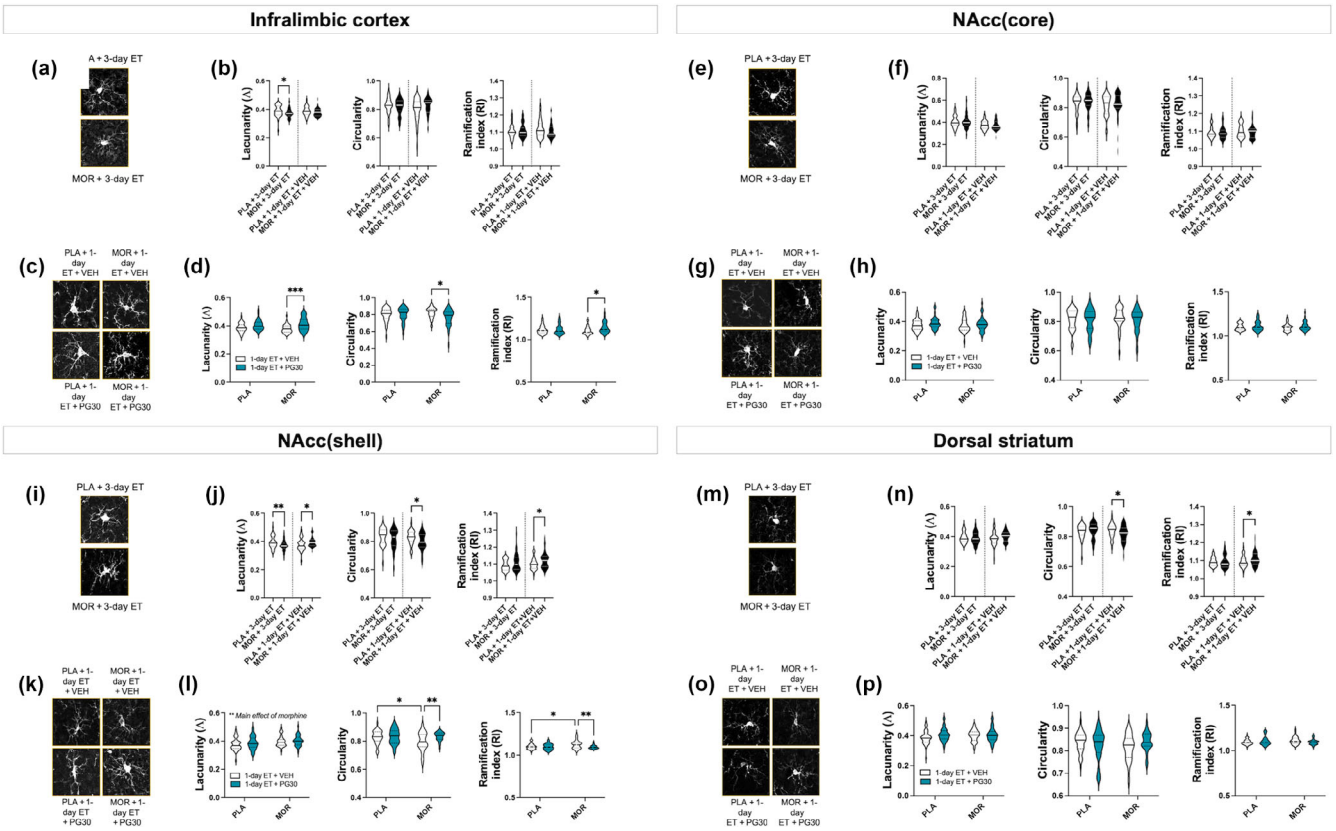

FIGURE 4 **Morphometric analysis of microglia after extinction paradigms in the infralimbic cortex (IL), core and shell of the nucleus accumbens (NAc) and dorsal striatum (DS).** (a) Representative detail of microglial staining in the IL after the 3-day extinction training (ET) experimental procedures. (b) Microglial lacunarity diminished after 3-day ET in morphine-dependent animals, but not after 1-day ET, in the IL. Neither circularity or ramification index (RI) were altered after 3-day ET nor 1-day ET in this area. (c) Representative detail of microglial staining in the IL after the 1-day ET experimental procedures. (d) PG01037 administration at 30 mg kg^{-1} altered microglial lacunarity, circularity and RI. (e) Representative detail of microglial staining after the 3-day ET experimental procedures in the core of the NAc. (f) Microglial morphological parameters were not altered after extinction paradigms in the NAc (core). (g) Representative detail of microglial staining after the 1-day ET experimental procedures in the NAc (core). (h) Microglial morphological parameters were not altered after PG01037 administration in the NAc (core). (i) Representative detail of microglial staining after the 3-day ET experimental procedures in the NAc (shell). (j) Microglial lacunarity diminished after conditioned place aversion (CPA) extinction in 3-day ET, but it increased after 1-day ET in morphine-dependent animals in the NAc (shell). Microglial circularity was diminished in animals that did not extinguish aversive behaviour (1-day ET), while RI increased. (k) Representative detail of microglial staining in the NAc (shell) after the 1-day ET experimental procedures. (l) Morphine increased microglial lacunarity in the 1-day ET paradigm in the NAc (shell). Morphine decreased microglial circularity, and PG01037 administration restored it to control levels in this area. Morphine also decreased microglial RI, and PG01037 administration restored it to control levels. (m) Representative detail of microglial staining in the DS after the 3-day ET experimental procedures. (n) Microglial lacunarity was not altered after 3-day ET. Microglial circularity was diminished in animals that did not extinguish aversive behaviour (1-day ET), while RI increased in the DS. (o) Representative detail of microglial staining after the 1-day ET experimental procedures in the DS. (p) PG01037 administration did not altered microglial lacunarity, circularity or RI in this area. * P <0.05, ** P <0.01, *** P < 0.001. n = 36–49 glial cells per group.

after a 3-day ET exhibited lower lacunarity (Λ) than placebo-treated rats in the IL and the NAc (shell), with no alterations of circularity or ramification index (Figure 4b,j). No significant differences were detected for Λ, circularity nor ramification index in the NAc (core) and DS between these experimental groups (Figure 4f,n). Microglia of opioid-dependent animals subjected to a 1-day ET exhibited no alterations in any of these parameters in the IL and NAc (core) (Figure 4b, f). Differently, microglia of morphine-treated animals exposed to 1-day ET displayed diminished circularity and increased ramification index in the NAc (shell) and DS and increased Λ in the NAc (shell) (Figure 4j,n). Finally, we evaluated the effect of the extinction of the morphine withdrawal-induced CPA by D_3 antagonism on microglia morphology. No effects of morphine, PG01037 nor their interaction were found in any of the parameters analysed in the NAc (core) (Figure 4h). We observed main effects of the opioid on Λ, circularity and ramification index in the NAc (shell) (Figure 4l). PG01037 had a significant effect on Λ in the IL, and on circularity and ramification index in the IL and NAc (shell) (Figure 4d,l). Finally, a main effect of the interaction morphine × PG01037 was discovered on circularity in the IL, NAc (shell) and DS, and on ramification index in the IL and NAc (shell) (Figure 4d,l,p). No statistical differences between groups were discovered for Λ, circularity nor ramification index in the NAc (core)

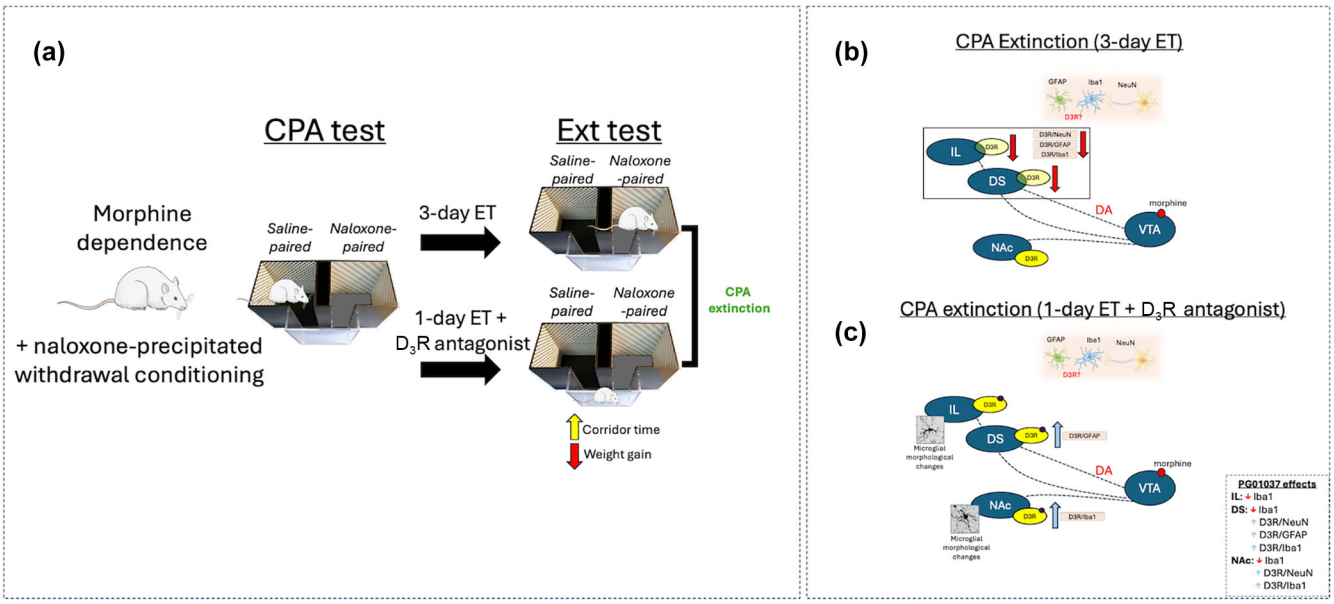

FIGURE 5 **Summary of findings.** (a) Morphine-dependent animals successfully extinguished the aversive behaviour induced by naloxone withdrawal, through 3-day extinction training (ET) and 1-day ET in conjunction with D_3 receptor (R) blockade. However, animals injected with the D_3 antagonist experienced decreased weight gain and increased time in the unconditioned compartment of the conditioning apparatus (the corridor). (b) D_3R was down-regulated in the infralimbic cortex (IL) and dorsal striatum (DS) of animals that extinguished the aversive behaviour through 3-day ET. Concordantly, D_3R colocalization levels with neuronal, astrocytic and microglial markers was diminished in these areas. (c) D_3R colocalization levels were up-regulated with glial markers in the DS and nucleus accumbens (NAc) of morphine-dependent animals receiving the D_3R blocker at 30 mg kg^{-1}, and its actions seem to alter microglial morphology in the IL and NAc.

and in the DS (Figure 4h,p). Microglia in the IL of morphine-treated rats that received the D_3 antagonist displayed increased Λ and ramification index, and decreased circularity when compared to those injected with vehicle (Figure 4d). In the microglial cells of the NAc (shell), no alterations between groups were detected for Λ. In contrast, these cells in morphine-dependent animals receiving vehicle exhibited significantly lower circularity and increased ramification index than controls, which may reflect a hyper-ramified phenotype, and PG administration significantly enhanced their circularity and diminished their ramification index (Figure 4l). Representative microglial staining from the different nuclei and groups are displayed in Figure 4a, c, e, g,i,k,m,o. Collectively, a summary of the molecular mechanisms underlying both extinction procedures in the aforementioned areas are shown in Figure 5b,c.

4 | DISCUSSION

The critical role of D_3 receptors in SUD and the ability of its antagonists to inhibit, augment the resistance to, or even accelerate the extinction of abused drug-seeking behaviours prompted by their rewarding effects or their reinstatements have been corroborated by several investigations (Ashby et al., 2015a, 2015b; de Guglielmo et al., 2019; Franco-Garcia et al., 2023; Galaj et al., 2016, 2015; Rocío Guerrero-Bautista et al., 2019; R. Guerrero-Bautista et al., 2020; R. Guerrero-Bautista et al., 2021; Hachimine et al., 2014; Jordan et al., 2019; Sun et al., 2016; Woodlief et al., 2023; You et al., 2019). D_3 blockade in the basolateral amygdala inhibited opiate withdrawal memory formation (Rosen et al., 2017) and the systemic administration of a D_3 antagonist also prevented the expression of naloxone-induced withdrawal after acute morphine administration in rats (Rice et al., 2012). Hence, D_3 antagonists have been proposed as potential cognitive enhancers that, concomitantly with extinction-based therapies, could help to inhibit and substitute the maladaptive behaviours responsible for drug use with others healthier for the individual (Ma et al., 2019; Nic Dhonnchadha & Kantak, 2011; Pich & Collo, 2015; Yan et al., 2013). While our results confirm the efficacy of PG01037 to accelerate the extinction of the aversive behaviour related with morphine withdrawal-associated contextual stimuli, they also allow to suggest that this effect might be at least partially because of decreased motivation. The reduced food intake or the increased time in the corridor, contemplated independently, might be considered as relatively weak signals of diminished motivation. However, in animals injected with the higher dose of the D_3 antagonist that resulted in the extinction of their CPA, this augmented time in the corridor was statistically significant, not only between tests but also in comparison with other experimental groups. Further, this fact co-occurred with the decreased food intake that, similarly, took place exclusively in rats that received the higher dose of PG01037 and extinguished the opioid withdrawal-induced drug-seeking behaviour. Concordantly with our data, D_3 receptor blockade diminished food intake (Thanos et al., 2008) and D_3 receptor deficient mice exhibited decreased sucrose CPP (R. Wang et al., 2025). Moreover, several recently published studies support D_3 receptor involvement in motivation

(Enriquez-Traba et al., 2024; Xi et al., 2024). Furthermore, D_3 receptor expression decreased in the NAc and mPFC of mice in a model of **LPS**-induced depression (J. Wang et al., 2018) and a depressive-like behaviour has been found after D_3 receptor blockade or D_3 receptor genetic deletion, as well as an anti-depressive effect of D_3 agonists on the same LPS-induced depression model (Moraga-Amaro et al., 2014; J. Wang et al., 2018, 2020, 2022). Altogether, these investigations support that diminished D_3 receptor function might underlie in unmotivated states. Nonetheless, additional behavioural tests would be beneficial to confirm the hypothesis of a diminished motivational state after D_3 antagonism.

Whereas some laboratories reported no effects of D_3 blockade or genetic deficiency on spatial memory (Favier et al., 2023; Moraga-Amaro et al., 2014; Thanos et al., 2008; Wang et al., 2018, 2025), others have found enhanced spatial learning and reduced memory deficits after D_3 antagonism (Tanyeri et al., 2015; Xing et al., 2010). Thus, improved cognition cannot be ruled out been involved in PG01037 effects on CPA extinction of morphine-dependent animals.

Distinct mechanisms could mediate in the effects of D_3 antagonism. PG01037 might block presynaptic D_3 receptor on DA neurons of the midbrain and/or inhibit postsynaptic D_3 signalling in dopaminoceptive neurons of the mesocorticolimbic system, thus, presumably, attenuating the enhanced DA firing in the ventral tegmental area caused by chronic morphine (Doyle & Mazei-Robison, 2021). In this sense, specific deletion of presynaptic D_3 receptors in DA neurons augmented pulse-evoked DA release in the NAc (Xi et al., 2024). Additionally, D_3 receptor in GABAergic interneurons of the hippocampal CA1 contributes to the disinhibition of excitatory neurotransmission in the ventral hippocampus to influence synaptic plasticity (Brown et al., 2024). Moreover, D_3 receptors in a specific glutamatergic cell population of the PFC appears to modulate the generation of high-frequency action potential bursts through a unique mechanism (Clarkson et al., 2017). Nonetheless, non-neuronal mechanisms might participate in PG01037 effects because our data show that animals that received the D_3 antagonist displayed alterations in glial cells that connect its behavioural outcomes with neuroinflammatory processes.

D_3 antagonism in control and morphine-treated animals attenuated the activation state of microglia, measured as Iba1-IR, and augmented D_3 receptor expression in these cells of the NAc (shell) and DS. Moreover, the extinction of the morphine withdrawal-induced CPA after D_3 blockade was accompanied by increased D_3 receptor expression in astrocytes of the DS and in microglia of the NAc (shell), and by modifications in microglial morphology in the IL and the NAc (shell). While D_3 antagonism in the IL induced a more ramified phenotype of microglial cells, which could be coherent with morphine-induced reactive microglia that turns into a homeostatic state after D_3 antagonism, in the NAc (shell) our data might be consistent with morphine-induced hyper-ramified microglial cells, which is a phase preceding their reactive state (Morrison et al., 2017), that revert to a homeostatic phenotype after PG01037 administration. Concordantly, increased levels of CD11b mRNA, a marker of microglial activation, were found in the NAc after chronic morphine treatment (Y. Zhang et al., 2011). As the activated phenotypes of microglia have been related with the release of proinflammatory molecules (Lannes et al., 2017; Lynch, 2009) and some of those inflammatory markers, in turn, have been postulated to mediate in abused substances effects (Eidson & Murphy, 2019; Lacagnina et al., 2017; Linker et al., 2019; Stellwagen et al., 2019; H. Zhang et al., 2020), the determination of proinflammatory cytokines levels in these regions would aid to elucidate this question.

Alterations in microglial activity induced by drugs of abuse, through their regulation of neurotransmission and synaptic plasticity, are crucial in behaviours prompted by their rewarding properties as well as in the somatic opioid withdrawal (Eidson & Murphy, 2019; Lacagnina et al., 2017; Linker et al., 2019; Stellwagen et al., 2019; H. Zhang et al., 2020). Moreover, microglia modulate the morphology and functionality of dopaminergic neurons, and vice versa (da Silva et al., 2023). Although D_3 receptor expression in glial cells *in vitro* has been controversial (Elgueta et al., 2017; Farber et al., 2005; Mastroeni et al., 2009; Miyazaki et al., 2004), some studies have detected D_3 receptor in astrocytes and microglia in rodents models for disorders such as Parkinson Disease and depression, and have attributed this receptor to have a main role in glial function in these conditions (Elgueta et al., 2017; Montoya et al., 2019; J. Wang et al., 2020). To our knowledge, our study reports for the first time the presence of D_3 receptor in glial cells in the context of SUD. Moreover, our findings reveal a cell-specific regulation of D_3 receptor expression after its blockade in mesocorticolimbic dopaminoceptive regions and suggest that D_3 receptor modulation of microglial activity might participate in the aversive behaviour of morphine withdrawal-associated contextual stimuli. Furthermore, the present data supports the proposal that a decreased motivational state might partake in the facilitated extinction of morphine-seeking behaviours prompted by its withdrawal caused by D_3 antagonists. In agreement, depressive-like behaviours have been associated with D_3 receptor modulation of microglial activation state (J. Wang et al., 2020). Additionally, selective deletion of D_3 in D_1 receptor- and **DAT**-expressing cells of the midbrain decreased opioids self-administration and brain-stimulation reward (Xi et al., 2024). As glial cells are known to express these proteins (Channer et al., 2023), glial D_3 receptor involvement in these behavioural outcomes cannot be ruled out. Our results show altered microglial morphology compatible with an activated state in the IL and the NAc (shell) of dependent animals that seems to turn into a homeostatic phenotype in animals that extinguished the drug-seeking behaviour through D_3 blockade. In this regard, both D_3 antagonism and genetic deficiency lessened microglial activation in midbrain after **lipopolysaccharide** (LPS) challenge (Montoya et al., 2019) and D_3 antagonism resulted in increased ramification density of microglia in the striatum in a model of Parkinson Disease (Elgueta et al., 2017). Also, decreased D_3 receptors in the mPFC and NAc parallelled with depressive-like behaviours in an LPS-induced model of depression (J. Wang et al., 2018). Nevertheless, these depressive-like behaviours were associated with increased proinflammatory cytokines levels and both reverted after the administration of a D_3 agonist but not after D_3 receptor blockade (J. Wang et al., 2018). Knockout D_3 receptor mice also exhibited depressive-like behaviours, increased proinflammatory cytokines and Iba1-labelled

microglia that ameliorated after the inhibition or ablation of microglia (J. Wang et al., 2020). These discrepancies may respond to the distinct experimental models and/or the rodent species used, among other factors. Nonetheless, all these investigations indicate that microglial activity regulation could represent an important mechanism of D_3 receptor functionality that needs to be addressed with further research to decipher its role in the pathophysiology of neurodegenerative and psychiatric disorders.

Besides behaviour, animals that extinguished the morphine withdrawal-induced CPA through cognition-based approaches presented other differences with those that extinguished the aversive behaviour after D_3 receptor blockade. Maybe as a result of the longer duration of morphine exposure and in agreement with what has been described before (Brazile et al., 2024), we found signs of what might be neuronal degeneration and/or death in the IL and the striatum of animals subjected to the 3-day ET. Also, GFAP levels decreased in the IL and NAc (core) of these rats. Although a number of investigations have reported astrogliosis after opioids treatment (H. Zhang et al., 2020), morphine analgesic effects have been associated with reduced GFAP in mPFC, hippocampus and amygdala (Asgharpour-Masouleh et al., 2023; Ghasemzadeh et al., 2021). In accordance with our data, and endorsing the astrocytes involvement in cognitive function and impairment in substance use disorders (Santello et al., 2019), decreased GFAP in the NAc (core) and amygdala were observed after the extinction of cocaine self-administration and of morphine-induced conditioned place preference (Lin et al., 2011; Scofield et al., 2016). Finally, D_3 receptor expression unspecifically diminished in neurons, astrocytes and microglia of the IL and DS of rats that extinguished their CPA after behavioural training alone, which points out that D_3 receptor could be involved in the extinction of morphine withdrawal-associated memories. Concordantly, D_3 receptor has been published to control the expression of genes involved in cognitive function (Castorina et al., 2013; D'Amico et al., 2013) and its overactivation in the mPFC occurred concomitantly with memory deficits (Chang et al., 2018, 2020). Also, D_3 antagonism increased social memory recognition and diminished **scopolamine**-induced memory deficits (Millan et al., 2007). Undeniably, more research is needed to better understand D_3 receptors role in cognition.

Research underlining the contribution of sex in different phases of substance use disorders is growing (McHugh et al., 2018; Towers et al., 2021, 2023). Because our work was based only on male rats, future investigation will be necessary to explore sex as a putative variable and its impact on the D_3 receptor-mediated extinction of opioids withdrawal-induced avoidance behaviours, as well as in its effects on the different brain cells populations in areas related to motivation and cognition. Also, despite several investigations reporting depressive-like states and diminished motivation evoked by D_3 blockade or D_3 receptor deficiency (Favier et al., 2023; Moraga-Amaro et al., 2014; Thanos et al., 2008; J. Wang et al., 2018), some of them paralleling with altered microglial activation state (J. Wang et al., 2020), our study would benefit from the administration of another D_3 antagonist to compare with the effects of PG01037 on the extinction of morphine withdrawal-induced CPA.

Summarising, while our results confirm that D_3 antagonists facilitate the extinction of morphine-seeking behaviours triggered by contextual stimuli associated with its withdrawal, this effect could be partly because of their ability to diminish motivation, which might limit their therapeutic use. This effect, as our and other studies suggest, may be at least partially mediated by non-neuronal mechanisms, and microglial cells might be one of the actors. Present data show that PG01037 had a significant effect diminishing Iba1-IR in the IL, NAc (shell) and DS, thus pointing out that D_3 blockade might decrease the activation of microglial cells in these brain regions. Among all these areas, only in the NAc (shell) D_3 antagonism in opioid-dependent animals, which extinguished their CPA, was accompanied by significantly increased microglial D_3 receptor expression and by morphological changes consistent with a more homeostatic state in comparison with vehicle-injected morphine-treated rats. Thus, our results point out that microglial D_3 receptor in the NAc (shell) might be a main actor for the activation of these cells and, consequently, for the aversive behaviour to the contextual stimuli associated with morphine withdrawal. More research is needed for a better characterisation of D_3 receptor function, which will permit this receptor to be a useful target in therapeutic strategies able to decline the relapses induced by opioids withdrawal-associated stimuli.

AUTHOR CONTRIBUTIONS

A. Franco-García: Investigation; visualisation; data curation; writing—original draft. **V. Gómez-Murcia**: Investigation; visualisation. **M. V. Milanés**: Conceptualisation; validation; formal analysis; data curation; funding acquisition; project administration; supervision; writing—original draft. **C. Núñez**: Conceptualisation; validation; formal analysis; data curation; funding acquisition; project administration; supervision; review and editing. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this manuscript.

DATA AVAILABILITY STATEMENT

The datasets of the current study are available from the corresponding author on reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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