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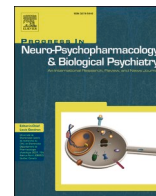


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# Progress in Neuropsychopharmacology & Biological Psychiatry

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## Traumatic-like fear memory recall causes persistent morphine conditioned place preference in drug withdrawn male mice

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### ABSTRACT

Post-traumatic stress disorders (PTSD) can lead to substance use disorders (SUD), and in particular opioid dependence. Although preclinical models of the literature focused on traumatic stress-induced potentiation of opioid dependence acquisition, none studied the effect of fear memories during opioid withdrawal. The goal of this study was thus to develop and describe a preclinical model of the PTSD/SUD comorbidity during this critical period in adult male mice. Classically, the traumatic-like memory was acquired by fear conditioning and was followed by morphine-conditioned place preference (CPP) to acquire the associative memory linked to the drug-reinforcing effect. Departing from this approach, we evaluated the effect of a stress on drug-induced CPP using regular re-exposures to a conditioned fear stimulus recall (FR), immediately followed by CPP tests, several days after the last morphine injection (from the 5th to the 21st day). Our data indicated that FR sessions induce a persistent morphine CPP, that is absent in morphine withdrawn mice not subjected to FR. This effect was prevented when antalarmin, a corticotropin-releasing factor receptor 1 antagonist, was administered during morphine withdrawal before each FR. Persistent morphine-induced CPP was concomitant with a FR-induced *kappa* opioid receptor mRNA upregulation in the prefrontal cortex, while *mu* opioid receptor mRNA expression was enhanced in control morphine withdrawn mice, an effect absent, however, in withdrawn mice subjected to FR. Surprisingly, in the amygdala, endogenous opioid-related mRNA expression changes in relation with the long-term persistence of drug-induced CPP were few, but Next Generation Sequencing revealed differential expression of numerous microRNAs in that brain area between morphine-control vs morphine-FR mice. The present study thus proposes an innovative behavioral model of the PTSD/SUD-like comorbidity with biological modulations in both the prefrontal cortex and the amygdala, paving the way to develop adapted treatments for this comorbidity in clinics.

### 1. Introduction

Post-traumatic stress disorder (PTSD) and substance use disorders (SUD) are frequently comorbid (María-Ríos and Morrow, 2020; Leconte et al., 2022) and this PTSD/SUD comorbidity is often associated with more severe clinical profiles compared with either diagnosis alone. The bidirectional relationship between PTSD and SUD suggests that trauma can lead to SUD as a maladaptive coping mechanism, while substance abuse can exacerbate PTSD symptoms. This vicious circle is partly explained by the self-medication hypothesis, as most patients report that drugs help alleviate their symptoms (Khantzian, 1985; Volpicelli et al., 1999; Danovitch, 2016). Among PTSD patients, opioid use disorders

(OUD) are widespread and highly correlated with PTSD symptoms (Mills et al., 2006; Dworkin et al., 2018). Opioids are among the substances of choice, as they can initially relieve several PTSD symptoms (e.g., insomnia, flashbacks, hypervigilance) and chronic pain frequently associated with PTSD (López-Martínez et al., 2023). Epidemiological studies report that 30%–50% of individuals with OUD also suffer from PTSD (Shiner et al., 2017).

While early and acute administration of morphine may have beneficial effects on PTSD outcomes in clinics (Saxe et al., 2001; Bryant et al., 2009), prolonged opioid use ultimately worsens PTSD symptoms (Danovitch, 2016; María-Ríos and Morrow, 2020). In preclinical studies, conditioned place preference (CPP) and fear conditioning (FC) usually

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performed to evaluate respectively, drug-induced reward and fear memory, have been used to investigate the vicious circle between stress and addiction (Bruchas et al., 2010; Leconte et al., 2022). First, morphine addiction can increase fear memory lability. For instance, contextual fear conditioning extinction has been shown to be impaired in adult male rats 9 days after morphine withdrawal (Gu et al., 2008), or when exposed to morphine prenatally (Tan et al., 2015). Conversely, stress can increase vulnerability to morphine addiction. Conditioned fear enhances morphine withdrawal behaviors (Abrahamsen et al., 1995) and tolerance to morphine-induced hyperthermia (Barsy et al., 2011). Additionally, conditioned cue-fear recall or a situational reminder of an electrical foot-shock have been shown to enhance opioid conditioned place preference acquisition (Jing Li et al., 2018; Yu et al., 2025). Thus, studies in the past investigated stress-induced enhancement of acquisition of morphine-induced CPP (i.e. models of stress-induced vulnerability to the development of an addiction). In contrast, our specific goal was to develop a model to explore the effect of a traumatic-like stress on morphine withdrawal symptoms. The novelty and significance of the present study is thus more related to how to trigger a stress-induced persistent CPP specifically during the morphine withdrawal period, which will be useful to address preclinically some questions of clinical and therapeutical interest.

There is a significant overlap in the biological, anatomical, and epigenetic mechanisms underlying both PTSD and SUD memories (Pizzimenti and Lattal, 2015). Each pathology is characterized by deficits in associative memory extinction that may be responsible for the persistence of drug-seeking behavior and traumatic fear responses. Common brain structures are involved in these two psychiatric conditions. The medial prefrontal cortex (mPFC) is notably crucial for the acquisition, expression and extinction of both (i) fear memory and (ii) compulsive substance abuse behaviors, through its projections, respectively, (i) to the amygdala and (ii) to the nucleus accumbens (NAc), two other key brain structures for these pathologies (Akirav and Maroun, 2007; Perry et al., 2011).

Endogenous opioid systems including precursors of neuropeptides (pro-opiomelanocortin, POMC; prodynorphin, pDYN) and receptors, as well as the corticotropin-releasing factor (CRF) and its receptors are among the most obvious targets in PTSD/OD comorbidity (Van't Veer and Carlezon, 2013; Leconte et al., 2022; Upadhyay et al., 2022). Since delta opioid receptors (DOR) and Nociceptin/Orphanin FQ Receptors (NOP) systems have a more moderate implication in morphine addiction, they were not studied yet in the present study. The *mu* opioid receptor (MOR) and subsequent biochemical and molecular changes are part of the main system mediating opioids positive reinforcement, driving euphoria and tolerance (Karkhanis et al., 2017), but also modulates fear memory, upregulating attention to a stimulus predictor of a threat (Iordanova et al., 2006). In opposition to MOR that mediate reward, *kappa* opioid receptors (KOR) and signalling mediate negative reinforcement, driving withdrawal, and stress-induced relapse (Karkhanis et al., 2017). KOR and pDYN were also claimed to play a major role in the pro-addictive effects of stress (Bruchas et al., 2010; Leconte et al., 2022). The stress effect partly occurs through its relationship with CRF-induced dysphoria: KOR activation influence CRF expression and corticosterone levels in rats (Iyengar et al., 1986). Furthermore, CRF synthesis is increased during withdrawal, while CRF Receptor 1 (CRFR1) antagonism or CRFR1 genetic deletion limits withdrawal symptoms and relapse in rats (Papaleo et al., 2007; Koob, 2009; Baidoo et al., 2025).

We thus investigated genes expression of the above neuropeptide precursors and receptors in the PFC, the amygdala and the NAc. Moreover, as many clinical studies have investigated the differential microRNA expression in PTSD stress axis dysregulation (Daskalakis et al., 2018) and in SUD neuroadaptations and synaptic plasticity-related changes (Occhipinti et al., 2023), we investigated amygdalar microRNAs regulations in our model using Next Generation Sequencing (NGS).

## 2. Material and methods

### 2.1. Animals, treatments and body temperature measurements

All care and experiments were done in accordance with the ethical approval (APAFiS#34465) stipulated by the animal experimental ethics committee (CEEA-059, Paris Centre et Sud), the French regulations and the European Union Council Directive of September 22, 2010 (2010/63/EEC) on the protection of animals for experimental use. Eight-week-old male C57Bl6/J mice supplied by Janvier Labs (Le Genet St Isle, France) were housed 4 per cage with nest, on a 12-h light/dark cycle (light 8:00 a.m.-8:00 p.m.) in a temperature-controlled room ( $21 \pm 2^\circ\text{C}$ ) with food and water available ad libitum.

This study uses 265 animals in total. Cohorts experimental schedules are illustrated at the top of each figure (Fig. 1A-7A). The mice were housed for one week, and thereafter were randomly assigned to groups subjected or not to fear conditioning (FC), with repeated saline injection or morphine injections (10 mg/kg/day, i.p.) for CPP acquisition. To avoid the beneficial effects of early and acute morphine administration on fear memory extinction (Morris and Gebhart, 1978), there was a five-day delay between FC and the beginning of chronic morphine administrations. Five days after the last morphine administration, mice were subjected or not to fear recall (FR) consisting in re-exposure to the conditioned fear stimulus (2.5 kHz sound) in the cage context (without shocks). Mice not subjected to prior FC underwent an exposure to the same 2.5 kHz sound alone ("sound" groups) in a neutral context. In one cohort, mice were injected with antalarmin (10 mg/kg/day, i.p.) or the vehicle (10 mL/kg) 2 h before each FR. Morphine hydrochloride (Francopia, Paris) dissolved in NaCl 0.9% solution, was daily injected during the two consecutive 4-day sessions of CPP. Antalarmin (Axon Medchem©) was prepared in 1% DMSO/NaCl 0.9% solution. This dose of antalarmin was selected from previous studies (Deak et al., 1999; Ducottet et al., 2003).

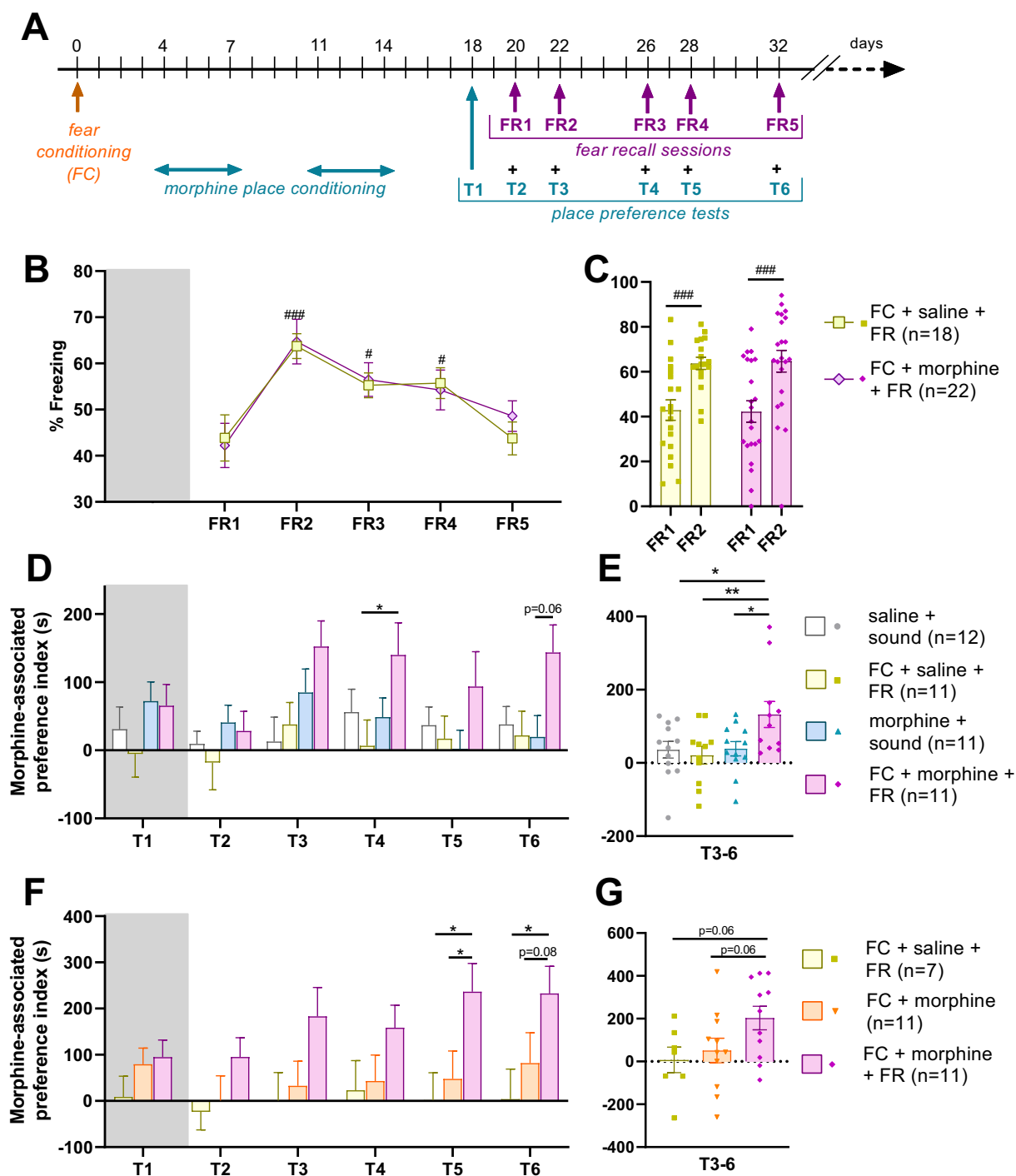
Body temperature was measured using a mouse rectal thermometer to evaluate morphine-induced hyperthermia 20 min after injections on day-7 (second morphine injection), day-9 (fourth morphine injection) and day-11 (fifth morphine injection after two days of morphine withdrawal). Body weight was measured once per week, and every day before any treatment.

### 2.2. Fear conditioning, fear recall and fear extinction

**Fear conditioning (FC)** was done as previously described (Corne et al., 2019). Briefly, on day-0, each mouse was put in a lit cage (19 × 22 cm, height 13 cm, 50 lx) with a grid floor connected to an electric foot-shock generator and a loudspeaker producing a tone (2.5 kHz, 85 dB) in a sound-attenuating cubicle. Stimuli were controlled from a computer system (MedAssociate, St-Albans, VT, USA). Cages were previously cleaned with a scented solution (0.1% vanilla in 75% alcohol) to define the conditioning context. During the conditioning phase, animals were exposed 6 times to the sound (30 s, conditioned stimuli, CS) immediately followed by a mild foot-shock (2 s; 0.5 mA; 2 min Inter-Trials Intervals; ITI). Fear response, behaviorally expressed as "freezing" (complete immobility except respiration), was recorded and analyzed using a video tracking software (Anymaze® vs 7.00).

**Fear recall (FR)** sessions were performed on days 20, 22, 26, 28 and 32 just before the place preference conditioning test, to assess the effect of a short re-exposure to the context and the CS (without foot-shock) on CPP during opioid withdrawal. Each mouse was placed in the FC context (lit cage with vanilla odor) for 6.5 min: 4-min baseline in the context, one CS tone (30s, 2.5 kHz, 85 dB), and 2-min post-CS recording. Fear behavior was calculated as the percentage of time spent freezing during the CS minus that measured during the prior 2-min baseline.

**Fear extinction (FE)** was done by performing two sets of experiments in order to first reduce contextual fear and then, to reduce the CS cue-induced fear in a new context. On day-27 or -33, contextual FE was



**Fig. 1.** Fear recall reveals persisting morphine place preference in morphine withdrawn mice in two different cohorts. (A) Experimental schedule. Results are represented as mean  $\pm$  SEM. (B) Percentage time freezing (during the “30 s cue-conditioned stimulus” minus the “prior 120 s contextual baseline”) during fear recall trials. Two-way ANOVA for repeated measurements followed by Sidak post-hoc test ( $^*p < 0.05$ ,  $^{###}p < 0.001$ : comparison with FR1 for both groups), and (C) with data distribution at FR1 vs FR2. (D, F) Morphine-associated preference index of the two distinct cohorts. The “sound” control mice were exposed to the neutral sound (not associated with footshock). Two-way ANOVA for repeated measurements followed by Dunnett post-hoc test ( $^*p < 0.05$ ). (E, G) Morphine-associated preference index averaged for each animal from T3 to T6. One-way ANOVA followed by Sidak post-hoc test ( $^*p < 0.05$ ,  $^{**}p = 0.01$ ). For the freezing data (B, C) the two cohorts were pooled.

performed by placing the mouse for 18 min in the FC apparatus previously cleaned with the scented vanilla solution, without applying any CS nor foot-shocks. Secondly, on day-28-29 or 34-35, mice were repeatedly exposed to the CS alone in a completely different cage (circular Plexiglas arena; diameter 19 cm, height 14.5 cm), in the dark without the vanilla odor. Both sessions consisted of 40 sound CS exposures (30 s, 5 s ITI) to

generate conditioned freezing extinction. The intra-session FE index was calculated as the freezing during the first 2 CSs minus that of the last 2 CSs of the extinction sessions. The inter-session FE index corresponds to the freezing during the first 2 CSs of the first day minus the freezing during the first 2 CSs of the last day. FE sessions were then followed by a last FR + CPP test session.

### 2.3. Conditioned place preference to morphine

Morphine rewarding effects were evaluated using a biased conditioned place preference (CPP) protocol as previously described (Requana Aradas et al., 2023). The CPP apparatus (Imetronic, France) included 2 lateral chambers (15 × 15 × 20 cm) connected by a central corridor (5 × 15 × 20 cm) and 2 sliding doors to separate the chambers from the corridor. In each chamber, 2 Plexiglas prisms with triangular bases (5 × 7 × 19 cm) were placed to form different patterns and cover the same surface of the chamber. They were used as conditioned stimuli along with 2 different types of embossed Plexiglas floors (one gridded and one striped).

The protocol consisted of 3 phases. First, the preconditioning phase (day-4): drug-naive animals had free access to both compartments for 18 min, the time spent and locomotor activity in each compartment were recorded, using the number of light beam breaks. Second, in the conditioning phase (8 days: days 5 to 8 and 11 to 14), animals were treated with saline and placed individually in one of the conditioning chambers for 18 min; 4 h later, mice received morphine and were placed for 18 min in the opposite compartment. This sequence was repeated every conditioning day. Third, during the CPP tests, animals had free access to both compartments for 18 min, and the time spent and locomotor activity were recorded in each compartment. Results were expressed as preference index (in seconds) calculated as the difference between “the time spent in the drug-paired compartment during the test phase” minus “the time spent in the same compartment during the preconditioning phase”.

### 2.4. Marble-burying test

This test is commonly used to evaluate anxiety and obsessive-compulsive-like behavior (Taylor et al., 2017). It was performed at day-5 (one hour after the first morphine administration), day-15 (20 h after the last morphine administration) and day-27 (24 h after FR3). Mice were placed individually in a cage (21 × 15 × 25 cm) with 12 marbles evenly spaced on a 10 cm layer of bedding material. For 20 min, mice were free to bury the marbles: more than two-thirds buried marbles were manually counted by the experimenter at fixed intervals (1, 2, 3, 4, 5, 10, 15 and 20 min).

### 2.5. Euthanasia, samples, quantitative RT-qPCR and NGS

To evaluate mRNA expression, animals were euthanized 30 min after the behavioral challenge: FR1 + T2 on day-20, or FR3 + T4 on day-26. To evaluate microRNA expression, animals were euthanized 3 h after the behavioral challenge at FR3 + T4 on day-26.

The amygdala, NAc, and PFC were punched in a RNase-free environment. Brains were dissected into 3 slices, 2 mm each, using a brain matrix. The first slice (2 to 0 mm from bregma) allowed dissection of the PFC and the NAc, the second slice (bregma: −1 to −3 mm) the dissection of the amygdala. Samples were stored at −80 °C. RNA was extracted using the miRNeasy Mini Kit (Qiagen™) according to the manufacturer's instruction. Total RNA quantity and purity were determined by spectrophotometry (Nanodrop).

Thereafter, cDNAs were obtained using the SYBR Green (Applied Biosystems™) high-capacity reverse transcription (rt-PCR). CRF, CRFR1, KOR, MOR, pDYN, and POMC (primers in supp. Table 1) mRNA expressions were quantified in the amygdala, the NAc, and the PFC. Each qPCR (using Quantitative Polymerase Chain Reaction SYBR Green kit; Applied Biosystems™) run was performed in a 384-well plate, in triplicates, with the following cycle: “95°C, 5min; [95C, 10s; 60°C, 30s] x 40 cycles”. Each run was analyzed with Biorad-CFX-maestro® software. mRNA expression level was normalized using two housekeeping genes, HPRT and PPIA (primers in supp. Table 1) and expressed as fold change of the “saline + sound” group.

Total RNA samples collected 3 h after FR3 in the amygdala,

containing small RNAs including microRNAs, were used for Next Generation Sequencing (NGS) performed and analyzed by the Genom'IC Platform (Cochin Institute, Paris). The quality of the RNA (RNA integrity number) was determined on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) as per the manufacturer's instructions. To construct the libraries, 1–5 ng of the small RNA fraction were processed using Qiaseq mir RNA library prep kit (Qiagen) according to manufacturer instructions. Briefly, 3' and 5' adapters are ligated to each end of RNA molecules and then reverse transcribed and amplified to generate a cDNA library. Libraries were quantified by Qubit (Invitrogen) and profiles were assessed using the DNA High Sensitivity kit on an Agilent Bioanalyzer. Libraries were sequenced on an Illumina Nextseq 500 instrument using 76 base-length reads in a single end mode. After sequencing, a primary analysis based on AOZAN software (ENS, Paris, Perrin et al., 2017) was applied to demultiplex and control the quality of the raw data (based of FastQC modules / version 0.11.5). QIAGENs Ingenuity Pathway Analysis (IPA) with the keywords “addiction” and “opioid-related disorders” was performed at the Proteom'IC Platform (Cochin Institute, Paris).

### 2.6. Statistics

Results were expressed as mean ± SEM except for microRNAs that were median ± min/max values. All statistical analyses were performed using GraphPad Prism® software (vs 8.0.2). The normality of behavioral and molecular data was verified using Shapiro-Wilk test. All data sets passed the normality test. In case when animals were repeatedly tested over time, normality was tested over the whole period of time for each group. After that, data were analyzed using a One-Way ANOVA or a Two-Way ANOVA for repeated measurements, followed by Dunnett (intergroup comparisons to a unique group) and/or Sidak (for inter-time comparisons in one group or intergroup comparisons) post-hoc tests. With repeated measurements, in cases when no time effect was observed, the average value could be calculated for a given time period for each animal. For microRNA raw data analysis and statistics were performed with the miRge3.0 (Patil and Halushka, 2021) and DESeq2 (Love et al., 2014).

## 3. Results

### 3.1. Fear recalls reveal persistent morphine-induced place preference

First of all, we carried out analyses of morphine-induced weight loss, hyperlocomotion and hyperthermia, as detailed in supplementary Fig. 1.

The effect of the fear-associated context and CS re-exposure was investigated in two different cohorts by subjecting mice to FR on D-20, D-22, D-26, D-28 and D-32 (Fig. 1A). FR induced similar conditioned freezing in both groups (Fig. 1B and C), indicating that morphine withdrawal did not influence fear behavior during FR. Interestingly, freezing behavior was enhanced from FR2 until FR4 compared to FR1 in both groups ( $p < 0.001$  FR2,  $p < 0.05$  FR3 and FR4; Fig. 1B, C), suggesting that repeated brief FRs induce reconsolidation of cue-induced fear.

When cohorts presented in Fig. 1D, F were pooled together at T1, before FR exposures, the place preference index was statistically enhanced in mice treated with morphine ( $78 \pm 16$  s,  $n = 44$ ) compared to those treated with saline ( $9 \pm 21$  s,  $n = 30$ ; Student's *t*-test,  $p < 0.01$ ). However, when cohorts were not pooled (Fig. 1D and F), this baseline CPP test did not reach statistical significance. Animal handling or the mouse strain chosen may somehow limit CPP amplitude (Cunningham et al., 1992). Altogether these results suggest that, in our conditions, the morphine-induced place preference is at the threshold limit.

The subsequent effect of FR on morphine CPP was investigated with FRs carried out immediately prior to the CPP tests, conducted regularly during the withdrawal period (Fig. 1A). The morphine control groups did not show any increase in place preference score during this time,

whether they had (Fig. 1F) or not (Fig. 1D) been subjected to FC two weeks before. In contrast, FC- and morphine-treated mice exposed to FR displayed the highest CPP index, starting from T3 until T6 (Fig. 1D, F). Indeed, the place preference index of the FC + morphine+FR groups was enhanced throughout time (2-way ANOVA for repeated measurements: Group effect  $p < 0.0001$ ; One-way ANOVA for repeated measurements, Time effect for FC + morphine+FR group only  $p < 0.05$ , Fig. 1D and F). CPP index was statistically enhanced at T4 and marginally significant at T6 for the first cohort ( $p < 0.05$ ,  $p = 0.06$ , Fig. 1D), as well as at T5 and T6 for the second cohort ( $p < 0.05$ , Fig. 1F). We also examined the overall morphine-associated preference index from T3 to T6 sessions, when there is no significant Time effect (One-way ANOVA for repeated measures; Fig. 1E, G). There was a significant difference between the FC + morphine+FR and the other control groups ( $p < 0.05$  vs. morphine+sound and saline+sound groups,  $p = 0.01$  vs. FC + saline+FR group, One way-ANOVA, Fig. 1E), and a marginally significant difference between the morphine alone and morphine+FR groups ( $p = 0.06$ , One-way ANOVA, Fig. 1G). Overall, these results suggest that FRs tend to make morphine-CPP scores significant or persistent during withdrawal.

We then examined in the marbles burying test whether a previous FC session (4 days or more before) or morphine withdrawal (1 day or 2 weeks after the last administration) generate in our conditions, an anxiety-like state (Fig. 2A). Mice have an innate propensity to compulsively bury potentially dangerous objects. FC session days before does not alter the typical kinetic of marble burying (data not shown). However, 1 h after the first morphine administration on D4, the number of visible marbles was increased (minute-3:  $p < 0.05$ , minute-4 and -5:  $p < 0.01$ , minutes 10 and 15:  $p < 0.001$ , minute-20:  $p < 0.01$ , FC + saline compared to morphine and FC + morphine groups, Fig. 2B), indicating that acute morphine administration induced an anxiolytic-like effect. Conversely, at D15, in acutely withdrawn mice, the number of visible marbles was decreased (minute-4 to minute-10:  $p < 0.05$ , FC + saline compared to morphine groups, Fig. 2C), meaning that early morphine withdrawal induced an anxiogenic effect. Similarly, late withdrawal, at day 27, produced an anxiogenic state (minute-5:  $p < 0.05$ , minute-10:  $p < 0.001$ , minute-15:  $p < 0.05$ , FC + saline compared to morphine groups, Fig. 2D), but there was no effect of the previously administered

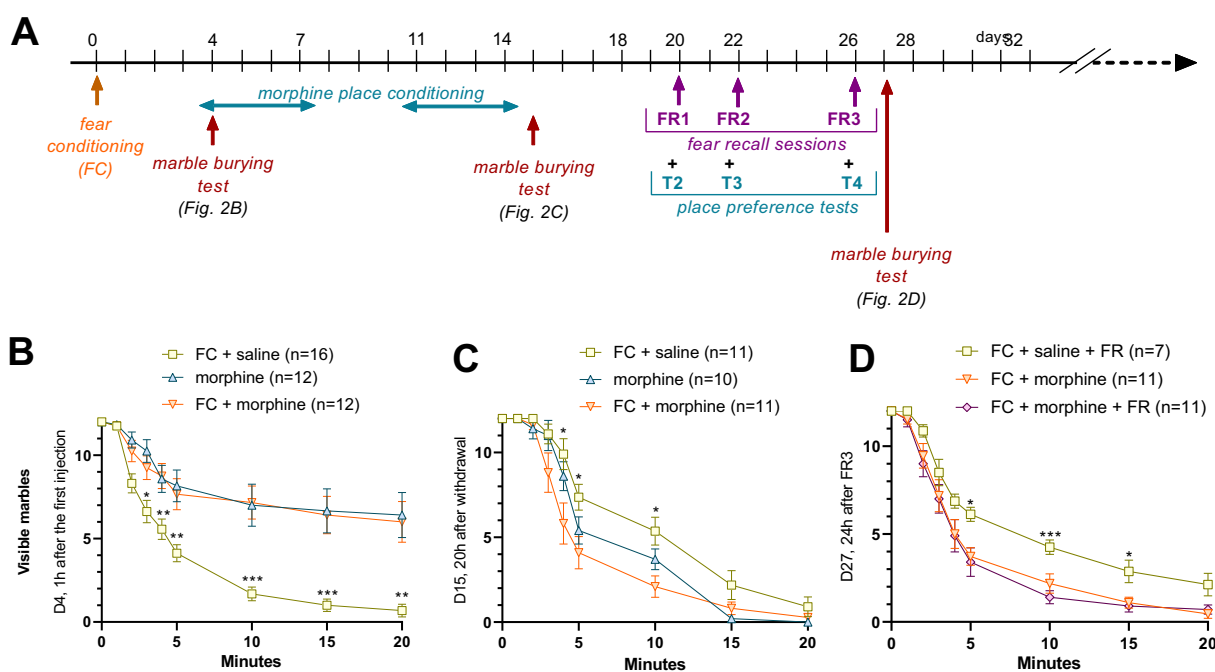
FR sessions (one day before) on the increased compulsive-like behavior generated by morphine withdrawal. Altogether these results indicate that although acute morphine administration is anxiolytic, consistently with the literature (Umathe et al., 2012; Kitanaka et al., 2015), morphine withdrawal produced in our conditions a mild but persisting anxiogenic-like state.

### 3.2. Fear and hormonal stress responses involvement in the FR-induced persistence of morphine-induced CPP

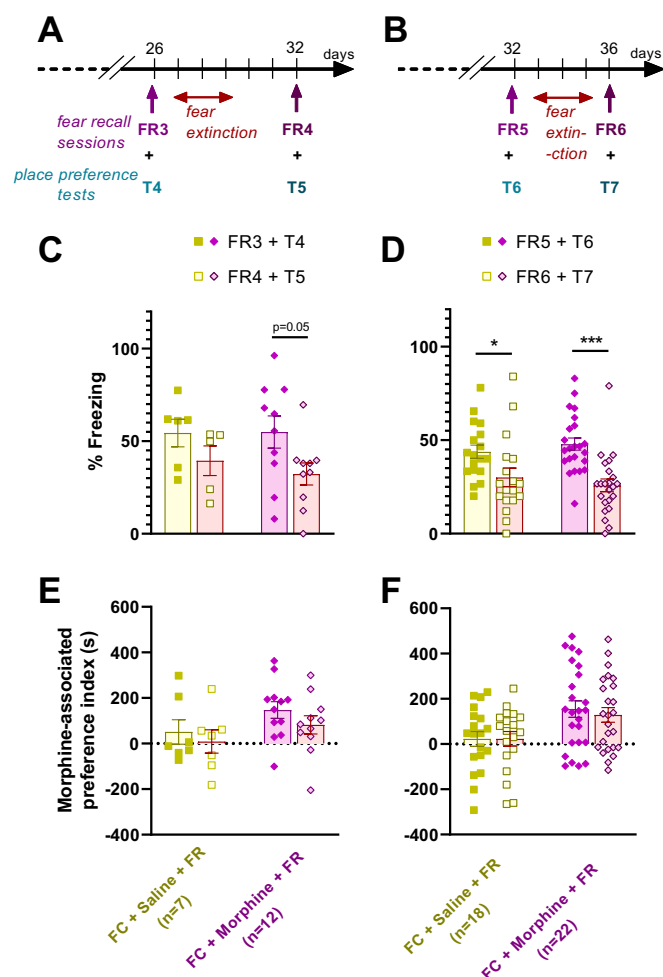
To explore the implication of CS-induced freezing behavior in the FR-induced persistent CPP, a fear extinction protocol was performed in mice after FR3 for one cohort (Fig. 3A), and after FR5 for another cohort (Fig. 3B). The fear extinction procedure was effective in all the groups: as presented in the supplemental Fig. 2 for a typical intra-session extinction, the percentage of time spent freezing decreased during the CS-extinction (time effect,  $p < 0.05$ , supp. Fig. 2A- B) and there was, as classically observed, an inter-session extinction between the first vs the second day of extinction (supp. Fig. 2C).

During the last FR session, freezing behavior was decreased when the fear extinction protocol was performed either at D27–29 ( $p = 0.05$ , FR4 compared to FR3 in FC + morphine+FR, Fig. 3C) or at D33–35 ( $p < 0.05$ , FR6 compared to FR5 in FC + Saline+FR group;  $p < 0.001$ , FR6 compared to FR5 in FC + morphine+FR group, Fig. 3D). However, FR-induced morphine place preference persistence was not modified by prior fear extinction (Fig. 3E, F).

To investigate the role of hormonal stress neurotransmission on FR-induced persistence of morphine-induced CPP, antalarmin, a CRFR1 antagonist, was used (10 mg/kg, 2 h before each FR session, Fig. 4A). Antalarmin modified neither the percentage of time spent freezing during FRs nor the amplitude of fear reconsolidation starting from FR2 (Fig. 4B, C). As shown earlier (Fig. 1D, F), morphine withdrawn mice subjected to FR exhibited significant FR-induced increase in morphine place preference index (significant time effect;  $p < 0.05$  on T2 and T3 compared to T1;  $p < 0.05$  on T3 and T4 FC + morphine+vehicle/FR compared to FC + saline+vehicle/FR, Fig. 4D). However, this was not the case in animals pretreated with antalarmin (no time effect),



**Fig. 2.** Effect of acute morphine injection and morphine withdrawal on anxiety. (A) Experimental schedule. Marble burying tests were done at (B) D4 (1 h after morphine), (C) D15 (20 h after morphine) and (D) D27 (24 h after FR3). Results are represented as mean  $\pm$  SEM. Two-way ANOVA for repeated measurements followed by Dunnett test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ : comparisons versus the FC + saline group).



**Fig. 3.** Fear extinction did not decrease the fear-recall-induced persistence of morphine conditioned place preference. (A, B) Experimental schedules of the two cohorts in which fear extinction (contextual extinction followed by  $2 \times 40$  cue-conditioned stimuli) was performed. Results are represented as mean  $\pm$  SEM. FC: Fear conditioning. Data obtained (C) during FR3 + T4 vs FR4 + T5 in one cohort, and (D) during FR5 + T6 vs FR6 + T7 in the second cohort. (C, D) Percentage time freezing (cue-conditioned stimulus - context) during FR before and after fear extinction. (E, F) FR-induced morphine place preference index persistence before and after fear extinction. Two-way ANOVA for repeated measurements followed by Sidak test (\* $p < 0.05$ , \*\*\* $p < 0.001$ ). The data in D and F are pooled from two similar cohorts.

indicating that CRFR1 antagonism prevents the FR-induced persistence of morphine-induced CPP. Overall, with the morphine-associated preference index average from T3 to T4, there were significant effects of FC + morphine-vehicle/FR group compared to other groups ( $p < 0.01$  vs. the FC + saline-vehicle/FR group;  $p < 0.05$  vs. the FC + morphine-antalarmin/FR group; Fig. 4E).

### 3.3. Molecular changes induced by contexts re-exposure

Molecular modulations in terms of both mRNA and microRNA expressions were then investigated. Prominent mRNAs of the endogenous opioid precursors and receptors and CRF/CRFR1 system were measured at different time points after FR- and CPP-associated contexts, in the PFC, the amygdala and the NAC, structures widely involved in fear, stress and addiction. Modulations of mRNAs are illustrated in Fig. 5 at FR1, and one week later in Fig. 6 at FR3, in both the amygdala and the PFC. No modulation of mRNAs was found in the NAC at either time points (supp. Fig. 3).

Concerning the potential morphine withdrawal effects at FR1, POMC mRNA expression was increased in the amygdala in all morphine withdrawn mice ( $p < 0.05$ , compared to their own control group, Fig. 5G). Similarly, MOR mRNA expression in the PFC was increased in the morphine+sound group ( $p < 0.01$ , compared to saline+sound group, Fig. 5J), an effect not found in the FC + morphine+FR group, suggesting that FR modified the withdrawal-induced expression of MOR in the PFC. Conversely, two mRNAs were found to be enhanced only in FC + saline+FR group compared to the saline+sound group - that is, pDYN in the amygdala ( $p < 0.05$ , Fig. 5F), and POMC in the PFC ( $p < 0.05$ , Fig. 5M). These effects were not observed in animals that were treated with morphine, subjected or not to FR. Finally, mRNAs coding for KOR and pDYN in the PFC appeared to be particularly relevant to the stress/addiction comorbidity. In the PFC, only the combination of fear and morphine context re-exposure enhanced KOR mRNA expressions (compared to FC + saline+FR mice  $p < 0.05$ , or morphine+sound mice  $p < 0.01$ , Fig. 5I) and pDYN (compared to FC + saline+FR mice  $p < 0.05$ , or morphine+sound mice  $p < 0.01$ , Fig. 5L). These data suggest that FR increases dynorphin/ $\kappa$  opioid system transmission only in morphine withdrawn mice.

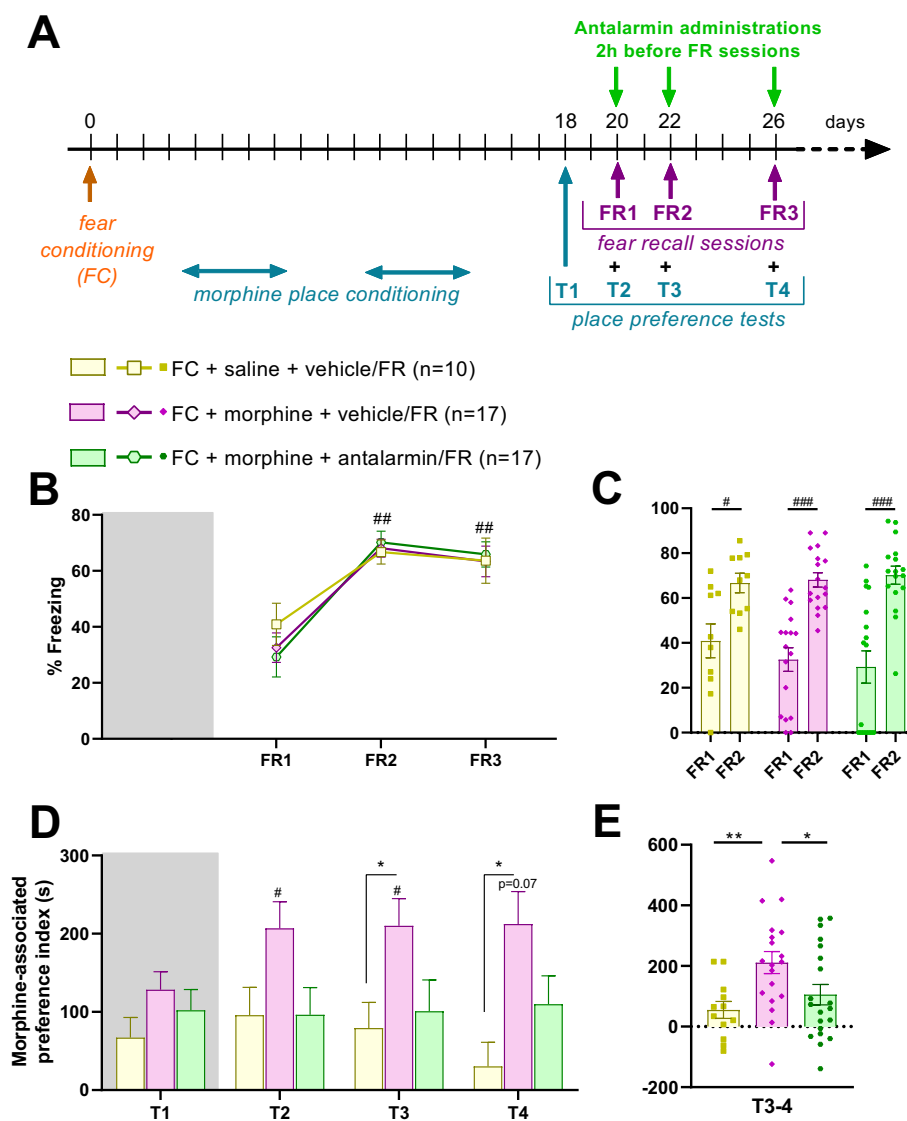
About a week later, although fear memory was well reconsolidated (Fig. 1B) and the persistence of drug-induced CPP remained apparent (Fig. 1C-D), the above mRNA modulations were absent (Fig. 6). After this time span, only KOR mRNA expression appeared enhanced in the FC + saline+FR group ( $p < 0.05$ , compared to the saline+sound group, Fig. 6I), suggesting that morphine withdrawal inhibited this FR3-induced mRNA modulation. Chronic intermittent re-exposure to fear and morphine associated contexts could trigger other long-term molecular changes involving microRNAs.

NGS was used to evaluate microRNAs expression after the third fear and morphine contexts re-exposure (D26, Fig. 7A) in the amygdala of animals that exhibited FR-induced persistence of morphine-induced CPP test (CPP index at FR3:  $4 \pm 11$  s morphine group vs.,  $136 \pm 24$  s morphine+FR group,  $n = 3-4$ ,  $p < 0.001$ ). NGS allowed detecting 579 distinct microRNAs. Pairwise comparisons indicated numerous microRNAs modulated by the combination of the conditioned sound and morphine associated context re-exposure compared to naive+sound animals (Fig. 7B; only values above 1 Log2 fold change are illustrated, 81 were up-regulated, 53 were down-regulated, adjusted  $p$  values are listed in supp. Table 2) and compared to morphine-controls (Fig. 7C; 66 were up-regulated, 52 were down-regulated,  $p < 0.05$  to  $p < 0.0001$ , adjusted  $p$  values are listed in supp. Table 3). None of the fold changes were particularly large but many microRNAs of the let-7 family were significantly modulated (in view of either their  $p$  values, or adjusted  $p$  values). One-way Anova revealed several intergroup differences in this particular family (supp. Fig. 4A-E). Other microRNAs implicated in addiction- and opioid-related disorders, according to an ingenuity pathway analysis (IPA) are indicated with red arrows on Fig. 7B-C or illustrated as histograms in supplementary Fig. 4. These results therefore suggest that chronic intermittent fear- and morphine-associated contexts re-exposure induce several long-term microRNA modulations in the amygdala, a structure well known to be involved in fear, but also in addiction.

## 4. Discussion

Altogether our study describes a model of the PTSD/OD comorbidity associated with concomitant molecular mechanisms regulations. It shows for the first time that a traumatic-like fear memory can trigger persistent drug-seeking behavior. In our conditions, morphine-induced CPP did not reach significance on baseline, but amplitudes of CPP scores were markedly increased by stress, starting from the second FR.

Fear-conditioning in rodents has been shown to enhance acute morphine dependence (Abrahamsen et al., 1995). In contrast, in our model, FC itself, delivered days before, did not produce any effect on place preference acquisition, but FR delivered immediately before, did



**Fig. 4.** Effect of antalarmin on fear recall-induced persistence of morphine-induced CPP. (A) Experimental schedule. Results are represented as mean  $\pm$  SEM. (B) Percentage time freezing during FR sessions. Two-way ANOVA for repeated measurements followed by Sidak test ( $^{##}p < 0.01$ : comparison versus FR1). (C) The freezing data distribution of FR1 vs FR2. (D) Morphine associated preference index on T1, and thereafter (FR + T2–4) after antalarmin or vehicle administration 2 h prior FR. Two-way ANOVA for repeated measurements followed by Sidak test ( $^{#}p < 0.05$ : comparison versus T1) or Dunnett test ( $^{*}p < 0.05$ ). (E) Morphine-associated preference index average from T3 to T4. One-way ANOVA followed by Dunnett test ( $^{*}p < 0.05$ ,  $^{**}p < 0.01$ ).

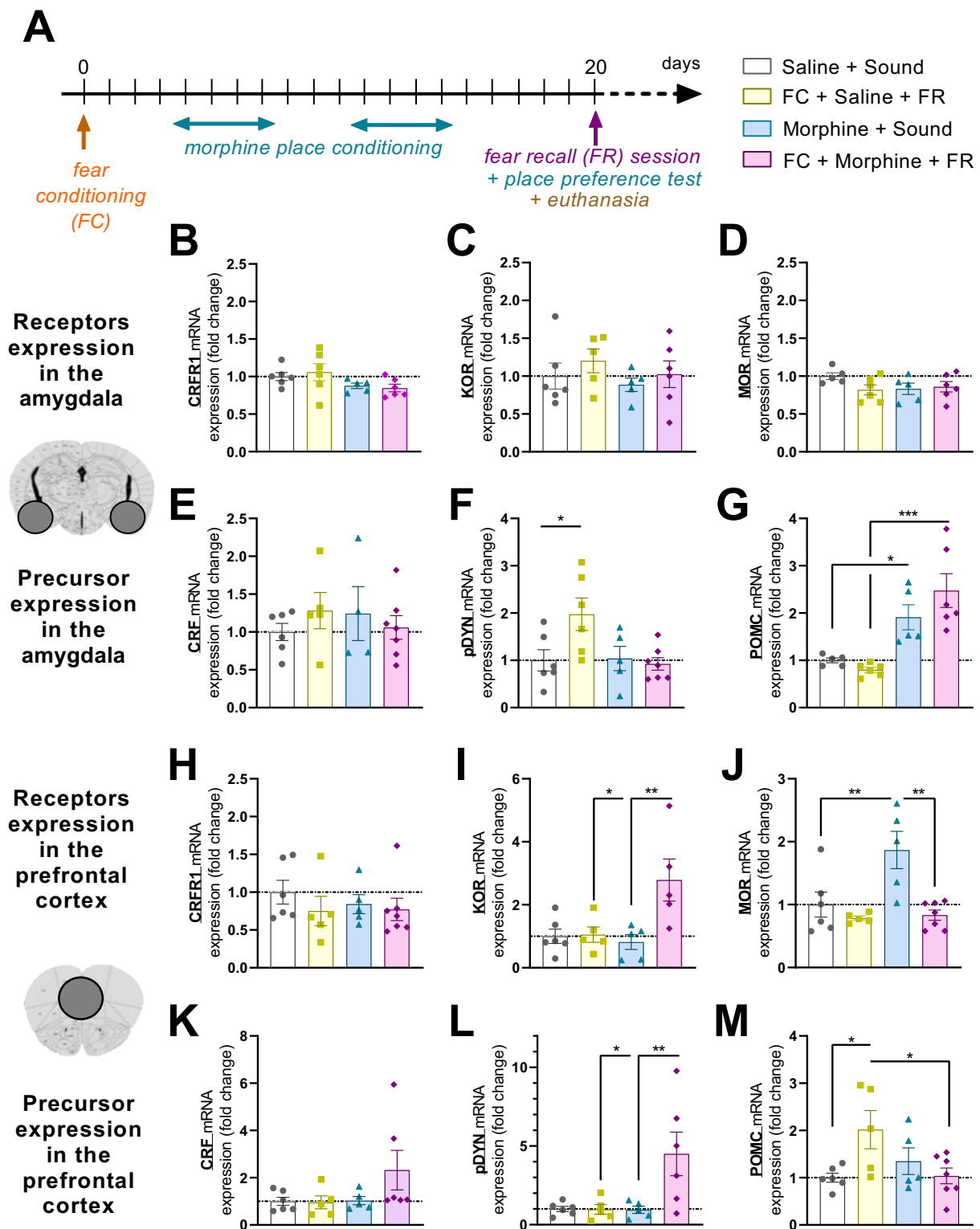
increase CPP expression. Clearly, a stress reminder is an important factor influencing addiction symptoms. It has indeed been previously shown that mice subjected to a strong foot-shock or FC + FR prior CPP exhibited a significantly increased acquisition of morphine CPP compared to mice subjected to FC alone (Jing Li et al., 2018; Yu et al., 2025). By contrast, in our conditions, FR alone was applied after CPP acquisition, i.e., during drug withdrawal, and the morphine-CPP was revealed significant and rendered persistent after FR exposure. The subsequent question addresses how FR exerts its impact during drug withdrawal.

As previously shown with the marble burying test in the literature, acute morphine administration induces an anxiolytic-like effect (Umathe et al., 2012; Kitanaka et al., 2015), and early morphine withdrawal (8–48 h) produces an anxiogenic-like effect (Umathe et al., 2012). In the present study, morphine withdrawal produced an anxiogenic-like effect even 13 days after drug withdrawal. Previous sessions of FC or FR days or hours before did not have any additional anxiogenic-like effect. We can thus conclude that treated mice were tested under a mild anxiety state produced essentially by morphine

withdrawal.

FR exposure delivered immediately before the CPP tests triggered a fear-memory associated stress, as indicated by freezing behavior during FR sessions. Cue-induced freezing got greater by the second FR, appearing as an apparent memory reconsolidation. The brief presentation of the cue-CS could transiently put back the associative memory in a labile state, sensitive to perturbations that can induce a reconsolidation process instead of a fear extinction when re-exposure to context is prolonged (Vaverková et al., 2020). We then investigated whether conditioned freezing plays a central role in the stress-induced persistence of morphine CPP. A standard fear extinction protocol was carried out and significant intra-session and inter-session extinctions of conditioned freezing were observed. When mice were placed back into the initial FC context, some (near 30%) freezing was still observed in response to another FR session, but this corresponds to significantly less conditioned freezing compared to the previous FR session. Nevertheless, despite the reduction of freezing, we failed to observe any reduction in the amplitude of the drug-induced CPP.

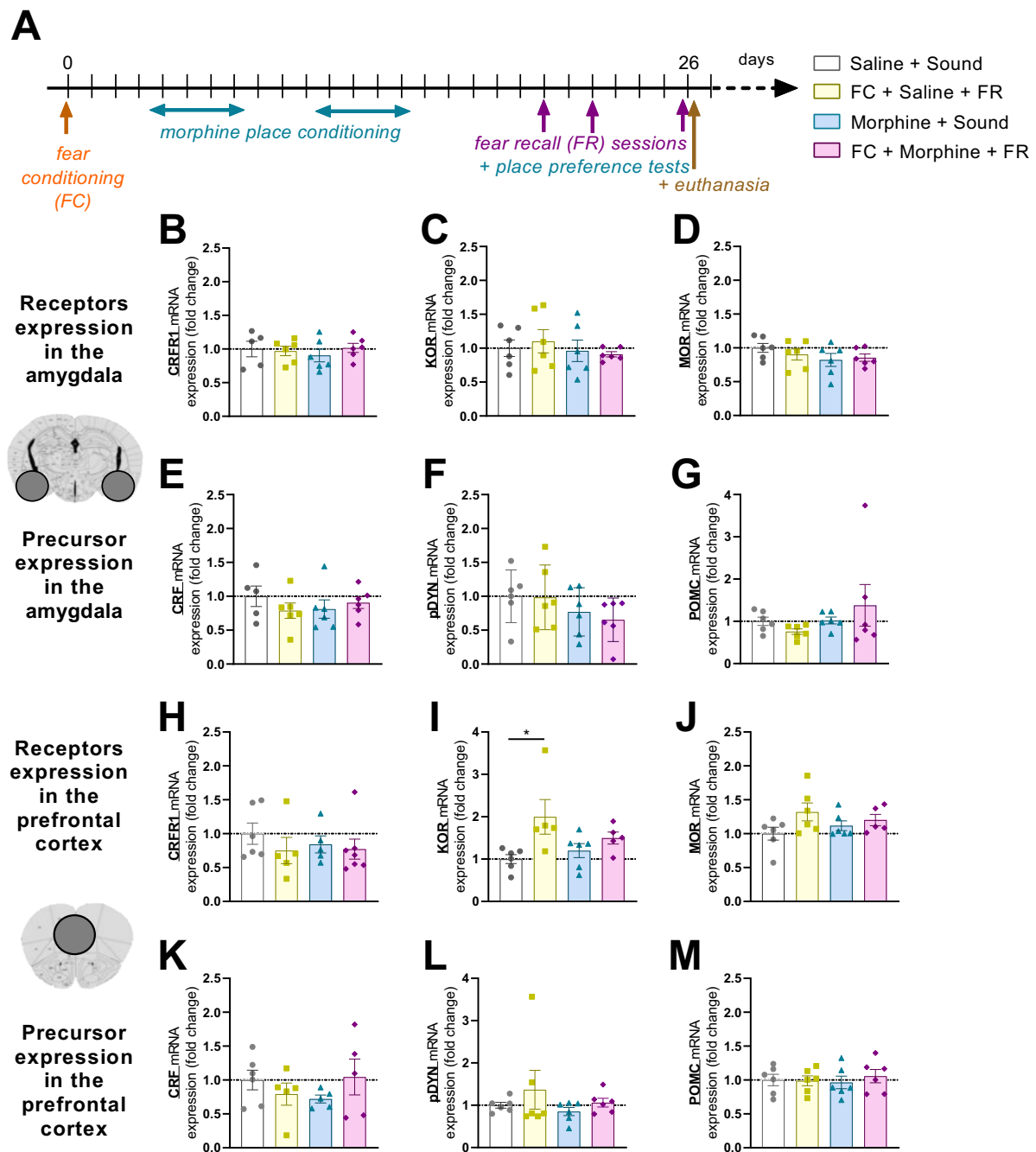
Thereafter, to suppress some of the hormonal stress responses, the



**Fig. 5.** Effect of a single fear- and morphine-associated contexts re-exposure on stress- and opioid-related mRNA expression. (A) Experimental schedule. Brains were collected 30 min after a single fear recall (FR1) + CPP test (T2) at D20. Results are represented as mean fold change  $\pm$  SEM. Amount of mRNA relative to the saline+sound group for (B, E, H, K) CRF and CRFR1, for (C, F, I, L) pDYN and KOR and for (D, G, J, M) POMC and MOR; in (B-G) the amygdala and (H-M) the PFC. One-way ANOVA followed by Sidak post-hoc test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

effect of a CRFR1 antagonist was tested. Administration of antalarmin before each FR session prevented the FR-induced enhancement of the CPP performance. However, as previously reported (Deak et al., 1999; Hubbard et al., 2007), blocking CRFR1 had no effect on cue-conditioned freezing or its reconsolidation. It therefore appears that the stress-

induced increase in morphine-CPP is not dependent on fear expression, at least that expressed through freezing, but maybe nevertheless dependent on CRFR1 activation. Our results are in line with the literature showing a role of CRF via CRFR1 in reducing escalated drug-taking behavior and adverse stress-like withdrawal symptoms in rodents



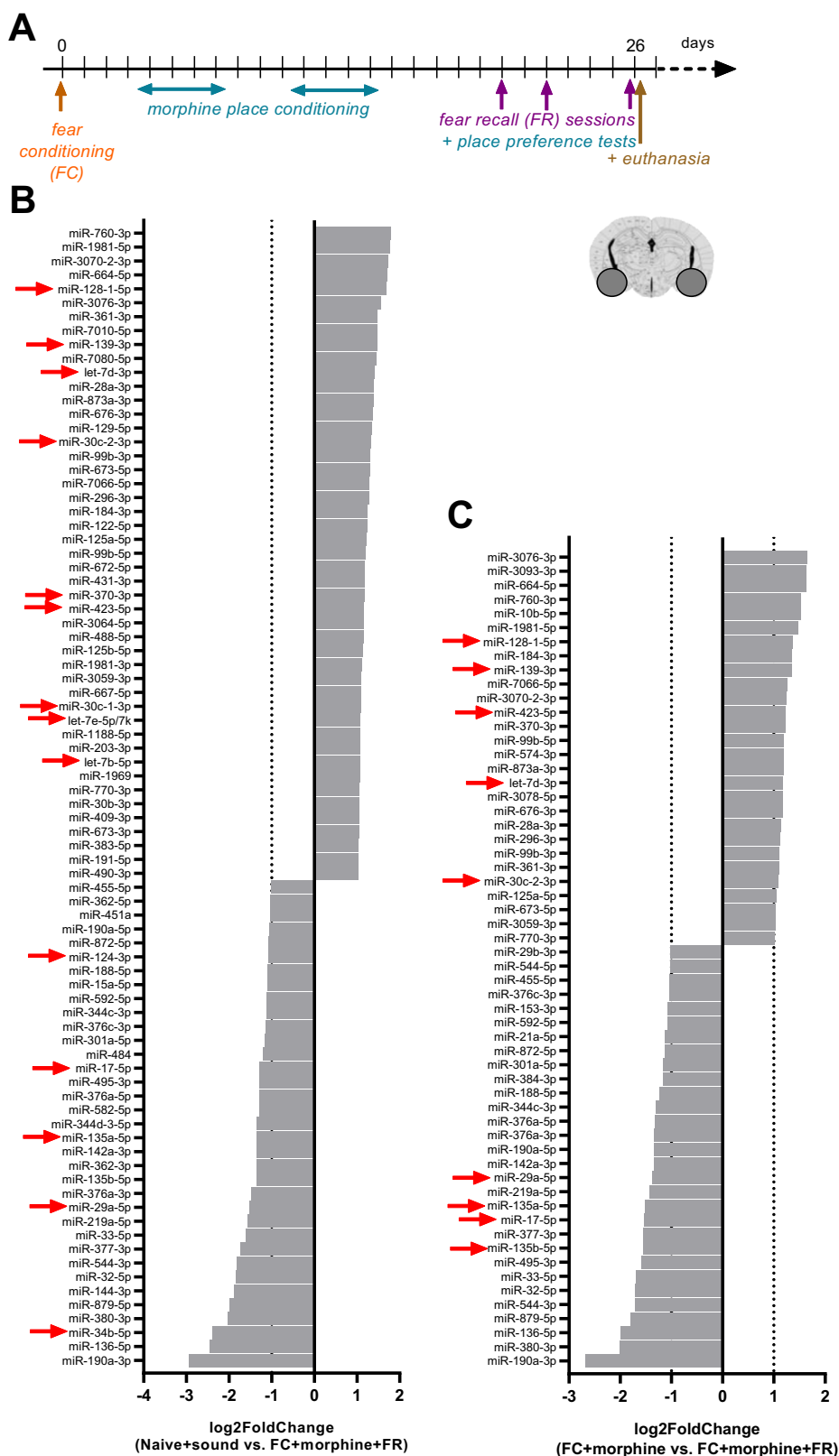
**Fig. 6.** Effect of repeated fear- and morphine-associated context re-exposure on mRNA expression. (A) Experimental schedule. Brains were collected 30 min after FR3 + T4 at D26. Results are represented as mean fold change  $\pm$  SEM. Amount of mRNA relative to the saline+sound group for (B, E, H, K) CRF and CRFR1, for (C, F, I, L) pDYN and KOR and for (D, G, J, M) POMC and MOR; in (B-G) the amygdala and (H-M) the PFC. One-way ANOVA followed by Sidak post-hoc test (\* $p < 0.05$ ).

(Shaham and De Wit, 2016).

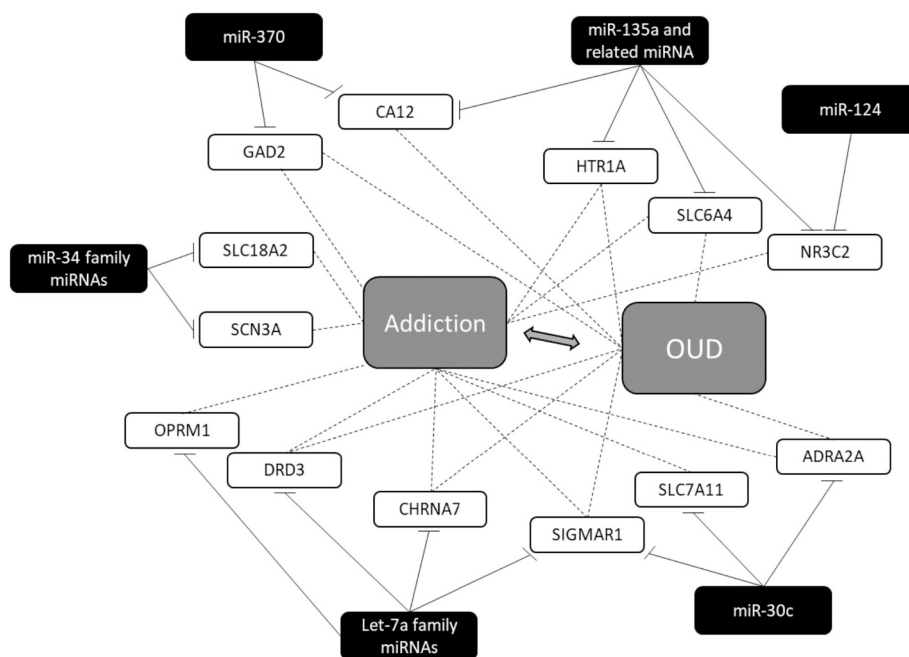
As mentioned above, the mPFC is crucial for the lability of both (i) fear memory and (ii) compulsive substance abuse behaviors, through its projections to respectively, (i) the amygdala and (ii) the NAc (Akirav and Maroun, 2007; Perry et al., 2011). Furthermore, the amygdala has been shown to be crucial for the fear-associative memory lability and consequently, reconsolidation (Ferrara et al., 2023), and conditioned freezing through its central nucleus and projections to the periaqueductal gray area (Keifer et al., 2015), and the hormonal stress response via the paraventricular nucleus of the hypothalamus (Herman et al., 2016). Thus, CRF precursor and CRFR1 mRNA expressions were measured in the amygdala, the PFC and the NAc, but no change in response to morphine context re-exposure or FR were observed in our

model. Nevertheless, in a preliminary study (Leconte et al., 2023; with 4 days of morphine conditioning), levels of CRF mRNA were found to be increased in the hypothalamus after either morphine- or FR-associated context exposure.

Interestingly, we found that the FR1 + T2 session increased KOR and pDYN mRNA in the PFC of morphine-withdrawn mice. This change in morphine withdrawn mice did not occur, however, in the amygdala, where there was instead an increase of pDYN in saline treated mice exposed to FR. A molecular interaction between CRF and DYN/KOR system may lead to behavioral adaptations to stress. For example, chronic stress could dysregulate KOR signalling via a p38 MAPK-dependent mechanism, suggesting that CRF can influence other signalling pathways connected to KORs during stressful conditions (Lemos



**Fig. 7.** Association of micro-RNA expression modulations with the persistence of morphine-induced CPP. (A) Experimental schedule. Brains were collected 3 h after FR3 + T4 at D26. Results are represented as mean fold changes, adjusted  $p$  values  $<0.05$  (data analysis and statistics performed with the miRge3.0 and DESeq2). (B–C) MicroRNA-seq results are represented only for micro-RNAs showing a significant increase superior to 1, or a significant decrease inferior to  $-1$  log 2 fold change, compared to (B) the naive-sound group and (C) the FC-morphine group. Arrows indicate microRNAs relevant to addiction or OUD according to the Ingenuity Pathway Analysis.



**Fig. 8.** Potential targets of some of the significant microRNAs using the Ingenuity Pathway Analysis. All predicted interactions have been experimentally confirmed. Targets are in majority receptors, DRD3: Dopamine Receptor D3, CHRNA7: Neuronal Acetylcholine Receptor Subunit 7, SIGMAR1: Sigma Non-Opioid Intracellular Receptor 1, ADRA2 A: Alpha-2A Adrenergic Receptor, SLC18A2: NR3C2: Mineralocorticoid Receptor, HTR1A: 5-HT-1A Receptor, OPRM1: Opioid Receptor Mu 1, and transporters, SLC7A11: Cystine/Glutamate Transporter, SLC18A2: Vesicular Monoamine Transporter 2, SLC6A4: Serotonin Transporter. There are also some enzymes, GAD2: Glutamate Decarboxylase 2, CA12: Carbonic Anhydrase 12, and a channel, SCN3A: Sodium Voltage Gated Channel Subunit.

et al., 2012). Others suggested that CRF-related pathways can induce dynorphin release, thereby implicating KOR signalling in stress-induced reinstatement of addiction (Funk et al., 2014). The DYN/KOR system is involved in addiction (Karkhanis et al., 2017), PTSD (Lanius et al., 2018) and their comorbidity (Leconte et al., 2022). Furthermore, KOR antagonism in rodents inhibits the stress-induced sensitization of cocaine reward and cocaine reinstatement (McLaughlin et al., 2003; Land et al., 2009). One role of KOR in the PFC is to trigger anxiogenic-like responses in rodents directly through its projection to the basolateral amygdala (Tejeda et al., 2015). Therefore, in view of our results and the beneficial effects of buprenorphine (KOR antagonist and MOR partial agonist) in substitution treatment for opioid disorders and in PTSD (Lake et al., 2019), testing such a compound in our model may be a valuable future strategy.

A week later, at FR3, the changes in KOR signalling were absent in the PFC of FR-exposed morphine withdrawn mice. Indeed, there was instead an increase of KOR in the PFC of saline treated mice that did not exist in morphine treated mice. In a preliminary study, we had previously observed the same pattern of modulations in the hippocampus, but after the first FR (Leconte et al., 2023).

As others before us, we observed that opioid withdrawal enhances *mu* opioid system mRNA expressions. Our data showed a withdrawal-induced increase in POMC expression in the PFC and in the amygdala, together with an increase in MOR mRNA expression in the PFC. Others had reported in rodents an increase in MOR expression after morphine withdrawal in the lateral hypothalamus, NAc and caudate putamen (Zhou et al., 2006), or after oxycodone withdrawal in the hippocampus (Blackwood et al., 2019). Surprisingly, but in line with a clinical study (Hsu et al., 2015), this withdrawal response appeared to be inhibited in the PFC by prior fear-associated context re-exposure, suggesting that MOR signalling could be differentially modulated in our comorbidity model, and this may depend on previous stress exposures.

As long-lasting molecular changes could explain the FR-induced persistence of CPP, we turned our attention on microRNAs expression. NGS performed after the third re-exposure to fear and morphine contexts revealed numerous microRNAs expression being either up- or

down-regulated in the amygdala (supp. Fig.4). There was no striking modulation in terms of fold change, but the IPA analysis indicated that many of these microRNAs had been previously involved in addiction or opioid-related disorders, as well as in stress (Figs. 7 and 8). Many were of the let-7 family: let-7a-1-3p, let-7b-5p, let-7e-5p, let-7d-3p, let-7c-5p and let-7i-5p. Considering that the latter had long been implicated in opioid addiction (He and Wang, 2012) and in many forms of addiction (Bahi and Dreyer, 2020; Chavoshi et al., 2020), it is not surprising to observe significant changes in 6 out of 10 members of the let-7 family in our conditions. For instance, an increase in let-7b has been noted in the plasma of heroin-dependent individuals (Liu et al., 2021). Interestingly, it has been reported that there is a direct interaction between many let-7 microRNAs and the mRNA coding for MOR, and a higher concentration of let-7 can lead to a decrease in MOR density (He and Wang, 2012). Furthermore, let-7i is predicted to interact with KOR, according to the Targetscan database (Mouse Targetscan 8.0. [www.targetscan.org](http://www.targetscan.org)).

Many other microRNAs stand out from this IPA analysis (Fig. 8): miR-124, one of the most abundant microRNA in the brain, was shown to be a modulator of immunosuppression after chronic morphine treatment (Qiu et al., 2015), and as let-7d, a modulator of cocaine CPP expression, extinction and reinstatement (Chandrasekar and Dreyer, 2011). The 3 members of the miR-34 family (miR-34 a,b,c) were shown to interact with CRFR1 mRNA and have a major influence on stress-related amygdala neuroplasticity (Andolina et al., 2016, 2018). Furthermore, miR-128 has been shown to be implicated in addiction (Li et al., 2021). It is known to regulate neuronal excitability and has been demonstrated to be an important factor in contextual fear extinction (Lin et al., 2011). It is also abnormally expressed in the brain in response to cocaine or methamphetamine administration (Hollander et al., 2010; Li et al., 2021). Several others have been implicated in stress and depression including miR-135, miR-139 and miR-423 (Chen et al., 2015; Wei et al., 2020; Yoshino et al., 2021; Mingardi et al., 2023).

As illustrated in Fig. 8, many microRNAs identified in our model are involved in key neurotransmitter systems, such as dopamine or GABA, as well as in the regulation of neuronal excitability and stress responses. Among the receptors of this long list of genes we can mention ADRA2

and HTR1A (linked to miR-30c and miR-135a, respectively) that are interesting as both 5-HT1A- and  $\alpha$ 2-autoreceptors located on raphe and locus coeruleus (LC) neurons and terminals, respectively, have increased sensitivity after morphine treatment and withdrawal (Sastre-Coll et al., 2002). Alpha2-adrenergic receptors and LC projections to the amygdala are crucial for fear memory consolidation (Davies et al., 2004; Haubrich et al., 2020) while morphine treatment has an inhibitory effect on LC firing rate that has a key role in the expression of reward-related behavioral manifestations including opioid seeking and withdrawal states (Van Bockstaele et al., 2010; Farahani et al., 2023). Noradrenergic ascending terminals in the amygdala illustrates one of many sites where CRF and noradrenaline, in stress, intersect with  $\mu$ - and  $\kappa$ -opioid systems mediating vulnerability to morphine abuse (Van Bockstaele et al., 2010). Incidentally, treatment with the  $\alpha$ 2-adrenergic receptor agonist clonidine may benefit addiction-PTSD comorbidity patients (Gold et al., 2024; Marchi et al., 2024) and may be studied with animal models such as the one developed here.

## 5. Conclusions

Our study presents a novel preclinical model of comorbid PTSD and OUD behaviors, demonstrating for the first time that re-exposure to a fear-associated cue and context after morphine withdrawal can reveal persistent morphine-CPP. The anxiety-like effect of morphine withdrawal would be compounded by the traumatic-like fear memory. This effect appears dependent on CRFR1 neurotransmission, but did not correlate with how much conditioned freezing behavior mice expressed in reaction to each FR.

We identified concomitant biological regulations of the stress-induced increase of CPP and found interesting changes in fronto-cortical and amygdalar DYN/KOR and endorphins/MOR opioid systems, although none appeared to be consistently present across both FR1 and FR3 sessions. Finally, NGS data identified altered expression of several microRNAs in the amygdala after fear and morphine context re-exposure, suggesting that multiple molecular mechanisms may explain the persistent drug-induced CPP observed in our model. Many are parts of integrated neurotransmitter pathways known to be involved in either addiction or opioid-related disorders. Note, however, that sexually dimorphic therapeutic targets identified here (such as KOR) were derived from male mice only and that future investigations in females will be valuable, especially as studies suggest that women may be at greater risk of developing OUD compared to men (Davis et al., 2021; Leconte et al., 2022).

Keeping in mind that the treatment of PTSD – opioid addiction is difficult to codify, and no pharmacological or behavioral treatment used for one pathology alone is effective enough in the SUD/PTSD comorbidity (Shorter et al., 2015; Flanagan et al., 2016), this present model, focusing on the persistence of morphine withdrawal symptoms in the context of a conditioned stress, may be valuable to evaluate new strategies to treat this comorbid situation.

## Ethical statement for solid state ionics

Hereby, I Claire Leconte consciously assure that for the manuscript, “Traumatic-like fear memory recall causes persistent morphine seeking behavior in drug withdrawn mice” the following is fulfilled:

- 1) This material is the authors' own original work, which has not been previously published elsewhere.
- 2) The paper is not currently being considered for publication elsewhere.
- 3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
- 4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
- 5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

The violation of the Ethical Statement rules may result in severe consequences.

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I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

## CRediT authorship contribution statement

**Claire Leconte:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Virginie Beray-Berthaut:** Writing – review & editing, Validation, Methodology, Funding acquisition, Data curation, Conceptualization. **Fanny Saulnier:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Derin Reha Ulusoy:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Amanda Patricia Sales:** Data curation. **Matilde Donzelli:** Data curation. **Laura Marques:** Data curation. **Isabelle Nondier:** Methodology, Data curation. **Corinne Canestrelli:** Data curation. **Florence Noble:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Raymond Mongeau:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

All authors declare that they have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pnpbp.2026.111611>.

## Data availability

Data will be made available on request.

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