KAPPA OPIOID RECEPTOR BLOCKADE IN NAc SHELL PREVENTS SEX-DEPENDENT ALCOHOL RELAPSE-LIKE BEHAVIOUR INDUCED BY INFLAMMATORY PAIN.

Jesús D. Lorente, Javier Cuitavi, Yolanda Campos-Jurado, Raquel Montón-Molina, Jose L. González-Romero, Lucía Hipólito[#].

Department of Pharmacy and Pharmaceutical Tech. and Parasit. University of Valencia, Spain

Corresponding author:

Lucia Hipólito,

Department of Pharmacy and Pharmaceutical Technology and Parasitology

Avda. Vicente Andrés Estellés s/n 46100 Burjassot, Valencia, Spain

Lucia.hipolito@uv.es

PHONE: (0034) 963544914

FAX: +34 96 3544911

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Abstract:

Background and Purpose

Pain-induced negative affect reduces life quality of patients by increasing psychiatric comorbidities, including alcohol use disorders (AUD). Indeed, clinical data suggest pain as a risk factor to suffer AUD, predicting relapse drinking in abstinent patients. Here, we analyse the impact of pain on alcohol relapse and the role of kappa opioid receptors (KOR) activation in mediating this pain-induced effects since KOR play an important role in pain-driven negative affect and AUD.

Experimental approach

Female and male Sprague Dawley rats underwent to two alcohol intermittent access periods separated by a forced abstinence period. The complete Freund adjuvant (CFA) model of inflammatory pain was introduced during abstinence and alcohol intake after alcohol reintroduction was assessed. Additionally, we used behavioural approaches to measure stress and memory impairment and biochemical assays to measure KOR expression in abstinence and reintroduction periods. Finally, KOR antagonist norbinaltorphimine (norBNI) was administered in the nucleus accumbens shell (NAcS) during abstinence to prevent pain-induced alcohol relapse-like phenomenon in CFA-female rats.

Key results

Only female CFA-treated rats increased alcohol intake during reintroduction period. Concomitantly, this group showed enhanced stress-like behaviour and increased KOR expression in the NAcS that was developed during abstinence and remained during reintroduction period. Finally, norBNI administered in the NAcS prevented pain-induced alcohol relapse-like behaviour in female rats.

Conclusions and implications

Our data evidenced that inflammatory pain constitutes a risk factor to relapse only in female rats, by the arise and maintenance of stress probably mediated by kappa opioid receptor (KOR) signaling in the NAcS.

Keywords: addiction, neuropharmacology, pain, kappa opioid receptor, nucleus accumbens, alcoholism, gender.

Bullet point summary

What is already known

- Failure of analgesic treatment is a predictor of alcohol relapse in abstinent patients
- Pain but also alcohol chronic exposure induces Dynorphin/KOR alterations in the NAcS.

What this study adds

- Inflammatory pain induces alcohol relapse-like behaviour only in female rats.
- Nor-BNI administered in NAcS impairs inflammatory pain induced alcohol relapse-like behaviour in female rats.

Clinical significance

- History of AUD should be monitored in pain patients, especially in women.
- KOR antagonists might be useful to prevent negative affect and alcohol relapse in pain patients.

1. Introduction

Chronic pain patients commonly suffer co-morbid psychological disorders as anxiety, stress and motivation loss that impact their quality of life (Cahill et al., 2014; Csupak et al., 2018; Tsang et al., 2008). Together with these psychopathological situations, clinical reports have revealed that around 38% of chronic pain patients consume high quantities of alcohol (Brennan et al., 2005; Riley & King, 2009). This behavioural maladaptive coping strategy puts these pain patients in risk of developing an alcohol use disorder (AUD) or, in the case of abstinent patients, raises the risk of relapsing (Brennan et al., 2005; Jakubczyk et al., 2016). Indeed, the intensity and the poor management of the painful situation correlated with a higher craving and risk of alcohol relapse (Boissoneault et al., 2019; Jakubczyk et al., 2016; Paulus et al., 2019; Vowles et al., 2018).

Additionally, pain syndromes are very common in alcohol abusers especially during abstinence (Brennan et al., 2005; Egli et al., 2012; Jakubczyk et al., 2016), converting the suffering of physical pain into a relevant risk factor of alcohol relapse specifically in these patients. In the last years, some preclinical approaches have analysed the effect of pain in alcohol intake by using rodent models of pain. In the majority of these reports, the presence of pain increased alcohol intake in a free bottle choice paradigm (Butler et al., 2017; Yu et al., 2019). More interestingly, this increase was reported for male mice and not for female mice, indicating that pain may impact alcohol drinking behaviour in a sex-dependent manner. Although clinical data point to pain as a factor that may induce alcohol relapse, only one preclinical research have actually focused on the relapse phase, probably because of the lack of appropriate rodent models

(Campos-Jurado et al., 2020). Additionally, most part of preclinical studies have not initiated the investigation of the neurobiological substrates underlying these pain-induced changes in alcohol-drinking behaviour.

Research has pointed to pain-induced alterations in dopamine signaling in the mesocorticolimbic system (MCLS) as key event that finally conduces to changes in motivation and in patterns of drug intake (Hipolito et al., 2015; Massaly et al., 2019). In fact, recent data revealed that the activation of dynorphinergic neurons in the nucleus accumbens (NAc), which controls local dopamine release (Karkhanis et al., 2016; Rose et al., 2016), is sufficient to cause inflammatory pain-induced loss of motivation (Massaly et al., 2019). Based on the involvement of dynorphin/kappa opioid receptors system (Dyn/KOR) in the neurobiological effects of alcohol (Karkhanis et al., 2016; Rose et al., 2016), it is feasible that the observed pain-induced hyperactivity of this system in the MCLS might also impact alcohol drinking behaviour and/or relapse. In this case, antagonising the KOR in the MSCL might constitute a target to impair pain-induced alcohol relapse (Lorente et al., 2020). In the light of these previous data, our research develops a novel combination of inflammatory pain and alcohol relapse rat models in males and females with two aims: to analyse the effect of pain on alcohol relapse-like behaviour in males and females and to initiate the analysis of the involvement of the Dyn/KOR in the pain-AUD interaction with the aim of identifying a successful anti-relapse pharmacological approach.

2. MATERIALS AND METHODS.

2.1. Animals.

Sprague Dawley rats, 90 females and 57 males, were used (Envigo®, Spain). Animals were kept in light/dark controlled cycles (12/12h, light on at 10:00 p.m., temperature 23 ± 1ºC, and 60% humidity). All behavioural tests were conducted during the dark cycle. Each animal was individually housed in standard plastic cages (42×27×18 cm^3) provided with shredded aspen bedding (Teklad, Spain) and cotton enrichment (iso-BLOXTM;Teklad). Food and tap water were available ad libitum throughout the experimental period. The protocols used were approved by the Animal Care Committee from University of Valencia, authorised by the regional government and were carried out in strict accordance with Spanish laws (RD 53/2013) and European Directive (EC 2010/63).

2.2 Experimental design

2.2.1 Experiment 1: inflammatory pain effect on alcohol relapse-like behaviour, stress, long-term memory and KOR expression in NAc, PFC and Amygdala.

Ethanol intermittent access model and pain induction

In this experiment, animals followed the classical ethanol intermittent access (IA) model as previously explained (Carnicella et al., 2014). In this procedure, rats had free access to 20% ethanol along with water three times a week for 24h, followed by 24 or 48h of non-access to ethanol. Fresh ethanol solution and water were always used. Ethanol bottles were introduced at 10:00 a.m., right after the lights turned off, each Monday, Wednesday and Friday and removed

24h later at the same time. The bottles were weighed before and after their introduction to measure total fluid intake and alcohol intake and rats were weighted every day before the introduction of the bottles to calculate ethanol and water consumption in g/kg/day. Alcohol intake data that were higher than 20 g/kg/day were excluded since this really high intake could be a result of a leaking from the bottle. Previous reports using this IA protocol have never show intake levels higher than 15 g/kg/day. When observed more than 20 g/kg/day, bottles were substituted for new bottles to prevent further leakage issues. The number of alcohol consumption data excluded was 15 of the 1479 consumption data from the experiment 1 (1.01% of the data obtained) and 7 of the 783 consumption data from the experiment 2 (0.89% of the data obtained). Besides, in order to assure that the alcohol consumption was not due to side preferences, the order of the water and ethanol bottles was alternated each time alcohol was introduced. This procedure was followed during the acquisition period of seven or eight weeks to obtain basal ethanol consumption data from each rat. After that, alcohol was removed for three weeks to force a period of abstinence (figure 1A).

The first day of the third week of abstinence, animals received 0.1 ml subcutaneous injection of the Complete Freund Adjuvant (CFA, Calbiochem) or sterile saline in the plantar surface of the hindpaw (Hipolito et al., 2015). The animal model of pain based in the administration of CFA has been broadly used for replicating human aspects of arthritis (Fischer et al., 2017). Following the three weeks of forced abstinence, alcohol was reintroduced in half of the animals following the same IA procedure previously described. Rats underwent five sessions of IA during the reintroduction period and 24 hours after the last

session animals were sacrificed by isoflurane overdose (for western blot analyses) or by perfusion (for immunohistochemical analyses). Besides, rats belonging to the abstinence groups were sacrificed after completing 3 weeks of abstinence, on the day when alcohol was supposed to be reintroduced (figure 1A).

Experimental groups

In experiment 1, 119 rats (57 male and 62 female) were used. The experiment was carried out in two different batches including all experimental groups to confirm the reproducibility of the data. According to the IA protocol, male and female rats were randomly assigned to one of the following experimental groups: Control (n= 10-12 /group, rats that only had access to water and did not follow IA schedule); CFA-A (n= 10-13/group, pain rats that were sacrificed immediately after abstinence period); CFA-R (n= 10-13/group, pain rats sacrificed after reintroduction period); SAL-A (n= 12-13/group, no-pain rats sacrificed immediately after abstinence period); SAL-R (n= 13/group, no-pain rats sacrificed after reintroduction period).

Mechanical nociception assessment

Mechanical nociception thresholds were measured by Von Frey test to ensure that a low mechanical nociception was maintained during all the protocol. The protocol started with a habituation period of 20 min to the behavioural boxes and the room were the test was performed. After this habituation period, we manually applied five filaments (Aesthesio®) following a simplified up-down method, as previously described (Bonin et al., 2014). The first Von Frey session was performed just before the CFA or saline injection (Monday of the third week

of abstinence) to obtain the basal mechanical nociception for each rat. The following sessions were performed every Thursday from the third week of abstinence period at least 3 hours after alcohol bottles were removed from cages to avoid the possible pharmacological action of the ethanol (see Figure 1A). Results were expressed in grams (g).

Stress and memory impairment behavioural tests.

Only female rats from the second batch were used for the stress and long-term memory impairment experiments. Abstinence groups were tested 2, 4 and 6 days after CFA or saline injection with the light-dark box (LDB) test and the novel object recognition (OR) test, while reintroduction groups were tested 2, 4 and 6 days after the alcohol reintroduction (Figure 1E).

Light-dark box test.

The apparatus consists in a box (64cm x 48cm x 24cm) divided in two different chambers: light chamber (two-thirds of the total size) and dark chamber (one third of the total size). The chambers are connected by a squared opening (8 cm x 8 cm). The light chamber has white walls and it is uncovered and illuminated by a 60-lux light, and the dark chamber has black walls and it is covered and with no appreciable illumination (i.e. < 2 lux). For the test, rats were placed at the centre of the light box, with the head facing opposite to the squared opening, and then the rats freely explored the chambers during 5 minutes (figure 1E). All the animals were recorded in the light box during the test for further analysis. The test was performed during the dark cycle at least 3 hours after alcohol bottles were removed from the cages. The analysed

parameters were latency to enter in the dark box, time in light box and latency to re-enter the light box. All the measures were expressed in second (s).

Novel object recognition test.

The apparatus consisted in an open field (90 cm x 90 cm x 40 cm). Two equal objects were used for the familiarisation phase, and one of them was replaced by a different object the day of the test (two days after familiarisation phase). Objects had no significance for rats and had never been associated with reinforcement. During the familiarisation phase and test, rats were placed at the centre of the apparatus, with the head facing opposite to the objects, and then rats explored freely during 5 minutes (figure 1E). The test was executed during the dark cycle and animals did not have access to the alcohol bottles at least 3 hours before the test. The behaviour analysed in the test was the ratio total time (s) exploring both objects / time (s) exploring the new object.

Biochemical analysis

Brains from the animals belonging to experiment 1 were removed after isoflurane or pentobarbital overdose and used to measure KOR expression by western blot and immunofluorescence, respectively.

Western Blot.

The tissue (0.5 mL of lysis buffer each 250 mg of tissue) was homogenated in cold lysis buffer (1% IGEPAL CA-630, 20 mM Tris-HCl pH 8, 130 M NaCl, 10 mM NaF and 1% proteases inhibitor cocktail (Sigma)). The homogenate extracts were kept in ice for 30 minutes. Afterwards, samples were straightaway centrifuged at 13225 G for 15 minutes at 4ºC and the supernatant was

collected. Furthermore, the concentration of the lysates was determined by using a Bradford protein assay kit (Bio-Rad). This procedure was adapted from a procedure we used before (Sánchez-Catalán et al., 2009).

For the immunoelectrotransference, 1 mm acrylamide gels at 10% were used. Loading buffer (350mM Tris pH 6.8, 30% glicerol, 30% mercaptoethanol, 100g/L Sodium Dodecyl Sulphate and 200 mg/L bromophenol blue) was added to the appropriate volume of each sample that contained 20 μg of total protein and they were heated at 70ºC for 20 minutes. Besides, the Bio-Rad Mini Protean buffer system (6g/L Trizma base, 2.88g/L glycine and 20g/L Sodium Dodecyl Sulphate) was used to carry out the electrophoresis at 120 V.

The proteins already separated by SDA-PAGE were transferred to nitrocellulose membranes (Bio-Rad) in a buffer (3g/L Trizma base, 1.44 g/L glycine and 20% methanol) with the help of a semi-dry system (Bio-Rad Trans-Blot® TurboTM) for 25 minutes at 25 V.

After that, nitrocellulose membranes were blocked for 60 minutes in 5% non-fat dried milk in TBS-Tween20 (TBS-T) 0.1% (20mM Tris and 500mM NaCl pH 7.5) and they were incubated overnight at 4ºC with the corresponding primary antibodies rabbit IgG anti-KOR (1:2000, Thermo Fisher) (Jolivalt et al., 2006; Zádor et al., 2015). Then, the membranes were washed three times with TBS-T 0.1% and were incubated for an hour at room temperature with the secondary antibody goat IgG anti-rabbit (1:2000, Bio-Rad). Finally, membranes were developed by chemiluminescence with CheLuminate-HRP PicoDetect (Panreac), the images were captured with ChemiDoc™ XRS+ System (Bio-Rad) and further quantified using Image J software. GAPDH conjugated with

HRP (1:1000, Thermo Fisher) was used to detect GAPDH as a protein loading control. The intensity of the bands was expressed as arbitrary units and normalised to GAPDH bands intensity. Relative protein levels to control were determined by setting the control group to 100% and calculating the respective percentages for each band. All samples were run in duplicate obtaining an average of the % from control for each sample. Then the relative KOR expression of each experimental group was expressed as mean ± SEM of the % from control group.

Immunofluorescence.

Animals were transcardially perfused with 200 mL of PBS and 300 mL of 4% formaldehyde in PB 0.1M. Brains were removed and 20 h post-fixed in 4% paraformaldehyde in PB 0.1M. After post-fixation brain were placed in 30% sucrose solution until sank. 40 µm brain slices were obtained on a freezing microtome (Leica). The slices selected were transferred to TBS and sequentially incubated (including 3 washing of TBS between incubations) in: (1) 1h in 30% normal donkey serum (Thermo Fisher) in TBS-Tx 0.3%, (2) primary antibody rabbit IgG anti-KOR (1:500, Thermo Fisher) (Jolivalt et al., 2006; Zádor et al., 2015) in TBS-Tx 0.03% with Normal donkey Serum (20µL/mL) at 4ºC overnight, (3) secondary antibody donkey IgG anti-rabbit Alexa Fluor® 594 (1:1000, Abcam) in TBS-Tx 0.03% with Normal Donkey Serum (20µL/mL) at RT for 2 hours. Slices were then washed three times for 5 minutes each in TBS, mounted on slides and covered with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories) and prepared for fluorescence microscopy (Leica Biosystems, Germany). Images of the NAc shell and NAc

core were obtained with a 40x objective (images size 333.97x246.52 µm). We obtained 2-6 images per area and subject for a posterior quantification. Finally, quantification of the KOR levels was conducted with the software FIJI. The measures of each animal were the mean of the different images obtained. The protein levels were determined by setting the control group to 1 and calculating the respective values for each subject. The protein levels were expressed as mean ± SEM.

2.2.2 Experiment 2: Effect of the nor-BNI administration in the NAc medial shell on CFA-induced relapse-like behaviour in female rats.

In experiment 2, 28 female rats underwent the IA protocol and the inflammatory pain model described in experiment 1. This experiment was designed to pharmacologically explore the implication of dynorphinergic system in the relapse phenomenon observed in experiment 1 and detect a possible target to impair pain-induced relapse. For that, animals received an infusion of artificial cerebrospinal fluid (aCSF) or the irreversible KOR antagonist nor-binaltorphimine (norBNI, 2 mg per side in 0.5 ml of aCSF, Tocris) (Massaly et al., 2019), directly into the posteromedial shell of the NAc, 10 days before alcohol reintroduction. Therefore, rats were randomly assigned to one of the following experimental groups: CFA/aCSF (n=9, inflammatory pain rats that received aCSF administration); CFA/NorBNI (n=9, inflammatory pain rats that received NorBNI administration); SAL/NorBNI (n=10, no-pain rats that received NorBNI administration).

Surgery and NorBNI administration

All surgeries were performed under isoflurane anaesthesia (1.5 MAC) and under aseptic conditions. Rats received moments prior to the surgery 1.8 mg/kg enrofloxacin (s.c.), 2.5 mg/Kg of carprofen (s.c) and 0.1% topic lidocaine in the surgical area and in the ears. After that, rats were mounted in the stereotaxic frame (Stoelting, USA) and once craniotomy was made, rats were injected with aCSF or NorBNI bilaterally into the posteromedial NAc. Microinjections were made with a stainless steel microinjector (33-gauge) attached to a PE-10 tubing and a 25 µL Hamilton syringe mounted on a syringe pump (Kd Scientific). The stereotaxic coordinates used were the following: +0.96 mm anteroposterior, ±0.8 mm mediolateral and -6.2 mm dorsoventral from bregma in a flat skull position (Massaly et al., 2019). After microinjections, craniotomies were covered with bone wax (Ethicon) and the animal's skin was sutured with a nylon monofilament suture (Ethilon). Once the surgery was finished animals were gently place in a recovery box provided with a heat blanket and a close monitoring was made until full recovery from the anaesthesia. During the following days after the surgery, rat's health was daily examined.

Micro-injection placement verification.

At the end of the experiments, rats were sacrificed and brains were collected and frozen in dry ice to determinate the injection placements. 35 µm coronal slices of the NAc were obtained by cryostat (Micron) and injection placements were verified with GAFP immunohistochemistry protocol described below and cresyl violet staining. Only one animal was excluded for the statistical analysis because the animal died after the surgery. To perform the GAFP immunohistochemistry, sections were fixed in 4% paraformaldehyde during 4 min. Then, sections were blocked with 5% bovine serum albumin (BSA,

ThermoFisher) in TBS-Triton X-100 0.3% for 1 hour and endogenous peroxidases activity blocked with 1% hydrogen peroxide in TBS for 30 min at room temperature. Subsequently, sections were incubated in: 1) GFAP primary antibody (1:500, ThermoFisher) in TBS with 0.3% Triton X-100 and 5% BSA, 2) biotinylated secondary antibody (1:200; Vector Labs) in the same buffer that primary antibody, and 3) avidin-biotin-peroxidase complex (ABC Elite Kit; Vector Labs) in TBS-Triton X-100 0.3%. The peroxidase activity was revealed with SIGMAFAST-DAB (3,3′-Diaminobenzidine tetrahydrochloride, Sigma-Aldrich) in TBS for 5 min. Then, sections were dehydrated with an alcohol chain of increasing concentrations (50-100% Ethanol and 100% xylene). Finally, sections were coverslipped with Eukitt mounting medium (Eukitt; O. Kindler).

2.3. Statistical analysis.

All the results are expressed as mean ± standard error of the mean (SEM). Sample size of each experiment was *a priori* calculated by using G Power software (University of Düsseldorf). Prior to perform the statistical analysis, the Kolmogorov-Smirnov test was used to confirm normal distribution of the data. When the groups present a normal distribution, we used one of the following tests: One-way ANOVA test or ANOVA for repeated measures followed by Bonferroni multiple comparisons for post-hoc analysis. When homogeneity of variances was violated in one-way ANOVA test, we used Brown-Forsythe analysis of equality of means. In the case that normality was not assured, we analysed data by means of Kruskal-Wallis non-parametric test followed by Bonferroni adjustment for pairwise comparisons.

Statistical analyses were performed with IBM SPSS statistics v24 software. Significance level was always set at $p < 0.05$.

3. Results

3.1. Alcohol intake in female rats is higher than in male rats during acquisition in AI protocol.

The alcohol drinking behaviour during the acquisition was evaluated every consumption day (Figure 1B). Indeed, the ANOVA for repeated measures detected a main effect of sex factor ($F(1,35)= 11.569$, $p= 0.002$) and a significant interaction for sex and alcohol intake variables ($F(1,35)= 180.811$, $p= 0.0000004$). Following, the Bonferroni *post-hoc* analysis for multiple comparisons revealed a significant difference of alcohol intake between male and female rats in several days during the acquisition period (consumption days: 2, 7, 18, 21, 23, 35, 37, 39 and 51). Based on all these differences in basal alcohol consumption, from this moment, male and female behaviour and KOR levels were analysed separately.

3.2. Only CFA-treated female rats increase alcohol intake after the abstinence period.

The IA procedure was used to evaluate the effect of inflammatory pain on alcohol intake after an abstinence period. For that, the average of the last five days of alcohol intake before the abstinence period (basal) was compared to the average of the five days of alcohol intake of the reintroduction period (Figure 1C). The ANOVA with repeated measures for the female rats detected a

significant difference in the within-subjects alcohol intake factor ($F(1,24)= 5.567$, $p= 0.027$), but did not detect differences in the between subjects group factor ($F(1,24)= 0.172$, $p= 0.682$) or in the interaction between intake and group factors ($F(1,24)= 0.594$, $p= 0.448$). The Bonferroni *post-hoc* analysis revealed that alcohol intake after the abstinence period was significantly higher compared to baseline in CFA-treated female rats ($p= 0.037$), but not in saline-treated female rats ($p= 0.273$). Finally, in the case of male rats, the ANOVA with repeated measures did not detect differences in the alcohol intake factor ($F(1,23)= 0.544$, $p= 0.468$), in the pain factor ($F(1,23)= 0.703$, $p= 0.412$) or in the interaction between intake and pain factors ($F(1,23)= 0.094$, $p= 0.762$). Therefore, only female rats under pain conditions increased their alcohol intake after the abstinence period, showing a relapse-like behaviour.

3.3. Mechanical nociception hypersensitivity is unaltered in CFA-treated rats during the experimental procedure.

The Von Frey test showed that mechanical nociceptive thresholds were lower in rats under pain condition in both female and male rats until the end of the experimental procedure (Figure 1D). ANOVAs with repeated measures detected significant differences in between-subjects effect of group in male ($F(1,13)= 15.288$, $p=0.002$) and female rats ($F(1,24)= 15.713$, $p=0.001$) and in the within-subjects effect of nociception thresholds (male, $F(3,39)= 4.182$, $p= 0.012$; female, $F(3,72)= 4.021$, $p= 0.011$). Bonferroni *post-hoc* revealed a significant decrease of the mechanical nociception in the days 80 (male: $p= 0.008$, female: $p= 0.001$) and day 87 (male: $p= 0.0001$, female: $p= 0.0001$) of CFA-treated rats in comparison with the saline-treated rats in both male and female rats.

3.4. CFA-treated female rats maintain stress-like behaviour induced by alcohol withdrawal during alcohol reintroduction phase without altering long term memory.

We analysed the impact of inflammatory pain on stress-like behaviour and long-term memory only in female rats. For this, we used the LDB test and the NOR test in different moments of the abstinence and the reintroduction phases (Figure 1E).

For the LDB test, the one-way ANOVAs did not show significant differences between groups neither in the latency time to enter in dark box ($F(4,27)= 1.205$, $p= 0.332$) nor in the transition between light and dark box ($F(4,27)= 2.130$, $p= 0.104$) (Figure 1F and 1I). However, when analysing the time spent in light box, the one-way ANOVA showed significant differences between groups ($F(4,27)= 4.721$, $p= 0.005$) (Figure 1G). Concretely, saline-treated rats spent significantly less time in the light box compared to the control group during the abstinence period ($p= 0.042$) but not during the reintroduction period ($p= 0.416$), whereas in CFA-treated rats time in light box was significantly lower than the control group in both during abstinence ($p= 0.004$) and reintroduction ($p= 0.026$) periods. Additionally, the Kruskall-Wallis test showed differences between groups in the time to re-enter the light-box ($F(4,32)= 14.797$, $p= 0.005$) (Figure 1H). In this case, only abstinence rats both CFA-treated ($p= 0.006$) and saline-treated ($p= 0.047$) spent more time to re-enter light-box when compared to the control group.

In the OR test, Kruskall-Wallis test showed differences between groups in the ratio to explore the new object (F(4,32)= 16.451, p= 0.002) (Figure 1J). Concretely, the CFA-treated and saline-treated rats in abstinence spent significantly less time to explore new object during the test day when compared to the control group (p= 0.012 and p= 0.03, respectively).

3.5. CFA-treated female rats show increased KOR expression in NAc during the reintroduction period.

Levels of KORs in PFC, NAc and amygdala were measured in all the experimental conditions by western blot. Moreover, levels of KORs in NAc shell (NAcS) and NAc core were analysed for both female and male rats by immunohistochemistry.

One-way ANOVAs did not show differences on KORs expression of male rats in any of the brain areas examined (Amygdala: Saline, F(2,17)= 0.129, p= 0.88; CFA, F(2,17)= 1.542, p= 0.246. PFC: saline, F(2, 16)= 0.065, p= 0.937; CFA, F(2,17)= 0.490, p= 0.622; NAc: saline, F(2,14)= 0.213, p= 0.811; CFA, F(2,16)= 0.18, p= 0.838) (Figures 2A, 2C and 2E).

Nonetheless, female rats showed changes in KOR expression that depended on treatment and period (abstinence and/or relapse periods), but also on the brain area studied. KOR expression of female rats in PFC was unaltered (PFC: saline, F(2,7.247)= 1.688, p= 0.25; CFA, F(2,21)= 2.375, p= 0.305) (Figures 2D). The expression of KOR in amygdala was unaltered in female saline-treated rats (Amygdala: saline, F(2,7.688)= 2.512, p= 0.145). In the case of the analysis of the expression of KOR in amygdala of female CFA-treated rats, the ANOVA detected differences (CFA, F(2,10.379)= 5.316, p= 0.026) but the post-

hoc could not detect differences between the groups (Figure 2F). Interestingly, the expression of KOR in the NAc was significantly increased in female CFA-treated rats ($F(2,15)= 5.227$, $p= 0.022$) only during reintroduction period ($p= 0.025$). It is also important to note that saline-treated and CFA-treated female rats also showed an increase of KOR level in NAc during abstinence, but the statistical analysis did not confirm the observed tendency (Saline: $F(2,15)= 0.905$, $p= 0.636$; CFA-treated rats $p= 0,140$) (Figure 2B).

The immunohistochemistry experiment corroborates our previous results in the NAc. Male rats did not show significant differences in KOR expression in NAc (One-way ANOVA: NAc shell, $F(4,21)= 1.773$, $p= 0.181$; Kruskall-Wallis NAc core, $F(4,21)= 3.639$, $p= 0.457$) (Figure 3A and 3B). Conversely, female CFA rats showed an increase in KOR expression during relapse period which specifically appeared in the NAcS (Kruskall-Wallis: $F(4,29)= 15.160$, $p= 0.004$). KOR levels in NAc core remained not different from control (Kruskall-Wallis: $F(4,31)= 6.498$, $p= 0.165$) (Figure 3C and 3D).

3.6. Blockade of KOR in NAcS reverses the inflammatory pain-induced relapse phenomenon on female CFA rats.

Based in the detected changes of KOR expression in female rats of experiment 1, we injected Nor-BNI in posteromedial NAcS of female rats to study the role of Dyn/KOR in the pain-induced relapse phenomenon observed and the possibility of targeting KOR to block pain-induced relapse-like behaviour in female rats. The ANOVA with repeated measures showed significant differences in intake factor ($F(1,24)= 9.949$, $p= 0.004$) and in the interaction intake and group factors ($F(2,24)= 4.721$, $p= 0.019$). Concretely, as in experiment 1, CFA-treated rats

that received aCSF (CFA/aCSF) showed a significant increase in consumption levels after reintroduction (p=0.0003), while CFA-treated rats injected with NorBNI (CFA/NorBNI), and saline-treated rats injected with NorBNI (Saline/NorBNI), showed similar alcohol intake levels before and after the abstinence period (Figure 4A).

In addition, the Von Frey test showed that nociception thresholds were significantly lower for CFA groups compared to saline group until the end of protocol (ANOVA with repeated measures, between-subjects group factor $F(2,23)= 20.828$, $p= 0.001$, within-subjects effect of nociception threshold factor $F(3,69)= 13.075$, $p= 0.001$; Bonferroni post-hoc test, day 73: Saline/NorBNI vs CFA/NorBNI $p=0.009$, Saline/NorBNI vs CFA/aCSF $p= 0.010$, CFA/NorBNI vs. CFA/aCSF $p=1.000$; day 80: Saline/NorBNI vs CFA/NorBNI $p=0.008$, Saline/NorBNI vs CFA/aCSF $p= 0.004$, CFA/NorBNI vs. CFA/aCSF $p=1.000$; day 87: Saline/NorBNI vs CFA/NorBNI $p=0.009$, Saline/NorBNI vs CFA/aCSF $p= 0.003$, CFA/NorBNI vs. CFA/aCSF $p=1.000$ Figure 4B).

4. Discussion.

In the present study, we further provide detail of the inflammatory pain and AUD interaction by combining an inflammatory pain with an alcohol consumption rat model. The development of an inflammatory pain condition during withdrawal precipitates the relapse-like behaviour only in female rats that seems to be mediated by the increase and maintenance of stress-like behaviours and KORs expression in NAc during the abstinence and the relapse phases. Indeed, the blockade of these receptors in the NAc medial shell of the CFA-treated females

effectively impaired the increase in the alcohol intake during reintroduction phase.

Clinical and epidemiological reports have shown that patients with chronic pain have high prevalence of alcohol consumption (Witkiewitz et al., 2015). Additionally, alcohol abstinent patients with chronic pain show an increased vulnerability to relapse, especially when their pain condition is not successfully managed (Jakubczyk et al., 2016). Although some clinical reports have undercover the effects of pain on mesolimbic dopaminergic transmission by using PET (DaSilva et al., 2019), the exact mechanisms of how pain impacts this system could not be dilucidated. In this context, our goal was to use an animal model to further analyse the concrete neurochemical alterations mediated by pain in the MCLS and its consequences in alcohol relapse-like behaviour.

AUD is a complex disorder and preclinical research has not always been translated into the clinical set up. Giving the limitations of preclinical animal models, the scientific community has advanced in the field increasing our knowledge of the alcohol-induced alterations in the MCLS that might underlie its reinforcing properties. IA to alcohol in rats is a widely used preclinical model of AUD that shows advantages over other models. This model allows rats to voluntary drink excessive and stable amounts of alcohol (5 g/Kg/day) that produce adaptations at molecular, functional and behavioural level showing *face*, *construct* and *predictive validity* (Carnicella et al., 2014). Additionally, this model confers us an important advantage to study the development of the relapse-like behaviour. In general, animal models of alcohol relapse induce either the increase in alcohol consumption over the alcohol pre-abstinence

levels (i.e. alcohol deprivation effect (ADE) model) or increased responding on manipulanda in an instrumental paradigm (Domi et al., 2018). Previous studies (George et al., 2012) and our results, showed that in male rats or no pain rats the application of a prolonged abstinence period did not evoke an increase in alcohol consumption levels. In our case, only female rats that developed the inflammatory pain condition during abstinence showed a significant increase from the alcohol levels consumed before the forced abstinence period (figure 1C). All in all, our results support the hypothesis that inflammatory pain is a factor that can precipitate alcohol relapse-like behaviour in a sex-dependent manner, since males and saline-treated females did not increase alcohol intake during the reintroduction period. Previous studies have confirmed that inflammatory and neuropathic pain rodent models showed altered alcohol intake patterns (Butler et al., 2017; Campos-Jurado et al., 2020; Yu et al., 2019), especially in males, but only one of them has analysed alcohol relapse-like behaviour (Campos-Jurado et al., 2020). In this study, both CFA- and saline-treated male rats showed ADE, thus not being able to detect pain-induced specific alterations. However, it is interesting to note that in this study only males were used. Moreover, the selected animal model to study alcohol relapse was ADE model that induces alcohol-relapse like behaviour *per se.* Consequently, this model makes difficult to assess increases in the occurrence of relapse-like behaviour (Campos-Jurado et al., 2020). Here, we provided a model where both male and control female did not develop a relapse-like behaviour allowing to investigate the effect of pain as a factor to precipitate alcohol relapse-like behaviour.

As mentioned before, different preclinical studies have shown differential effects regarding the sex-dependent effect of pain in alcohol intake patterns, although clinical studies revealed that chronic pain is a risk factor for AUDs in both sexes (Boissoneault et al., 2019; Jakubczyk et al., 2016). In fact, previous data has shown that only males were susceptible to pain alterations in alcohol intake. However it is important to note that, in this study, acquisition but not relapse-like behaviour was analysed in mice (Yu et al., 2019). These differences can be explained based in the addiction phase studied since our research analyses relapse phase and the aforementioned studies were performed in animals without previous history of alcohol consumption. Moreover, it is important to highlight that sex-specific patterns of alcohol consumption may be explained by the sex differences of possible alcohol-induced analgesia and inflammatory response (Pohorecky & Shah, 1987). It is well demonstrated that CFA produces hyperalgesia more rapidly in females than in males, and analgesic drugs are less effective in reducing inflammation in females (Alfonso-Loeches et al., 2013; Cook & Nickerson, 2005; Pascual et al., 2017). Thus, it is possible that alcohol produces a more effective analgesia in male CFA-treated rats than in female CFA-treated rats. If so, female CFA-treated would consume more alcohol to achieve the same effects as in male CFA-rats. Although we cannot discard this possibility it seems unlikely since both male and female CFA-treated rats persisted with a low mechanical nociception during all the procedure and alcohol analgesia has shown to fade after 10 days of exposure (Gatch & Lal, 1999).

Stress is a crucial factor to produce alcohol relapse (Koob, 2013) and moreover, it is well known that pain and alcohol withdrawal induce stress like-behaviour by

themselves (Jarman et al., 2018; Li et al., 2019; Lorente et al., 2020; Narita et al., 2006; Parent et al., 2012; Somkuwar et al., 2017). Indeed, our behavioural data in female rats confirms the appearance of anxiety-like behaviour and impairment on long-term memory during alcohol withdrawal in both CFA-treated and saline-treated groups. These behavioural patterns have been previously shown after an alcohol intake period (Jarman et al., 2018; Li et al., 2019; Marco et al., 2017; Somkuwar et al., 2017), although it has never been assessed under pain conditions. Very interestingly, the reintroduction of alcohol beverages reversed the anxiety-like behaviour only in the saline treated group, suggesting an anxiolytic effect of ethanol. Thus, CFA-treated female rats showed a negative state maintained even when alcohol was reintroduced and it is well known that the negative-affect can promote alcohol intake and relapse (Jakubczyk et al., 2016; Karkhanis & Al-Hasani, 2020; Lopez et al., 2016). In our model, the combination of pain and withdrawal seems to aggravate the negative state driven by both, because of the persistence of the anxiety-like behaviour showed CFA-female rats during the reintroduction period. Additionally, pain showed to exert an effect specifically on the anxiety-like behaviour in females, since the reintroduction of alcohol reversed the long-term memory impairment in both CFA-treated and saline-treated female rats.

Pain and addiction share neural pathways (Apkarian et al., 2013; Egli et al., 2012). In the MSCL, the activation of KOR reduces dopamine release in NAc (Karkhanis et al., 2016; Margolis et al., 2003; Rose et al., 2016) and the exposure to alcohol induces NAc KOR hypersensitivity, leading also to a reduction in dopamine release (Karkhanis et al., 2016; Rose et al., 2016). On the other hand, it has also been described that inflammatory pain induces a loss

of motivation mediated by the increase of Dyn mediated transmission in the NAc shell (Massaly et al., 2019). Furthermore, the blockage of Dyn/KORs is able to reduce alcohol intake as well as stress-like behaviours (Anderson et al., 2019; Knoll et al., 2011; Shirayama et al., 2004; Walker & Koob, 2008). Based on these premises, we analysed the KOR expression in amygdala, PFC and NAc of male and female rats at two time points: abstinence and reintroduction period. Taking into account the limitations of the biochemical techniques, we found that pain increased KOR expression specifically in NAc shell of female CFA-treated rats that serves us to pinpoint a possible pharmacological target. Indeed, our experimental results fully supported our hypothesis and the blockade of the KOR by administering NorBNI in the medial NAc shell, impaired the significant increase of alcohol intake during the relapse phase showed by CFA-treated females.

In conclusion, we describe a new model to explore the inflammatory pain-induced alcohol relapse in which the female rats are vulnerable to pain-induced relapse. This model also might show *face validity* because of its similarities in pain-induced relapse showed in clinical set up; *construct validity* because during abstinence and relapse phase we described the development and maintenance of anxiety like behaviour together with alterations in the KOR expression in the NAc shell; and, hopefully, *predictive validity*. Indeed, by means of classical pharmacology, we demonstrated that the blockade of NAc KOR impaired the development of the pain-induced alcohol-relapse like behaviour. This finding supports the relevant role of the Dyn/KORs on pain-induced negative affect and reveals its role in sex-dependent pain-induced alcohol compulsive intake during relapse phase. Further analysis of neurochemical changes provoked by pain

will definitively help to disentangle pain-induced alterations in the MSCL that promote affective and addictive behaviour comorbidities.

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The authors declare no conflict of interest.

6. Authors contribution

Conceptualization, L.H; Methodology, J.D.L, J.C, Y.C-J, L.H; Formal Analysis, J.D.L, J.C, L.H; Investigation, J.D.L, J.C, Y.C-J, R.M-M, J.L.G-R; Writing – Original Draft, J.D.L, Y.C-J; Resources, L.H; Supervision, L.H. Writing – Review & Editing, J.D.L, J.C, Y.C-J, L.H.

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Figure legends.

Figure 1

Pain induces alcohol relapse only in female CFA-treated rats. A: Schematic of the alcohol and pain experimental design; B: Alcohol intake during acquisition period. Data are mean and SEM of each consumption days (n=25-26/group); * and ** denotes significant differences between groups (ANOVA for repeated measures followed by Bonferroni multiple comparisons, $p < 0.05$ and $p < 0.01$ respectively); C: on the left female and on the right male mean and SEM of total alcohol intake (g/Kg/day) of the 5 days pre- (basal, empty bar) and post-abstinence (filled bars) shown in black for the saline-treated groups (n=12-13/group) and in red for the CFA-treated groups (n=13/group). * denotes significant differences between groups (ANOVA for repeated measures followed by Bonferroni multiple comparisons, $p < 0.05$); D: on the left female and on the right male mean and SEM of nociception threshold (g) of saline-treated rats (black, n=7-13/group) and CFA-treated rats (red, n=8-13/group). ** and *** denotes significant differences between groups (ANOVA for repeated measures followed by Bonferroni correction for multiple comparisons, $p < 0.01$ and $p < 0.001$ respectively). E: Schematic of the behavioural experimental design in the second batch of female rats; F: Mean and SEM of the latency to enter in the light box (s) (n=5-7/group), in green for control group and black and red for saline-treated and CFA-treated rats respectively, during abstinence (empty bars) and after abstinence (filled bars) periods; G: Mean and SEM of time spent

in light box (s) (n=5-7/group), in green for control group and black and red for saline-treated and CFA-treated rats respectively, during abstinence (empty bars) and after abstinence (filled bars) periods. * and ** denotes significant differences between groups (One-way ANOVA followed by Bonferroni, $p < 0.05$ and $p < 0.01$ respectively); H: Mean and SEM of time to re-enter in light box (s) (n=5-7 per group), in green for control group and black and red for saline-treated and CFA-treated rats respectively, during abstinence (empty bars) and after abstinence (filled bars) periods. * denotes significant differences between groups (One-way ANOVA followed by Bonferroni, $p < 0.05$); I, Mean and SEM of number of transition between boxes (n=5-7/group), in green for control group and black and red for saline-treated and CFA-treated rats respectively, during abstinence (empty bars) and after abstinence (filled bars) periods.; J: Mean and SEM of the exploration ratio of the new object (s) (n=6-7/group), in green for control group and black and red for saline-treated and CFA-treated rats, respectively, during abstinence (empty bars) and after abstinence (filled bars) periods. ** and *** denotes significant differences between groups (One- way ANOVA followed by Bonferroni, $p < 0.01$ and $p < 0.001$ respectively). Abbreviations: Von Frey test (VF), saline (SAL), Complete Freund Adjuvant (CFA), abstinence period (A), reintroduction period (R), light-dark box test (LDB), novel object recognition (OR).

Figure 2

Pain induces changes on the expression of KORs in the MSCL. The data are expressed as mean and SEM of KORs levels in % from control

(n=4-6/group). Green bars represent control group and black and red bars represent saline-treated and CFA-treated rats respectively, during abstinence (empty bars) and after abstinence (filled bars) periods. A: NAc of male rats; B: NAc of female rats; C: PFC male rats; D: PFC female rats; E: Amygdala male rats; F, Amygdala female rats. * denotes significant differences between groups, $p < 0.05$ (One-way ANOVA followed by Bonferroni: male (NAc saline, NAc CFA, amygdala saline, amygdala CFA, PFC saline and PFC CFA) and female (NAc CFA, amygdala saline, amygdala CFA, PFC saline); or Kruskall-Wallis followed by Bonferroni: NAc saline and PFC CFA). Abbreviations: complete Freund Adjuvant (CFA), saline (SAL), abstinence period (A), reintroduction period (R), prefrontal Cortex (PFC), nucleus accumbens (NAc) and kappa opioid receptors (KORs).

Figure 3

Pain induces changes on the expression of KORs in NAc. The data are expressed as mean and SEM of the KORs levels in ratio from control (n=4-7/group), in green for control group and black and red for saline-treated and CFA-treated rats, respectively, during abstinence (empty bars) and after abstinence (filled bars) periods. A, NAc shell female; B, NAc shell male; C, NAc core male; D, NAc core female. All panels include one representative image of each group taken with 40x objective. * denotes significant differences between groups (One way ANOVA or Kruskall-Wallis followed by Bonferroni, $p < 0.05$). Abbreviations: complete Freund Adjuvant (CFA), saline (SAL), abstinence

period (A), reintroduction period (R), nucleus accumbens (NAc) and kappa opioid receptors (KORs).

Figure 4

KORs blockade with NorBNI in NAcS impairs pain-induced alcohol relapse. A: Mean and SEM of total alcohol intake (g/Kg/day) of the 5 days pre- (basal, empty bar) and post-abstinence (filled bars) shown in black for the saline-treated group injected with NorBNI (n=10), in red for the CFA-treated group injected with aCSF (n=9) and in yellow for the CFA-treated group injected with NorBNI (n=8). * denotes significant differences between groups (ANOVA for repeated measures followed by Bonferroni correction for multiplie comparisons, $p < 0.05$). B: nociception threshold (g) shown as mean and SEM in black for the saline-treated group injected with NorBNI (n=10), in red for the CFA-treated group injected with aCSF (n=9) and in yellow for the CFA-treated group injected with NorBNI (n=8). ** denotes significant differences in nociception thresholds of CFA/aCSF group versus SAL/NorBNI group and ## denotes significant differences in nociception thresholds of CFA/NorBNI versus SAL/NorBNI group (ANOVA for repeated measures followed by Bonferroni multiplie comparisons, $p < 0.01$). Abbreviation: Norbinaltorphimine (NorBNI), saline (SAL), complete Freund Adjuvant (CFA) and artificial cerebrospinal fluid (aCSF).