Pharmacology, Biochemistry and Behavior 183 (2019) 22–31

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Alcohol-induced conditioned place preference is modulated by CB2 cannabinoid receptors and modifies levels of endocannabinoids in the mesocorticolimbic system

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ARTICLE INFO

Keywords:
Conditioned place preference
Alcohol
Endocannabinoids
CB2R cannabinoid receptors

ABSTRACT

The endocannabinoid (eCB) system is a particularly important neuronal mechanism implicated in alcohol use disorders. Animal models are key to broadening our knowledge of the neurobiological mechanisms underlying alcohol dependence. This study has two main aims: i) to assess how eCB levels in different brain areas are modified by alcohol-induced conditioning place preference (CPP), and ii) to study how cannabinoid type 2 receptor (CB2R) is involved in alcohol-rewarding properties, using pharmacological manipulation in C57BL/6 mice. Our results suggest that the eCB system is dysregulated throughout the mesocorticolimbic system by repeated alcohol exposure during the CPP paradigm, and that levels of anandamide (AEA) and several other *N*-acylethanolamines are markedly decreased in the medial prefrontal cortex and ventral midbrain of alcohol-CPP mice. We also observed that the administering an antagonist/inverse agonist of the CB2R (AM630) during the acquisition phase of CPP reduced the rewarding effects of alcohol. However, activating CB2R signalling using the agonist JWH133 seems to reduce both alcohol- and food-rewarding behaviours. Therefore, our findings indicate that the rewarding effects of alcohol are related to its disruptive effect on AEA and other *N*-acylethanolamine signalling pathways. Thus, pharmacological manipulation of CB2R is an interesting candidate treatment for alcohol use disorders.

1. Introduction

Alcohol misuse results in various diseases and contributes to > 3 million deaths per year worldwide (Friedmann, 2013; World Health Organization, 2018). Harmful alcohol drinking is diagnosed as an alcohol use disorder (AUD) (American Psychiatric Association, 2013), and is the second most important psychiatric disorder (Collins et al., 2011). These disorders are characterized by compulsive seeking and consumption of the drug despite its negative consequences (American Psychiatric Association, 2013; Koob and Le Moal, 2008). Furthermore, an important clinical issue is that AUD patients have great difficulty in maintaining abstinence, which causes the majority to relapse within the first year of sobriety (Sinha, 2011). These alarming data emphasize the importance of understanding the neuronal mechanisms underlying AUD.

Early studies suggested that mesocorticolimbic dopaminergic neurons were involved in the reinforcing effects of drug of abuse, including alcohol (Brodie et al., 1990; Gessa et al., 1985; Spanagel and Weiss,

Abbreviations: 2-AG, 2-arachidonoyl glycerol; 2-LG, 2-linoleoyl glycerol; 2-OG, 2-oleoyl glycerol; AEA, anandamide; AUD, alcohol use disorder; CB1R, cannabinoid type 1 receptor; CB2R, cannabinoid type 2 receptor; CPP, conditioning place preference; D-LEA, *N*-linoleoylethanolamine; DA, dopamine; DEA, *N*-docosatetraenoylethanolamine; DHEA, *N*-docosahexaenoylethanolamine; eCB, endocannabinoid; i.p., intraperitoneally; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine; POEA, *N*-palmitoleoylethanolamine; SEA, *N*-stearoylethanolamine; VTA, ventral tegmental area

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https://doi.org/10.1016/j.pbb.2019.06.007
Received 4 February 2019; Received in revised form 6 June 2019; Accepted 16 June 2019
Available online 17 June 2019
0091-3057/

1999). In the mesocorticolimbic system, several actors (genes, proteins, signalling pathways) have been proposed to play a role in the neuronal mechanisms that underlie alcohol abuse (Ahmadiantehrani et al., 2014; Alasmari et al., 2018; Pava and Woodward, 2012; Ron and Messing, 2013; Wang et al., 2011). Among these factors, the endocannabinoid (eCB) system is particularly significant (Pava and Woodward, 2012). During the last decade, eCBs have been identified as a novel system that could be altered in patients with substance use disorders, including alcoholic patients. Genetic variants and dysregulation in cannabinoid genes were found to be associated with higher risk of drug dependence. In fact, a single polymorphism in fatty acid amide hydrolase (Bühler et al., 2014; Zhou et al., 2016) – the enzyme that catabolizes *N*-acylethanolamines – is present in some alcoholic patients (Hirvonen et al., 2013). Other genetic alterations are also contributing to alcohol intake behaviour in animal models (Kleczkowska et al., 2016; Pava and Woodward, 2012). The eCB system consists mainly of two inhibitory G protein-coupled receptors (cannabinoid type 1 and type 2 receptors, CB1R and CB2R) and several eCB ligands, including anandamide (AEA) and 2-arachidonoyl glycerol (2-AG). CB1R is widely expressed in the brain (Herkenham et al., 1990), and its activation leads to transient or chronic negative regulation of neurotransmission in both excitatory and inhibitory synapses (Alger, 2012; Castillo et al., 2012; Younts and Castillo, 2014). Notably, deregulation of CB1R signalling has been associated with psychiatric disorders (Mechoulam and Parker, 2013), including AUD (Pava and Woodward, 2012). CB2R is mainly expressed in the brain periphery (Munro et al., 1993), and is also found on both neurons and glial cells in different regions of human (Liu et al., 2009; Núñez et al., 2004) and rodent brains (Brusco et al., 2008; Gong et al., 2006; Onaivi, 2006; Zhang et al., 2017).

In particular, CB2R is expressed on dopaminergic neurons in different regions of the mesocorticolimbic system, such as the medial prefrontal cortex (mPFC), the nucleus accumbens (NAc), and the ventral tegmental area (VTA) (Aracil-Fernández et al., 2012; García-Gutiérrez et al., 2010; Gong et al., 2006; Zhang et al., 2017). Interestingly, the systemic activation of CB2R signalling inhibits dopaminergic neuron firing in the VTA (Zhang et al., 2017, 2014), and cocaine-enhanced dopamine release in the NAc in a dose-dependent manner (Xi et al., 2011). These studies show that CB2R activation prevents cocaine-induced dopamine (DA) release in the rodent NAc (Xi et al., 2011; Zhang et al., 2017). In fact, systemic administration (Zhang et al., 2015), and intra-VTA (Zhang et al., 2014), or intra-NAc (Xi et al., 2011) infusion of a CB2R agonist (JWH133) inhibited motivation to self-administer cocaine in rodents. Similarly, the locomotor (Delis et al., 2017; Xi et al., 2011) or rewarding effects (Delis et al., 2017; Ignatowska-Jankowska et al., 2013) of cocaine were reduced after systemic administration of JWH133. Moreover, in a mouse model of self-administration, the genetic deletion of CB2R increased alcohol intake and resulted in higher alcohol-conditioned place preference (CPP) scores compared with wild-type controls (Ortega-Álvaro et al., 2015). Together, these results suggest that CB2R signalling in mesocorticolimbic dopaminergic neurons could be essential for countering the abuse properties of drugs such as alcohol and may thus be protective against drug addiction effects.

The two major aims of the present study were: i) to investigate eCB alterations in different brain areas due to alcohol-induced CPP, and ii) to study the involvement of CB2R in alcohol-rewarding properties using pharmacological manipulation of male C57BL/6 mice. The mice underwent the alcohol-induced CPP procedure and eCB levels were measured in the mPFC, striatum, hippocampus, and ventral midbrain. Furthermore, a CB2R agonist (JWH133) and antagonist (AM630) were administered to assess the effects of CB2R ligands on the development of alcohol-induced CPP.

2. Materials and methods

2.1. Animals

Eight-week old male C57BL/6 mice were purchased from Charles River (Barcelona, Spain). Mice were housed in groups of 4 under a regular 12 h light/dark cycle, with lights on between 8 AM and 8 PM, and with *ad libitum* access to food and water. The housing room was maintained at an average temperature of 21 ± 1 °C and humidity of 55% ± 10%. Every effort was made to minimize animal suffering and to reduce the number of animals used. All procedures were conducted in accordance with national (BOE-2013-1337) and EU (Directive 2010-63EU) guidelines regulating animal research and were approved by the Animal Experimentation Ethics Committee of the Barcelona Biomedical Research Park (CEEA-PRBB).

2.2. Drugs

We prepared the alcohol solution from anhydrous absolute ethyl alcohol (190 proof), diluted to 10 or 20% (v/v) in saline solution (0.9% NaCl, w/v), and administered intraperitoneally (i.p.) 1.26 ml per 100 g of body weight. We habituated mice to the i.p. procedure using a single daily injection of saline for 3 days before beginning the experiments.

We dissolved JWH133 (CB2R agonist, Tocris, United Kingdom) in 31% (v/v) Tocrisolve™ 100 (Tocris, United Kingdom) in saline. The CB2R antagonist/inverse agonist AM630 (Tocris, United Kingdom) was dissolved in 20% (v/v) DMSO (Sigma-Aldrich, Spain) in saline. Control solutions were either 31% Tocrisolve™ or 20% DMSO in saline solution (0.9% NaCl, w/v). We administered both the agonist (JWH133) and the antagonist (AM630) as 1 ml i.p. per 100 g of body weight.

2.3. Conditioned place preference apparatus

The CPP apparatus (Cibertec, SA, Madrid, Spain) consisted of a plastic box divided in two large and equally sized compartments (length 30.7 cm, width 31.5 cm, and height 34.5 cm) interconnected by a small square corridor (length 13.8 cm, width 13.8 cm, and height 34.5 cm). The different compartments could be isolated from the rest by a manual guillotine door. Both large compartments differed in terms of wall colour and floor texture: one had black walls and a smooth black floor and the other had white walls and a rough black floor. The corridor compartment had no special characteristics. Boxes had lateral tracking sensors that detected and recorded the position of the subject throughout the sessions. The apparatus tracking system and data recording were controlled using MONPRE 2Z software (Cibertec, SA, Madrid, Spain).

2.3.1. Alcohol-induced conditioned place preference

Alcohol-induced CPP was adapted from Lim et al. (2012). On the first day (pre-conditioning day), we opened the guillotine doors and the mice had free access to all compartments for 30 min. During this phase, the time spent in each compartment was recorded and mice who spent > 70% or < 30% of the time in either of the compartments were excluded from the study. We used an unbiased design (unbiased apparatus and unbiased assignment). For the conditioning, we injected mice i.p. once a day with saline or with 2.0 g/kg alcohol, and then immediately confined them to one of the two large compartments for 5 min (guillotine doors closed). Each mouse received four pairings with alcohol (days 2, 4, 6, and 8) and saline (days 3, 5, 7, and 9). The control group received eight pairings with saline. Twenty-four hours after the last conditioning session, we tested the mice's place preference (day 10, post-conditioning day). We opened the guillotine doors and mice had free access to all compartments for 30 min. The time spent in each chamber was recorded. The apparatus was thoroughly cleaned after each test. The CPP score was calculated as the difference between the time spent in the saline- or alcohol-paired compartment during the

post-conditioning day and the time spent in the saline- or alcohol-paired compartment during the pre-conditioning day.

2.3.2. Food-induced conditioned place preference

We used a procedure adapted from that of Maldonado et al. (1997). To ensure motivation, we restricted access to food and maintained the mice at 90% of their free-feeding body weight during the entire procedure. During the pre-conditioning day (day 1), we gave mice access to the entire CPP box (guillotine doors open) for 20 min and recorded the time spent in each compartment. Mice showing an excessive spontaneous preference for one compartment (spending > 70% or < 30% of the time in either of the compartments) were excluded from the study. We conducted the conditioning sessions using an unbiased design. We conditioned mice to food by giving them access to a palatable chow ("Cheerios", Nestlé®) on days 3, 5, and 7 in one of the large compartments for 30 min. Conversely, on days 2, 4 and 6, we confined them to the other large compartment without food. Control animals were alternately placed in each large compartment with no food available at any time. On the next day, (day 8, post-conditioning day), we carried out the post-conditioning session as in the pre-conditioning phase. The CPP score was equal to the time spent in the no-food or food-paired compartment during the post-conditioning session minus the time spent in the no-food or food-paired compartment during the pre-conditioning session.

2.4. Locomotor activity

We assessed the locomotor activity using the LE 8816 IR motor activity monitor (Panlab s.l.u., Barcelona, Spain). The test chambers (length 25 cm, width 25 cm, and height 20 cm) had a black plastic floor and clear plastic walls, with two-dimensional (x- and y-axis) tracking sensors composed of 16 × 16 infrared beams for subject detection. During the test, the subjects were shielded from external noise and illuminated with indirect white light (20 lx). Horizontal locomotion was measured from photocell beam interruptions using the SEDACOM software (Panlab s.l.u., Barcelona, Spain).

2.5. Quantification of eCBs and related compounds using liquid chromatography–tandem mass spectrometry

We measured the levels of eCBs in the different brain areas that constitute the mesocorticolimbic system, i.e. the mPFC, striatum, and ventral midbrain, and in the hippocampus since this brain area is directly involved in contextual memory mediated by hippocampal CB2R (Li and Kim, 2016). Mice underwent the alcohol-induced CPP procedure, and immediately after the post-conditioning session, samples were collected from saline- and alcohol-paired mice.

eCBs and related compounds were quantified using previously described methodology (Busquets-Garcia et al., 2013; Pastor et al., 2014; Portero-Tresserra et al., 2018), which was adapted for the extraction of eCBs from brain tissue. The following 2-acyl glycerols and *N*-acylethanolamines were quantified: 2-arachidonoyl glycerol (2-AG), 2-linoleoyl glycerol (2-LG), 2-oleoyl glycerol (2-OG), *N*-arachidonoylethanolamine or anandamide (AEA), *N*-docosatetraenoylethanolamine (DEA), *N*-docosahexaenoylethanolamine (DHEA), *N*-linoleoylethanolamine (LEA), *N*-oleoylethanolamine (OEA), *N*-palmitoleoylethanolamine (POEA), *N*-palmitoylethanolamine (PEA), and *N*-stearoylethanolamine (SEA). Pooled brain tissue samples from 7 mice were placed in a 1 ml Wheaton glass homogenizer and spiked with 25 µl of a mix of deuterated internal standards dissolved in acetonitrile. The mix contained 0.005 µg/ml AEA-d4, 0.005 µg/ml DHEA-d4, 0.005 µg/ml LEA-d4, 0.05 µg/ml OEA-d4, 0.05 µg/ml PEA-d4, 0.05 µg/ml SEA-d3, 5.0 µg/ml 2-AG-d5, and 5 µg/ml 2-OG-d5. The tissue was homogenized on ice with 700 µl of a mixture of 50 mM Tris–HCl buffer (pH 7.4): methanol (1:1) and the homogenates were transferred to 12 ml glass tubes. The homogenizer was washed twice with 0.9 ml of the same mixture and the contents were combined in the tube, giving an approximate final volume of 2.5 ml of homogenate. The homogenization process took < 5 min per sample and the homogenates were kept on ice until organic extraction to minimize the ex vivo generation of eCBs. Next, homogenates were extracted with 5 ml chloroform and the tubes were centrifuged. The lower organic phase was transferred to a clean glass tube, evaporated under a nitrogen stream in a warm water bath, and the extracts reconstituted in 100 µl of a mixture of water:acetonitrile (10:90, v/v) with 0.1% formic acid (v/v) and transferred to glass microvials for purification by high-performance liquid chromatography. The eCBs were separated using an Agilent 6410 triple quadrupole Liquid-Chromatograph equipped with a 1200 series binary pump, a column oven, and a cooled autosampler (4 °C). Chromatographic separation was carried out using a Waters C18-CSH column (3.1 × 100 mm, 1.8 µm particle size) maintained at 40 °C with a mobile phase flow rate of 0.4 ml/min. The composition of the mobile phase was: A: 0.1% (v/v) formic acid in water; B: 0.1% (v/v) formic acid in acetonitrile. The eCBs and related compounds were separated by gradient chromatography. The ion source was operated in positive electrospray mode. The selective reaction monitoring mode was used for the analysis. Quantification was done by isotope dilution with the response of the internal standards. The internal standards used for the quantification of eCBs by liquid chromatography-tandem mass spectrometry were purchased from Cayman Chemical (Michigan, USA), except for 2-OG-d5, which was purchased from Toronto Research Chemicals (Ontario, Canada).

2.6. Schedule of the experiments

2.6.1. Alcohol-induced CPP and alcohol-induced reinstatement after extinction in CPP

Male mice (n = 33) underwent the alcohol-induced CPP protocol as described above (Fig. 1A). Each mouse received four pairings of alcohol 2 g/kg (days 2, 4, 6, and 8) and saline (days 3, 5, 7, and 9). The control group (n = 10) received saline in all pairing days. Twenty-four hours after the last conditioning session, we tested the mice's place preference. Mice with a CPP score of > 10% of the time spent in the drug-paired compartment during pre-conditioning went on to the next phase of the procedure (extinction sessions). After the pre-conditioning phase, three mice were excluded because they showed an innate preference for one of the compartments during the pre-conditioning day, and two mice were excluded from the experiment because they did not meet the criteria to move on to the extinction phase. The final number of animals included in the statistical analyses was 28 for the alcohol-CPP group and 10 for the saline-CPP group.

Subsequently, for a 5-day period, animals went through a daily session of extinction in which they had free access to all compartments for 30 min (guillotine doors open). Only mice that decreased their CPP score by at least 25% were considered to have achieved extinction, and they were kept testing reinstatement. To promote reinstatement, mice (n = 11) received an i.p. administration of 1 g/kg alcohol (10% v/v) immediately before being placed in the CPP box for 30 min with free access to all compartments (guillotine doors opened). In this case, one animal did not meet the extinction criteria for the alcohol-induced CPP, so the final number of mice considered for the statistical analysis was n = 10 for alcohol-CPP group. Mice receiving saline (0.9% NaCl, w/v) were used as control (n = 11).

2.6.2. Determination of eCB levels in different brain areas after alcohol-CPP induction

After the post-conditioning session, we dissected the mPFC, striatum, hippocampus, and ventral midbrain of mice (n = 7 per group) that had acquired the alcohol-induced CPP and determined their eCBs levels.

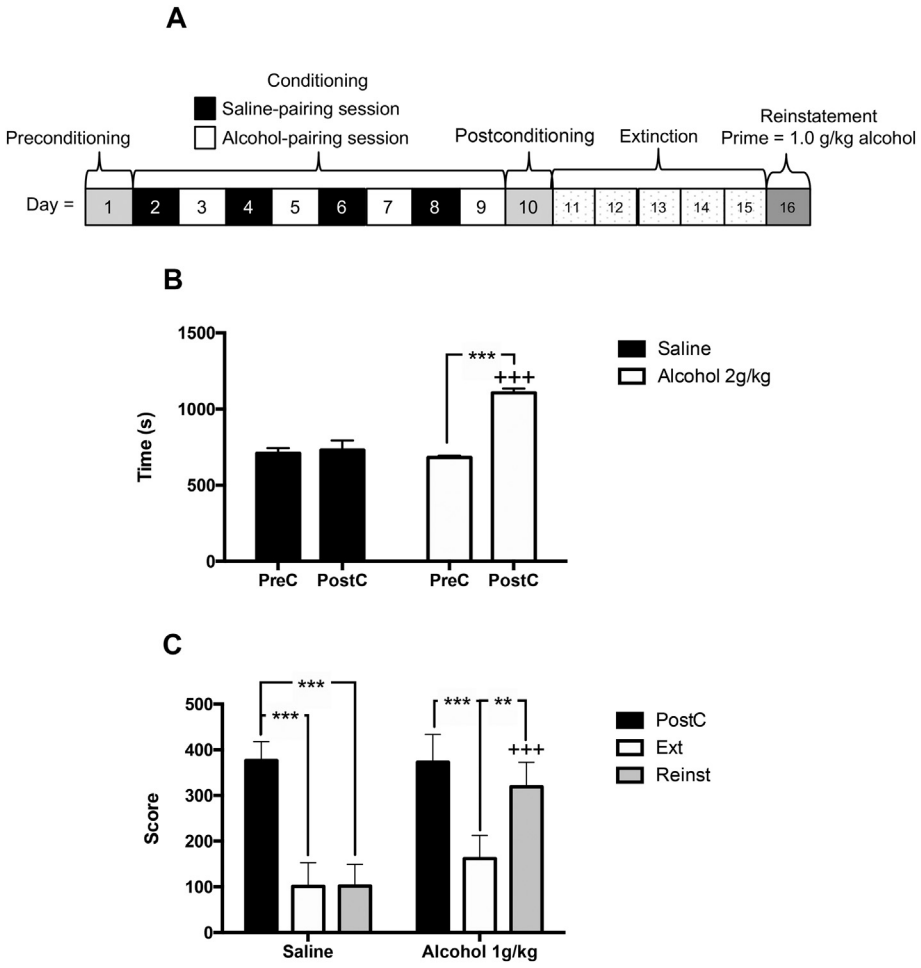

Fig. 1. Alcohol-induced conditioning place preference, extinction, and reinstatement. (A) Experimental schedule of the procedure conducted to assess the effect of alcohol-induced conditioned place preference, extinction, and reinstatement. (B) Bars represent the time (s) spent in the saline- (n = 10) or drug-paired (n = 28) compartment during pre-conditioning (PreC) and post-conditioning (PostC). (C) Bars represent the CPP score (time spent in the alcohol-paired compartment during the experiment) as the mean ± SEM of three phases: Post-conditioning (PostC, black bars), Extinction (Ext, white bars), and Reinstatement (Reinst, grey bars) in alcohol- or saline-treated animals. Only animals that achieved alcohol CPP (n = 10–11 mice/group) progressed to the extinction phase. There were no significant differences between groups in the extinction of alcohol-induced CPP. Reinstatement was induced by an injection of 1 g/kg alcohol, but not for saline solution. **p < 0.01, ***p < 0.001, +++p < 0.001 (Bonferroni post-hoc test).

CPP-induced by alcohol 2 g/kg (n = 28)	Extinction sessions (n = 11 per group)	Reinstatement saline (n = 11) Reinstatement alcohol 1 g/kg (n = 10)
	Brains eCB levels (n = 7 per group)	
CPP-induced by saline (n = 10)	Brains eCB levels (n = 7 per group)	

2.6.3. Effects of CB2R ligands on the expression of alcohol-induced CPP

We used a different group of mice to determine whether activation or inhibition of CB2R signalling alters the expression of alcohol-induced CPP. Saline- or alcohol-conditioned mice received an i.p. administration of a CB2R agonist (JWH133), antagonist (AM630), or their respective control solutions at a dose of 5 or 10 mg/kg, 1 h before beginning the post-conditioning session (Fig. 2A; n = 10–13).

2.6.4. Effects of CB2R ligands on the development of alcohol-induced CPP

A set of mice was used to evaluate whether the acquisition of a place preference for alcohol would also be disturbed by activation or inhibition of CB2R signalling. Mice received JWH133, AM630, or their respective control solutions 1 h before the saline- and alcohol-paring sessions in the CPP (Fig. 2D; n = 10–14). In this experiment, which was conducted 24 h after the last conditioning session, alcohol-induced place preference was tested without any further treatment.

2.6.5. Effects of CB2R ligands on locomotor activity in naïve mice

These experiments were designed to determine whether activation or inhibition of CB2R signalling in mice exposed to alcohol-induced CPP could be caused by an alteration of the animal's motility (Fig. 3A). Drug-naïve mice (n = 10–12/each group) were administered i.p. with either JWH133, AM630, or their respective control solutions 1 h before monitoring their locomotor activity during 30 min.

2.6.6. Effects of CB2R ligands on food-induced CPP

To evaluate whether the effects of the activation or inhibition of CB2R signalling observed in the CPP could be generalized to natural rewards, we evaluated food-induced CPP after the administration of AM630 or its control solution 1 h before beginning the post-conditioning session (Fig. 4A; n = 11–14), and JWH133 or its control solution 1 h before the no-food and food-paring sessions (Fig. 4C; n = 10–13). Three animals were excluded because they showed an innate preference for one of the compartments during the pre-conditioning day.

3. Statistical analysis

We used repeated measures ANOVA to analyse the data from the experiment of alcohol induced-CPP evaluating the acquisition, with *conditioning* (pre-conditioning and post-conditioning) and *alcohol administration* (saline and alcohol) as variables. We also used repeated measures ANOVA to analyse the different phases of the CPP (acquisition, extinction, and the reinstatement), with *phases* (post-conditioning, extinction and reinstatement) and *alcohol administration* (saline and alcohol groups) as variables. To study the effects of CB2R signalling in alcohol and food-induced CPP, we conducted two-way ANOVA, with alcohol or food administration (saline and alcohol groups, or food and no-food, respectively) and *treatment* (vehicle and JWH133 at different

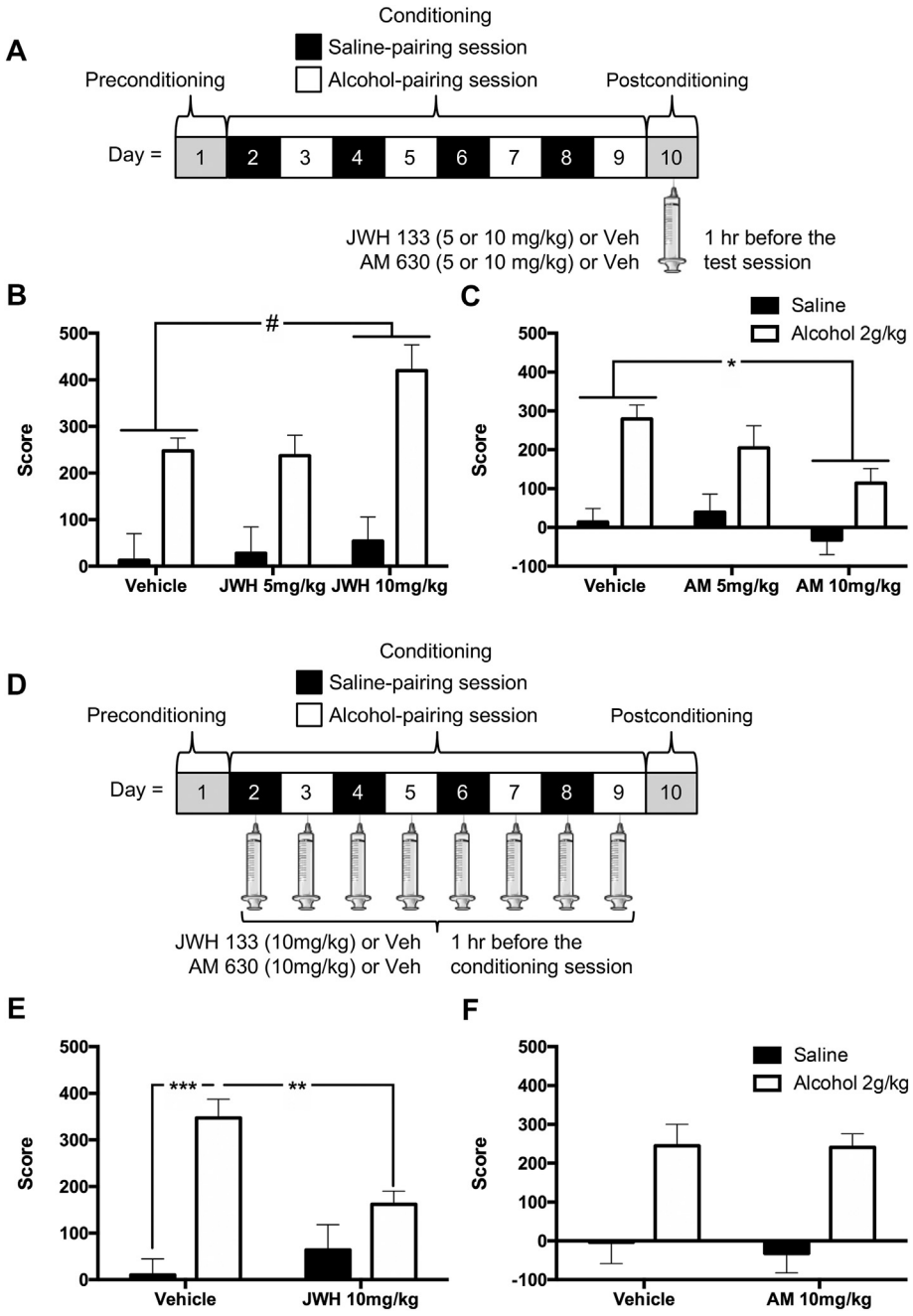

Fig. 2. Involvement of CB2R in both expression and development of alcohol conditioning place preference. (A) Schematic representation of the procedure to evaluate the effect of a single injection of JWH133 and AM630 on the expression of saline- or alcohol-induced CPP. (B) Bars represent the CPP score (time spent in the alcohol-paired compartment during the experiment) for saline- (black bars) and alcohol-treated animals (white bars) as mean ± SEM of the Vehicle, JWH133 at 5 and 10 mg/kg (n = 10–13 mice/group). (C) Bars represent the CPP score of saline- (black bars) and alcohol-treated (white bars) animals as mean ± SEM of the Vehicle or AM630 at 5 and 10 mg/kg (n = 10–13 mice/group). (D) Schematic representation of the procedure to evaluate the effects of JWH133 and AM630 on the acquisition of alcohol-induced CPP. (E) Bars represent the CPP score of saline- (black bars) and alcohol-treated animals (white bars) as mean ± SEM of the Vehicle and JWH133 at 10 mg/kg (n = 10–14 mice/group). (F) Bars represent the CPP score of saline- (black bars) and alcohol-treated animals (white bars) as mean ± SEM of the Vehicle and AM360 at 10 mg/kg (n = 10–13 mice/group). #p = 0.063 (trend toward significance), *p < 0.05, (ANOVA values). **p < 0.01, ***p < 0.001 (Bonferroni post-hoc test).

doses, or vehicle and AM360 at different doses, respectively) as variables. We used an unpaired two-tailed Student *t*-test to study differences in the amount of food consumed by the experimental groups during the food-CPP test. Data from locomotor activity were analysed using one-way ANOVA for the total number of counts, with treatment as the variable. Finally, we used an unpaired two-tailed Student *t*-test to analyse differences between eCB compounds in mice exposed to alcohol and control mice in the CPP for each brain area evaluated (mPFC, striatum, hippocampus, and ventral midbrain). Where necessary, we adjusted the results post-hoc using the Bonferroni correction. Differences are considered statistically significant when the p values were < 0.05 ($p < 0.05$). Quantitative variables are presented as mean and standard error of the mean (mean ± SEM). Statistical analyses were performed using SPSS (SPSS Statistics for Windows, Version 23.0., IBM Corp., Armonk, NY).

4. Results

4.1. Alcohol-induced CPP and reinstatement after extinction

Fig. 1A is a schematic representation of the experimental phases of CPP with extinction and reinstatement. For the alcohol-inducing CPP, the repeated measures ANOVA showed an effect of *conditioning* ($F_{1,36} = 66.28$, $p < 0.001$), *alcohol administration* ($F_{1,36} = 18.30$, $p < 0.001$), and also a *conditioning* × *alcohol administration* interaction ($F_{1,36} = 54.39$, $p < 0.001$; Fig. 1B). The *conditioning* × *alcohol administration* interaction indicates that alcohol- and saline-treated animals spent the same time during pre-conditioning in the drug- or saline-paired compartment. Moreover, the Bonferroni post-hoc test showed that alcohol-treated animals spent more time in the compartment associated with alcohol during the post-conditioning day than during the pre-conditioning day ($p < 0.001$). Finally, the post-hoc analysis showed differences between saline- and drug-paired animals during

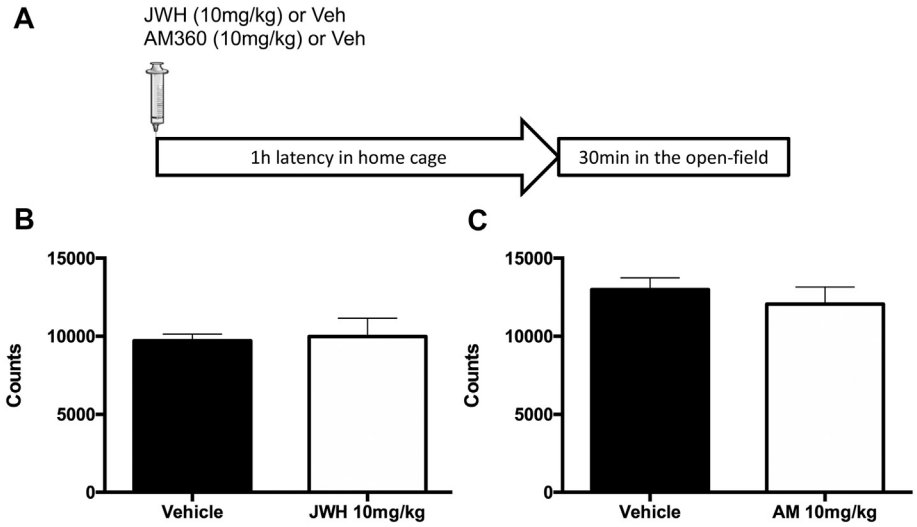

Fig. 3. Effects on mouse locomotor activity of acute administration of JWH133 or AM630. (A) Schematic representation of the procedure conducted to evaluate the effects of JWH133 (10 mg/kg) and AM630 (10 mg/kg) on locomotor activity. (B) Horizontal activity was evaluated in mice after agonist treatment. Black bars represent vehicle-treated animals, and white bars represent JWH133-treated animals (C) Horizontal activity was evaluated in mice after antagonist treatment. Black bars represent vehicle-treated animals, and white bars represent AM630-treated animals. The results are expressed as mean ± SEM (n = 10–12 mice/group).

post-conditioning day ($p < 0.001$).

After acquisition, the animals that had developed CPP were moved to the extinction and reinstatement phases. The repeated measures ANOVA for those phases showed a significant effect of phase ($F_{2,38} = 33.87$, $p < 0.001$) and a *phase* × *alcohol administration* interaction ($F_{2,38} = 7.08$, $p < 0.01$; Fig. 1C). The post-hoc analysis showed that the CPP behaviour disappeared after five days of extinction sessions ($p < 0.001$). However, after a single priming injection (saline or alcohol 1 mg/kg), only mice that received alcohol reinstated the previous alcohol place preference ($p < 0.01$) with a similar score to that observed in the post-conditioning session (Fig. 1C). In contrast, saline-primed animals did not reinstate the previously acquired CPP, as indicated by the fact that we observed statistical differences between the scores obtained during the CPP post-conditioning and reinstatement session ($p < 0.001$), but not between extinction and reinstatement. Finally, the scores obtained for alcohol-primed animals during reinstatement were higher than those for saline-primed animals ($p < 0.001$), indicating that the effect was due to the single dose of alcohol received (Fig. 1C).

4.2. Brain levels of eCBs following alcohol-induced CPP

We collected brain samples from mice exposed to place preference for alcohol and from control mice (paired with saline) (see Table 1) in order to analyse the levels of 2-AG, AEA, and related compounds using liquid chromatography-tandem mass spectrometry (Table 1).

For the mPFC, mice exposed to alcohol showed a significant decrease in levels of 2-AG ($t = 2.95$, $df = 10$, $p < 0.05$) and 2-OG ($t = 3.04$, $df = 10$, $p < 0.05$) compared to the control group. Similarly, among *N*-acylethanolamines the level of AEA was significantly reduced ($t = 3.78$, $df = 11$, $p < 0.01$), as were the concentrations of DEA ($t = 4.62$, $df = 11$, $p < 0.001$), DHEA ($t = 2.66$, $df = 11$, $p < 0.05$), LEA ($t = 2.45$, $df = 10$, $p < 0.05$), OEA ($t = 2.88$, $df = 11$, $p < 0.05$) and POEA ($t = 2.24$, $df = 11$, $p < 0.05$) after alcohol-induced CPP.

For the striatum samples from mice exposed to alcohol, the amounts of 2-AG and AEA were not significantly different to those in controls, while *N*-acylethanolamines levels were lower than the control. Levels of DEA, DHEA and OEA were also markedly lower in the alcohol-induced CPP group ($t = 2.89$, $df = 11$ $p < 0.05$; $t = 2.22$, $df = 11$, $p < 0.05$; $t = 2.63$, $df = 9$, $p < 0.05$, respectively). However, in the hippocampus, we did not find statistically significant differences for any of the compounds analysed.

Regarding the ventral midbrain, only levels of AEA ($t = 3.72$, $df = 10$, $p < 0.01$) and LEA ($t = 2.58$, $df = 10$, $p < 0.05$) were lower in mice exposed to alcohol than in control animals.

4.3. Effects of JWH133 and AM360 on the expression of alcohol-induced CPP

The procedure is represented schematically in Fig. 2A. Analysis of the effect of the CB2R agonist JWH133 on alcohol-induced CPP showed an effect of *alcohol administration* ($F_{1,61} = 44.25$, $p < 0.001$) and *treatment* ($F_{2,61} = 3.26$, $p < 0.05$), but without interaction *alcohol administration* × *treatment* (Fig. 2B). The Bonferroni post-hoc analysis revealed that there were no statistical differences between animals treated with JWH133 at 5 mg/kg and control-treated animals ($p > 0.05$). However, the JWH133 at doses of 10 mg/kg tended to enhance place preference for alcohol compared to control animals (#$p = 0.063$.; Fig. 2B).

Analysis of the effect of the CB2R antagonist AM630 showed a significant effect of *alcohol administration* ($F_{1,63} = 31.23$, $p < 0.001$) and *treatment* ($F_{2,63} = 3.44$, $p < 0.05$), but no interaction between *alcohol administration* × *treatment* (Fig. 2C). These data evidenced that AM630 at a dose of 10 mg/kg diminished the alcohol-induced CPP in comparison with saline-treated animals ($p < 0.05$) (Fig. 2C).

4.4. Activation but not inhibition of CB2R during conditioning impairs the development of alcohol-induced CPP

The procedure is represented schematically in Fig. 2D. Analysis of the administration of CB2R agonist JWH133 during CPP development showed an effect of *alcohol administration* ($F_{1,48} = 29.93$, $p < 0.001$) and an *alcohol administration* × *treatment* interaction ($F_{1,48} = 9.06$, $p < 0.01$), but not an effect of *treatment* effect (Fig. 2E). The Bonferroni post-hoc analysis showed that vehicle-treated animals had higher scores in alcohol-conditioned mice than in saline-conditioned mice ($p < 0.001$). In contrast, JWH133 treatment prior to the alcohol-pairing sessions attenuated alcohol-induced CPP compared to control-solution-treated mice conditioned with alcohol ($p < 0.01$; Fig. 2E).

In the case of AM630 treatment during CPP development, the two-way ANOVA analysis showed an *alcohol administration* effect ($F_{1,44} = 27.74$, $p < 0.001$) but no effect of *treatment* and no *alcohol administration* × *treatment* interaction (Fig. 2F). Therefore, AM630 did not show any effect on alcohol-induced conditioned place preference.

4.5. Effect of JWH133 or AM630 on spontaneous locomotor activity in mice

To assess whether the effects of CB2R signalling on alcohol-induced

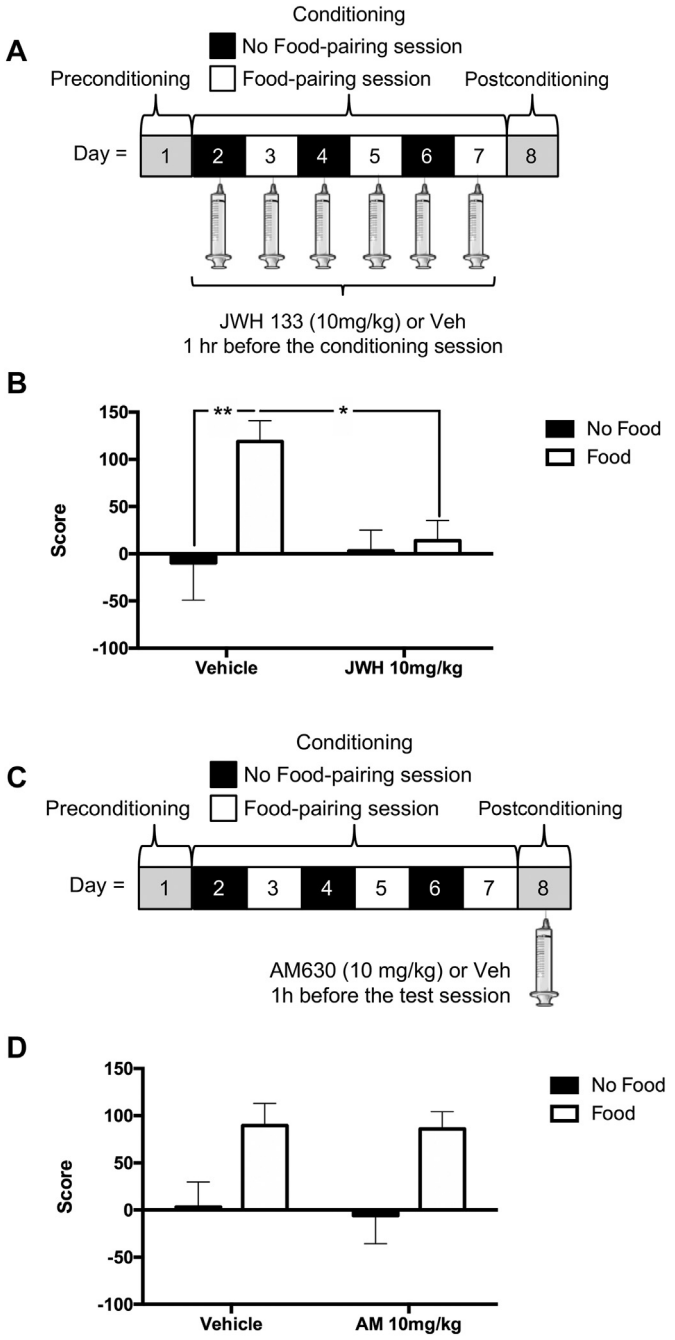

Fig. 4. Effects on food-induced conditioning place preference of JWH133 and AM360. (A) Schematic representation of the procedure conducted to assess the effect of JWH133 (10 mg/kg) on the rewarding effects of food. (B) Black bars represent no-food-conditioned animals, and white bars represent food-conditioned mice. Bars show the CPP score (time spent in the food-paired compartment during the experiment) as mean ± SEM of the control solution or JWH133 10 mg/kg (n = 10–13 mice/group). (C) Schematic representation of the procedure conducted to assess the effect of AM360 (10 mg/kg) on the rewarding effects of food. (D) Black bars represent no-food-conditioned animals and white bars represent food-conditioned mice. Bars show the CPP score as the mean ± SEM of the control solution or AM360 10 mg/kg (n = 11–14 mice/group). *p < 0.05, **p < 0.01 (Bonferroni post-hoc test).

CPP could be due to impaired mice locomotion, we administered the CB2R agonist JWH133 (10 mg/kg) and the CB2R antagonist AM630 (10 mg/kg) 1 h before measuring spontaneous locomotor activity (Fig. 3A). One-way ANOVA analysis showed no differences between treatments (Fig. 3B and Fig. 3C, respectively).

4.6. Activation but not inhibition of CB2R reduces the reward effects of food

To evaluate whether CB2R is important for natural reward, we conducted a food-induced CPP in mice treated with JWH133 (10 mg/kg; Fig. 4A) or AM630 (10 mg/kg; Fig. 4C). For the CB2R agonist JWH133, the analysis showed an effect of *food administration* ($F_{1,43} = 6.32$, $p < 0.05$) and a significant *food administration* × *treatment* interaction ($F_{1,43} = 4.52$, $p < 0.01$; Fig. 4B), but no effect of *treatment*. Post-hoc analysis revealed that vehicle-treated animals showed greater food-induced CPP ($p < 0.01$) than those that did not receive food during the pairing sessions. Administering the CB2R agonist JWH133 (10 mg/kg) prior to the food-pairing session inhibited the development of food-induced CPP ($p < 0.05$). Importantly, this effect was not due to an anorexigenic effect of JWH133 since treated mice consumed similar amounts of food to the control animals during the food-conditioning sessions (22.4 g/kg/30 min and 22.5 g/kg/30 min, respectively; data not shown). For the CB2R antagonist AM630, the analysis showed an effect of *food administration* ($F_{1,45} = 13.46$, $p < 0.001$) but not an effect of *treatment*, nor a significant interaction between these factors (Fig. 4C–D).

5. Discussion

The results of the present study confirm that eCB system may be implicated in the alcohol rewarding properties, by CB2R signalling pathway. In this study, we have shown that the eCBs are dysregulated throughout mesocorticolimbic system following repeated alcohol exposure in the CPP paradigm. We evaluated levels of 2-AG and AEA, and also of other 2-acyl glycerols (2-LG, and 2-OG) and different *N*-acylethanolamines (DEA, DHEA, LEA, OEA, PEA, POEA and SEA) that may be involved in CBR signalling. We observed that levels of AEA, together with those of several other *N*-acylethanolamines, are markedly decreased in the mPFC, striatum, and ventral midbrain of mice receiving alcohol in the CPP paradigm (Table 1). Consistent with previous studies (Ferrer et al., 2007; Rubio et al., 2007), our data suggest a decrease in AEA and the other *N*-acyl-ethanolamines (such as DEA, DHEA, and OEA) levels in the striatum in alcohol-exposed animals. In this sense, this robust effect of alcohol exposure could be due to a general disruptive action of alcohol on the metabolism and/or catabolism of AEA and other *N*-acylethanolamines. Interestingly, AEA and other *N*-acylethanolamines can activate non-cannabinoid receptors, such as the transient receptor potential vanilloid type 1 (TRPV1) or the family of peroxisome proliferator-activated receptor (PPAR) (Fezza et al., 2014). Therefore, altered levels of eCBs and related compounds may contribute to the rewarding effects of alcohol in CPP by altering the function of a different receptor, such as TRPV1, PPARα or PPARγ.

In contrast, alcohol-induced CPP seems to generate a more specific pattern of alteration in 2-AG levels. In fact, the levels of 2-AG and related compounds decreased in the mPFC and ventral midbrain, but not in the striatum of mice treated with alcohol. These results are in contrast with those reported by Malinen et al. (2009), who did not observe differences in the 2-AG concentrations in the mPFC of male mice following prolonged daily access to alcohol. Similarly, Pavón et al. (2018) recently reported an absence of changes in 2-AG levels after prolonged exposure to alcohol, with no differences between animals chronically treated with alcohol and alcohol-naïve animals. However, these differences were revealed only after an alcohol challenge that promoted a decrease in extracellular levels of 2-AG in the NAc of mice that had previously been chronically treated with alcohol. These discrepancies are likely due to differences in the experimental procedures used to analyse the eCB levels, and in the doses of alcohol to which the animals were exposed. Generally, we argue that 2-AG signalling is distinctively altered in different brain areas following alcohol exposure.

Recent studies have shown that the eCB system is intimately linked to memory consolidation (Nasehi et al., 2017), contextual memory (Li and Kim, 2016), and spatial learning (Çakır et al., 2019), which are

Table 1
Brain levels of endocannabinoids and related compounds in alcohol-induced conditioned place preference mice. Comparison of eCBs and related compounds levels (unpaired *t*-test) from medial prefrontal cortex, striatum, hippocampus, and ventral midbrain, from saline- (Sal) and alcohol-induced CPP (Alc) mice (n = 7 per group). The results are expressed as mean ± SEM.

	Medial prefrontal cortex		Striatum		Hippocampus		Ventral midbrain	
	Sal	Alcohol	Sal	Alcohol	Sal	Alcohol	Sal	Alcohol
2-AG nmg/g of tissue	15.56 ± 1.30	**10.76 ± 0.98***	10.11 ± 0.94	12.85 ± 2.0	16.45 ± 1.37	19.33 ± 2.19	9.08 ± 0.64	9.71 ± 0.54
2-LG nmg/g of tissue	0.68 ± 0.06	0.62 ± 0.11	0.41 ± 0.07	0.39 ± 0.05	0.49 ± 0.05	0.49 ± 0.07	3.91 ± 0.45	2.99 ± 0.15
2-OG nmg/g of tissue	3.13 ± 0.47	**1.58 ± 0.20***	2.40 ± 0.39	2.39 ± 0.57	2.96 ± 0.47	2.73 ± 0.65	6.14 ± 0.72	4.46 ± 0.43
AEA pmg/g of tissue	6.69 ± 0.41	**4.53 ± 0.39****	6.45 ± 0.45	5.09 ± 0.43	12.31 ± 1.10	10.84 ± 1.48	8.75 ± 0.36	**6.95 ± 0.32****
DEA pmg/g of tissue	1.08 ± 0.04	**0.81 ± 0.04*****	1.95 ± 0.20	**1.33 ± 0.10***	1.49 ± 0.09	1.34 ± 0.11	3.64 ± 0.23	3.23 ± 0.29
DHEA pmg/g of tissue	3.19 ± 0.13	**2.45 ± 0.23***	5.21 ± 0.51	**3.95 ± 0.30***	3.98 ± 0.26	3.45 ± 0.39	15.17 ± 1.02	14.52 ± 0.84
LEA pmg/g of tissue	3.46 ± 0.09	**2.50 ± 0.32***	3.33 ± 0.32	2.75 ± 0.40	3.61 ± 0.17	3.51 ± 0.49	9.76 ± 0.91	**7.61 ± 0.30***
OEA pmg/g of tissue	26.0 ± 0.9	**22.3 ± 0.9***	55.6 ± 5.0	**43.1 ± 0.8***	35.6 ± 2.25	29.88 ± 1.24	292.8 ± 13.7	260.3 ± 12.7
PEA pmg/g of tissue	37.5 ± 1.4	37.7 ± 2.3	93.9 ± 9.3	81.2 ± 5.8	54.6 ± 5.28	46.03 ± 1.41	736.0 ± 31.0	646.3 ± 34.8
POEA pmg/g of tissue	3.93 ± 0.17	**3.12 ± 0.30***	6.62 ± 0.85	6.13 ± 0.54	6.93 ± 0.74	5.62 ± 0.45	38.5 ± 2.1	35.5 ± 2.7
SEA pmg/g of tissue	18.43 ± 1.05	18.27 ± 0.97	39.7 ± 3.0	38.9 ± 1.3	21.6 ± 1.90	17.78 ± 0.25	285.0 ± 17.1	258.1 ± 12.4

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

mediated by hippocampal CB2R (Gong et al., 2006; Li and Kim, 2016). In this sense, variations in the eCB system could reflect impaired establishment of new hippocampal memories. Both AEA and 2-AG have differential implications for memory; the inhibition of fatty acid amide hydrolase, the enzyme that metabolizes AEA, has been found to impair short- and long-term memory in mice, whereas inhibition of monoacylglycerol lipase, which metabolizes 2-AG, does not (Busquets-Garcia et al., 2011). However, we found no differences in eCB levels in the hippocampus, probably because the observed effects of pharmacological manipulation of CB2Rs on CPP may be associated with the effects of these receptors on reward rather than on the memory process. Together, these results indicate that the development of alcohol-induced place preference is associated with a slight alteration in levels of eCBs and related compounds in the different brain regions studied. In fact, all of the observed changes in the eCB system due to alcohol-induced CPP could explain a putative increase in vulnerability to reinstate the conditioning behaviour after an acute injection of alcohol (Fig. 1B).

Although the involvement of CB2R in different types of hippocampal memories has been extensively demonstrated, its role in drug-reinforcing processes remains unclear. On the one hand, wild-type and CB2R knock-out mice show no differences in intravenous cocaine-self administration (Xi et al., 2011), while on the other hand, CB2R knock-out mice showed enhanced predisposition to alcohol-induced CPP, and increased alcohol intake in both voluntary alcohol consumption and oral self-administration paradigms (Ortega-Álvaro et al., 2015). Importantly, the deletion of CB2R in midbrain dopaminergic neurons (using Cre-Lox technology) reduces the rewarding effects of alcohol (Liu et al., 2017). In this sense, our results also highlight the fact that CB2R signalling may be important in the neuronal mechanisms underlying the rewarding effects of alcohol. Specifically, we observed that alcohol-induced CPP is either facilitated or hindered by the CB2R agonist JWH133, depending on its acute or repeated systemic administration (Fig. 2B and E). Notably, the alcohol-induced CPP, but not the food-induced CPP, could be diminished by a single dose of the CB2R antagonist/inverse agonist AM630 (Fig. 2C) 1 h before the post-conditioning session. In this regard, CB2R inactivation appears to specifically interfere with drug-induced CPP induction, but not when a natural reinforcer is presented, such as food.

Our results show that the effects of the agonist and antagonist depend on when they are administered during the learning process in the CPP paradigm. Thus, we found that acute administration of JWH133 before the post-conditioning session did not enhance the rewarding effects of alcohol in the CPP (Fig. 2B), although there is a trend toward significance when JWH133 is administered at 10 mg/kg ($p = 0.063$). In contrast, JWH133 had the opposite effect when administered repeatedly 1 h before the alcohol pairing sessions (Fig. 2E). These contrasting results suggest that CB2R could modulate both the expression and development of alcohol-induced CPP in different ways. In line with the attenuation of the rewarding effect of alcohol by JWH133, previous studies also found that CB2R activation protects against drug-related behaviours when activated before the paring sessions in the CPP (Al Mansouri et al., 2014; Delis et al., 2017). In fact, consistent with our results, administering either of the two CB2R agonists, JWH133 (10 mg/kg) (Delis et al., 2017) or β-caryophyllene (50 mg/kg) (Al Mansouri et al., 2014), before a cocaine (20 mg/kg) or alcohol (1.5 g/kg) conditioning session reduced reward in the CPP (Fig. 2E). Notably, JWH133 administered systemically or directly infused into the NAc reduced DA levels and prevented cocaine-induced DA release into the rodents NAc (Xi et al., 2011; Zhang et al., 2017). Accordingly, both *ex vivo* and *in vivo* experiments show that JWH133 treatment reduces mesocorticolimbic dopaminergic neuron firing (Zhang et al., 2014). Thus, the effects of JWH133 on mesocorticolimbic dopaminergic function could partly explain the general effects of this CB2R agonist we have observed on the enhancement of alcohol-induced CPP.

Administering the CB2R antagonist only impaired the alcohol-induced CPP when it was injected 1 h before the post-conditioning session (Fig. 2C), whereas a systematic administration during the alcohol-pairing session had no apparent effect (Fig. 2F). Our data contrast with previous results from Al Mansouri et al. (2014) and Delis et al. (2017), who showed that the systemic and repeated administration of AM630

(5 mg/kg) before conditioning does not affect the acquisition of cocaine-induced CPP in rats. Under similar conditions, we did not find that alcohol-induced CPP was impaired when AM630 was administered during pairing sessions. In contrast, we observed that administering CB2R antagonist (10 mg/kg) 1 h before the preconditioning phase suppresses alcohol-induced CPP, which is consistent with the reported effect of administering the CB2R inverse agonist SR144528 during conditioning sessions with nicotine in the CPP (Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013). These treatments (AM630 and SR144528) also prevented the acquisition of the place preference to nicotine (Ignatowska-Jankowska et al., 2013; Navarrete et al., 2013). Similarly, these authors also reported that complete CB2R deletion in mice blocks nicotine-induced CPP (Ignatowska-Jankowska et al., 2013), suggesting that CB2R modulates the rewarding effects of abuse drugs, including alcohol and nicotine.

In contrast to our behavioural findings, Powers et al. (2015) reported that administering AM630 (10 and 20 mg/kg) or JWH133 (10 and 20 mg/kg) before the post-conditioning session was insufficient to alter the expression of alcohol-induced CPP in selectively bred high-alcohol-preferring mice. The discrepancy with our results may be due to differences in experimental procedures or mice strains.

Despite the effects of JWH133 and AM630 on alcohol-induced CPP, CB2R did not appear to regulate basal locomotor activity (Fig. 4B and C). Our results are consistent with previous studies indicating that AM630 treatment does not impair motor behaviour in rats (Blanco-Calvo et al., 2014). These observations suggest that our mice did not deficient locomotion that could interfere with the learning process during the CPP experiments, which confirms that the absence or presence of alcohol-induced reward is directly due to the effects of the treatment on rewarding responses.

It is somewhat surprising that JWH133 and AM630, two drugs with opposite mechanisms of action, have protective effects on the detrimental effects of alcohol. Our results suggest that the effects of AM630 are specific, whereas those of JWH133 are not. This lack of specificity indicates that both drugs are likely to alter alcohol-related behaviour through distinct pathways. Thus, the convergent effects of JWH133 and AM630 drugs could actually reflect differential involvement of CB2R signalling between acquisition and expression of alcohol CPP.

Therefore, the present study is particularly interesting from a translational perspective because the CB2R antagonist/inverse agonist AM630 seems to show anti-alcohol rewarding (i.e. CPP expression) effects. In this sense, AM630 and JWH133 could be a potential candidate treatment to attenuate AUD. Importantly, from a therapeutic perspective, we observed that AM630 did not impede either the expression of food-induced CPP nor locomotion. This AM630 selectivity contrasts with the effects of current treatments for AUD, such as naltrexone and acamprosate, which repress water and sucrose intake (Beczkowska et al., 1992; Czachowski and Delory, 2009; Escher and Mittleman, 2006; Steensland et al., 2007), which could probably explain their compliance issues (Bouza et al., 2004; Johnson, 2008).

In conclusion, this study sheds light on the possible contribution of CB2R to preventing alcohol-induced CPP, and its protective effect against alcohol-related behaviours. Our results suggest that CB2R signalling regulates alcohol-related behaviours mediated by the eCB system. Importantly, our data show that inhibition (through antagonist/inverse agonist AM630) and activation (through agonist JWH133) of CB2R signalling reduces alcohol-rewarding properties through different mechanisms. AM630 seems to modulate the expression of alcohol-induced CPP, whereas JWH133 could regulate its development. Thus, our findings suggest that pharmacological manipulation of CB2R could be a new candidate for treating AUD.

Acknowledgements

This work was supported by the Ministerio de Economía y Competitividad (Spanish Ministry of Health" by "Ministerio de Sanidad, Servicios Sociales e Igualdad grant number SAF2016-75966-R-FEDER), and the Spanish Ministry of Health (Retic-ISCIII-RD16/0017/0010 and Plan Nacional sobre Drogas 2018/007). SM-R received a postdoctoral fellowship from the Conselleria d'Educació, Investigació, Cultura i Esport (APOSTD/2017), Generalitat Valenciana, Spain. The Department of Experimental and Health Sciences (UPF), "Unidad de Excelencia María de Maeztu" was funded by the MINECO (Ref. MDM-2014-0370). The authors have no conflicts of interest to declare.

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