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RESEARCH PAPER

# Effects of cannabidiol plus naltrexone on motivation and ethanol consumption

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## BACKGROUND AND PURPOSE

The aim of this study was to explore if the administration of naltrexone together with cannabidiol (CBD) may improve the efficacy in reducing alcohol consumption and motivation rather than any of the drugs given separately.

## EXPERIMENTAL APPROACH

The effects of low doses of naltrexone (0.7 mg·kg$^{-1}$, p.o.) and/or CBD (20 mg·kg$^{-1}$·day$^{-1}$, s.c.) on ethanol consumption and motivation to drink were evaluated in the oral-ethanol self-administration paradigm in C57BL/6 mice. Gene expression analyses of the opioid μ receptor (*Oprm1*) in the nucleus accumbens (NAc), tyrosine hydroxylase (TH) in the ventral tegmental area (VTA) and the 5-HT$_{1A}$ receptor in the dorsal raphe nucleus (DR) were carried out by real-time PCR. The role of 5-HT$_{1A}$ receptors in the ethanol reduction induced by the administration of CBD + naltrexone was analysed by using the 5-HT$_{1A}$ receptor antagonist WAY100635 (0.3 mg·kg$^{-1}$, i.p.).

## KEY RESULTS

The administration of CBD + naltrexone significantly reduced motivation and ethanol intake in the oral self-administration procedure in a greater proportion than the drugs given alone. Only the combination of both drugs significantly reduced *Oprm1*, TH and 5-HT$_{1A}$ gene expressions in the NAc, VTA and DR respectively. Interestingly, the administration of WAY100635 significantly blocked the actions of CBD + naltrexone but had no effects by itself.

## CONCLUSION AND IMPLICATIONS

The combination of low doses of CBD plus naltrexone were more effective than either CBD or naltrexone alone at reducing ethanol consumption and the motivation to drink. These effects appear to be mediated, at least in part, by 5-HT$_{1A}$ receptors.

## Abbreviations

AUD, alcohol use disorders; CB receptor, cannabinoid receptor; CBD, cannabidiol; DR, dorsal raphe nucleus; NAc, nucleus accumbens; OEA, oral ethanol self-administration; *Oprm1*, opioid μ receptor gene; VTA, ventral tegmental area



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## Introduction

The limited efficacy of the current pharmacological treatments for alcohol use disorders (AUD) justifies the development of alternative drugs. In this respect, **cannabidiol** (CBD), one of the main compounds present in the plant *Cannabis sativa*, which lacks activity as a drug of abuse (Fusar-Poli *et al.,* 2009; Manzanares *et al.,* 2016; Martin-Santos *et al.,* 2012; Mechoulam *et al.,* 2002; Manzanares and Garcia-Gutierrez, 2017; Winton-Brown *et al.,* 2011; Zlebnik and Cheer, 2016), has been pointed out as a new potential therapeutic drug for the treatment of drug use disorders due to its anxiolytic (Guimaraes *et al.,* 1990; Moreira *et al.,* 2006; Resstel *et al.,* 2006; Lemos *et al.,* 2010; de Mello Schier *et al.,* 2014; Blessing *et al.,* 2015), antidepressant (El-Alfy *et al.,* 2010; Zanelati *et al.,* 2010), antipsychotic (Zuardi *et al.,* 1991; Moreira and Guimaraes, 2005; Long *et al.,* 2006; Leweke *et al.,* 2012; Levin *et al.,* 2014; Peres *et al.,* 2016) and neuroprotective properties (Hamelink *et al.,* 2005; Campos *et al.,* 2016).

Interestingly, recent evidence revealed that CBD reduces **heroin** craving and relapse (Ren *et al.,* 2009) and **cocaine** intake (Weiss *et al.,* 2016). Furthermore, our group has demonstrated that CBD also decreases **ethanol** intake and ethanol preference in the two-bottle choice paradigm in mice. In addition, a single administration of a controlled release formulation of CBD (30 mg·kg$^{-1}$·day$^{-1}$, s.c.) that lasted for up to 2 weeks significantly decreased motivation to drink and ethanol consumption in the oral ethanol self-administration (OEA) paradigm. CBD also reduced ethanol-induced relapse but had no effect over non-reinforcing substances, such as water. These behavioural effects were associated with alterations in key targets and brain regions closely related with alcohol consumption, such as **TH** in the ventral tegmental area (VTA), the opioid **μ receptor** (*Oprm1*) and cannabinoid receptors (**$CB_1$**, **$CB_2$**, **GPR55**) in the nucleus accumbens (NAc), both critical regions for reward, goal-directed behaviour and habit formation (Viudez-Martinez *et al.,* 2018). Altogether, these results supported the potential therapeutic use of CBD in the treatment of AUD.

Despite the devastating impact of AUD on society, current options for treatment are scarce and have limited efficacy (Lee and Leggio, 2014). To date, there are just three drug-based treatments approved for AUD by the Food and Drug Administration and the European Medicines Agency: **naltrexone**, **disulfiram** and **acamprosate** (Rosner *et al.,* 2010; Jarosz *et al.,* 2013; Skinner *et al.,* 2014). Other drug-based therapies are usually employed off-label, such as **topiramate**, an anticonvulsant drug with a broad-spectrum activity that seems effective in treating alcohol dependence (Johnson *et al.,* 2003). Nevertheless, naltrexone is still the most effective drug available for the treatment of AUD since it reduces heavy drinking and ethanol craving by antagonizing the **β-endorphin**-stimulated **dopamine** release in the NAc induced by alcohol (Nicholson *et al.,* 2018).

The combination of different drugs is also a commonly used procedure for the treatment of AUD to achieve a greater effect than individual drug therapies by using lower doses of each drug than the ones employed in monotherapy. This strategy also prevents certain dose-related side effects. In this respect, the combination of naltrexone with other drugs, such as **gabapentin** or **5-HT (serotonin)** reuptake inhibitors, showed a greater clinical outcome in several clinical trials (Anton *et al.,* 2011; Froehlich *et al.,* 2013) and animal studies (Froehlich *et al.,* 2013). However, no combination has been proposed to be superior due to the variability among studies (Lee and Leggio, 2014).

In the present study, we further explored whether CBD improves the efficacy of naltrexone to reduce alcohol consumption and motivation to drink in mice. With this aim, the effects of a sub-effective dose of naltrexone (0.7 mg·kg$^{-1}$, p.o.), CBD (20 mg·kg$^{-1}$·day$^{-1}$, s.c., poly-ε-caprolactone spherical microparticles with small pores providing a continuous controlled release during 3 weeks) or their combination were employed. Dose selection was made according to published evidence showing that naltrexone (0.7 mg·kg$^{-1}$, p.o.), a lower dose than the one commonly used in most studies, is able to reduce ethanol intake in mice (Navarrete *et al.,* 2014), although it is not always effective (Oliva and Manzanares, 2007). For CBD, a lower dose than the one our group previously reported as effective (Viudez-Martinez *et al.,* 2018) was evaluated in the OEA paradigm in C57BL/6J male mice. Subsequent real-time PCR experiments were performed to analyse gene expression changes in *Oprm1* in the NAc, TH in the VTA and **$5\text{-}HT_{1A}$ receptor** in the dorsal raphe nucleus (DR) respectively.

Furthermore, to explore the role of $5\text{-}HT_{1A}$ receptors, one of the main targets for CBD (Blessing *et al.,* 2015; Ibeas Bih *et al.,* 2015), in the effects of CBD plus naltrexone, the $5\text{-}HT_{1A}$ antagonist, **WAY100635** (0.3 mg·kg$^{-1}$, i.p.), was administered previously to CBD and naltrexone in the OEA paradigm. To this purpose, the dose of WAY100635 was chosen according to published studies showing that administration of this compound (0.5 mg·kg$^{-1}$, i.p.) seems to prevent the reduction of ethanol intake produced by $5\text{-}HT_{1A}$ receptor agonists, such as **8-OH-DPAT**, in male C57BL/6J mice (Kelai *et al.,* 2006), but did not present effects on its own. Additionally, another group reported that the antipanic-like effects of CBD were blocked when WAY100635 (0.3 mg·kg$^{-1}$, i.p.) was given to male Swiss mice (Twardowschy *et al.,* 2013) and was without effect when given alone.

## Methods

### *Mice*

One hundred and forty C57BL/6J male mice from Charles River (Lille, France), 70 for each experiment, weighing 20–25 g, were housed in groups of six per cage (40 × 25 × 22 cm) under controlled conditions (temperature, 23 ± 2°C; relative humidity, 60 ± 10%; 12 h light/dark cycle, lights on from 8:00 to 20:00 h). The strain and gender of mice were selected based on the results previously reported by our group (Viudez-Martinez *et al.,* 2018). Behavioural analyses were initiated 1 week after acclimatization to the animal room and were performed by placing the home cage in the operant-task room during the development of conditioning experiments. All the studies were conducted in compliance with the Spanish Royal Decree 1201/2005, the Spanish Law 32/2007 and the European Union Directive of the 22nd of September 2010 (2010/63/UE) regulating the care of experimental animals, and the University Miguel Hernández Research Ethics Committee approved the experiments. Animal studies are reported in compliance

with the ARRIVE guidelines (Kilkenny *et al.,* 2010; McGrath and Lilley, 2015).

## *Behavioural analyses*

All the experiments were analysed by observers blind to treatment.

*Experiment 1: to evaluate the effects of the combination of CBD plus naltrexone on ethanol consumption and motivation to drink*

Oral ethanol self-administration paradigm. Our group performed the OEA following a published protocol (Navarrete *et al.,* 2014). The OEA was carried out in 18 modular operant chambers (Panlab) placed inside 18 noise isolation boxes equipped with a chamber light, two levers, one receptacle to drop liquid solution, one syringe pump, one stimulus light and one buzzer. Packwin software (Panlab) controlled the stimulus and fluid delivery and recorded operant responses.

Pressing on one of the levers did not have any consequences (inactive lever), whereas pressing the other lever (active lever) delivered 36 μL fluid combined with a 0.5 s bright stimulus and a 0.5 s, 2850 Hz, 85 dB buzzer beep, followed by a time-out period of 6 s, in which no fluid was delivered. After the 6 s time out, an intertrial interval started, the duration of which depended on each subject's spontaneous waiting time before an active lever press. The experiment was divided into three phases: training, saccharin substitution and ethanol 8% ($v·v^{-1}$) consumption (see Figure 1).

- Training phase (9 days): Two days before beginning the experiment, standard chow was restricted to only 1 h access a day. Before the first training session, water access was restricted for 24 h to increase the motivation for lever pressing during the first training session according to protocols previously described (Cohen *et al.,* 1999; Middaugh and Kelley, 1999; Orru *et al.,* 2012; Navarrete *et al.,* 2014; Garcia-Gutierrez *et al.,* 2016; Viudez-Martinez *et al.,* 2018). During this deprivation period, no behavioural or physical decline was observed. The body weight fluctuation was never higher than 10% (see Supporting Information Data S1). Food allotment was provided 1 h prior to the 60 min session to also increase the motivation for lever pressing. During the subsequent 4 days, water was provided *ad libitum* except during food access for 1 h before beginning each session, in which the water bottle was removed from the cages (postprandial). The following 5 days and during the rest of the experiment, food access was provided for 1 h after the end of each daily session, and water was available *ad libitum* to avoid ethanol consumption due to thirst (preprandial). C57BL/6J mice were trained to press on the active lever to receive 36 μL of 0.2% ($w·v^{-1}$) saccharin reinforcement.
- Saccharin substitution (9 days): The saccharin (Sac) concentration was gradually faded out as the ethanol concentration was gradually increased (Grant and Samson, 1986). Each solution combination was fixed to three consecutive sessions per combination (0.15% Sac–2.5% EtOH, 0.10% Sac–5% EtOH, 0.05% Sac–8% EtOH).
- Basal 8% ($v·v^{-1}$) ethanol consumption (11 days): The number of responses on the active lever, the 8% ethanol ($v·v^{-1}$) consumption and the motivation to drink in C57BL/6 mice without pharmacological treatment were measured. There were three phases: (i) Fixed ratio 1 (FR1), mice responding on the active lever to obtain 8% ethanol and no saccharin were evaluated using an FR1 reinforcement schedule during five daily consecutive 1 h sessions; (ii) FR3, after FR1 mice underwent five daily 1 h sessions using an FR3 reinforcement schedule (mice have to respond three times on the active lever to achieve one reinforcement); and (iii) progressive ratio (PR), on the day subsequent to FR3, a PR session was carried out. In this session, the response requirement to earn reinforcements escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The PR session lasted for 2 h and the 'breaking point' (the maximum number of lever presses each animal was able to perform to achieve one reinforcement) was determined in each animal.
- Effects of pharmacological treatment on 8% ethanol consumption (9 days): Once the animals underwent the FR1, FR3 and PR phases, they were selected according to the following learning task criteria: (i) reaching ≥70% of preference for the active lever; (ii) ≥10 reinforced trials by session in FR1 and FR3, and ≥5 reinforced trials in PR; (iii) ≤30% deviation in the number of reinforced trials, during the last three consecutive days (FR1 and FR3); (iv) mean 8% ethanol consumption ≥500 μL (1.5 $g·kg^{-1}$) in FR1, ≥300 μL (0.9 $g·kg^{-1}$) in FR3 and ≥117.5 μL (0.35 $g·kg^{-1}$) in PR; and (v) a breaking point ≥12 in PR. Mice reaching these criteria were randomly distributed into the following groups: vehicle + vehicle (VEH + VEH) ($n$ = 11), vehicle +

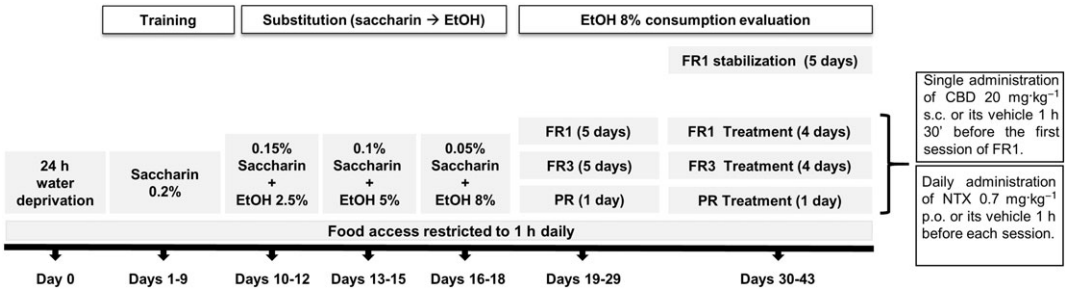



**Figure 1**

Schematic diagram of the ethanol oral self-administration schedule including the different experimental phases. FR1; FR3; PR.

naltrexone (0.7 mg·kg$^{-1}$, p.o) (VEH + NTX) ($n$ = 11), CBD (20 mg·kg$^{-1}$, s.c.) + vehicle (CBD + VEH) ($n$ = 11) and CBD (20 mg·kg$^{-1}$, s.c.) + naltrexone (0.7 mg·kg$^{-1}$, p.o) (CBD + NTX) ($n$ = 11). Selected mice underwent an FR1 stabilization phase (5 days), in order to stabilize the ethanol intake after the PR stage. Then they were exposed again to FR1 (4 days), FR3 (4 days) and PR (1 day) stages receiving the corresponding treatment as explained in Figure 1. For all the different stages, the ethanol left in the receptacle was detracted from the total amount of ethanol delivered, getting the real amount of ethanol consumed [Ethanol solution intake = 37 μL volume delivered – volume left on the receptacle].

Gene expression studies by real-time PCR. Mice were killed by cervical dislocation 2 h and 30 min after the vehicle or corresponding drug administration of the last OEA session. Brains were removed from the skull and frozen at −80°C. Briefly, brain sections were cut (500 μm) in a cryostat (−10°C) containing the regions of interest (NAc, VTA and DR) according to Paxinos and Franklin (2001), mounted onto slides and stored at −80°C. Sections were microdissected following the method described by Palkovits and previously performed by our group (Palkovits, 1983; Garcia-Gutierrez *et al.,* 2010). Total RNA was obtained from brain micropunches with TRI Reagent extraction reagent (Applied Biosystems, Madrid, Spain). Reverse transcription was carried out following the instructions of the manufacturer (Applied Biosystems, Madrid, Spain). Quantitative analysis of the relative abundance of TH (Mm00447546_m1), *Oprm1* (Mm01188089_m1) and 5-HT$_{1A}$ receptor (Mm00434106_s1) gene expressions was performed on the StepOne Sequence Detector System (Applied Biosystems, Madrid, Spain). All of the reagents were obtained from Life Technologies, and the manufacturer's protocols were followed. The reference gene used was 18S rRNA (Mm03928990_g1). Data for each target gene were normalized to the endogenous reference gene, and the fold change in target gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

*Experiment 2: to evaluate the role of 5-HT$_{1A}$ receptors in the effects exerted by the combination of CBD plus naltrexone on ethanol consumption and motivation to drink.* A further OEA experiment was carried out following the protocol described above. For this experiment, once the animals underwent the FR1, FR3 and PR phases, they were selected according to the learning criteria previously specified and randomly distributed into the following groups: (i) vehicle + vehicle (VEH + VEH) ($n$ = 10); (ii) WAY 100635 (0.3 mg·kg$^{-1}$, i.p.) + vehicle (WAY + VEH) ($n$ = 10); (iii) WAY 100635 (0.3 mg·kg$^{-1}$, i.p.) + CBD (20 mg·kg$^{-1}$, s.c.) + naltrexone (0.7 mg·kg$^{-1}$, p.o.) (WAY + CBD + NTX) ($n$ = 10); and (iv) vehicle + CBD (20 mg·kg$^{-1}$, s.c.) + naltrexone (0.7 mg·kg$^{-1}$, p.o) (VEH + CBD + NTX) ($n$ = 10). The selected mice underwent the FR1 (4 days), FR3 (4 days) and PR (1 day) stages receiving the corresponding treatment as explained in the Materials section.

## Group sizes

Group sizes were determined after performing different power calculations. The results of these tests showed that, in order to obtain a power >0.8, between 8 and 10 subjects were needed for each group. Taking into account that only around a 60% of mice that underwent the OEA would match the learning and consumption criteria needed to undergo the treatment evaluation phase, we employed 70 mice for each experiment. After undergoing the training, substitution, FR1, FR3 and PR phases, 44 mice matched the selection criteria ($n$ = 11 per group) for receiving treatment in experiment 1 and 40 subjects ($n$ = 10 per group) in experiment 2.

## Statistical analyses

Statistical analyses were performed using two-way ANOVA with repeated measures followed by the Student–Newman–Keuls test to compare the treatment and control groups at different time points on the OEA paradigms; *post hoc* tests were only applied when ANOVA ($F$ value) indicated significance. The data obtained from the gene expression studies and PR phase in OEA were statistically analysed using the two-way ANOVA test. Statistical analyses were performed with SigmaPlot v11.0 (Systat Software Inc., Chicago, IL, USA) software. Differences were considered significant if the probability of error was less than 5%. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.,* 2018).

## Materials

Poly-ε-caprolactone spherical microparticles with small pores providing a CBD continuous controlled release (20 mg·kg$^{-1}$·day$^{-1}$, s.c.) that lasts for more than 2 weeks and its respective vehicle (empty controlled release microparticles) were obtained from the Pharmaceutical Technology Department (Complutense University of Madrid, Madrid, Spain) (Viudez-Martinez *et al.,* 2018), suspended in PBS 1X (pH 7.4) + 1% Pluronic F-68 (wv$^{-1}$) and then administered (0.4 mL). After the fixed ratio 1 (FR1) stabilization phase, CBD continuous controlled release or its vehicle were administered only once on the first day after the stabilization phase, half an hour after administering WAY 100635 or VEH and half an hour before administering naltrexone or VEH (1 h and a half before starting the experimental session).

Naltrexone (naltrexone hydrochloride, Accord Healthcare S.L.U., Barcelona, Spain) was daily dissolved in tap water to obtain the desired concentration (0.7 mg·kg$^{-1}$, p.o., 0.3 mL). The naltrexone solution or its vehicle was administered once daily after the FR1 stabilization phase, 1 h before the beginning of each session.

WAY 100635 (WAY 100635 maleate; TOCRIS, Madrid, Spain) was daily dissolved in saline 0.9% wv$^{-1}$ to obtain the required concentration (0.3 mg·kg$^{-1}$, i.p., 0.3 mL). The WAY 100635 solution or its vehicle was administered once daily after the FR1 stabilization phase, 2 h before the beginning of each session and 1 h prior the administration of naltrexone or VEH.

For the oral self-administration procedure, absolute ethanol (Merck, Spain) and saccharin sodium salt were dissolved in tap water [8% (vv$^{-1}$) ethanol solution (EtOH)].

### *Nomenclature of targets and ligands*

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

## Results

### *Experiment 1: to evaluate the effects of the combination of CBD plus naltrexone on ethanol consumption and motivation to drink*

*Oral ethanol self-administration.* During the stabilization phase, no significant differences were observed in the number of active lever presses (Figure 2A) ($P > 0.05$, two-way ANOVA repeated measures) nor in the ethanol intake (Figure 2B) ($P > 0.05$, two-way ANOVA repeated measures) between the different groups before being treated.

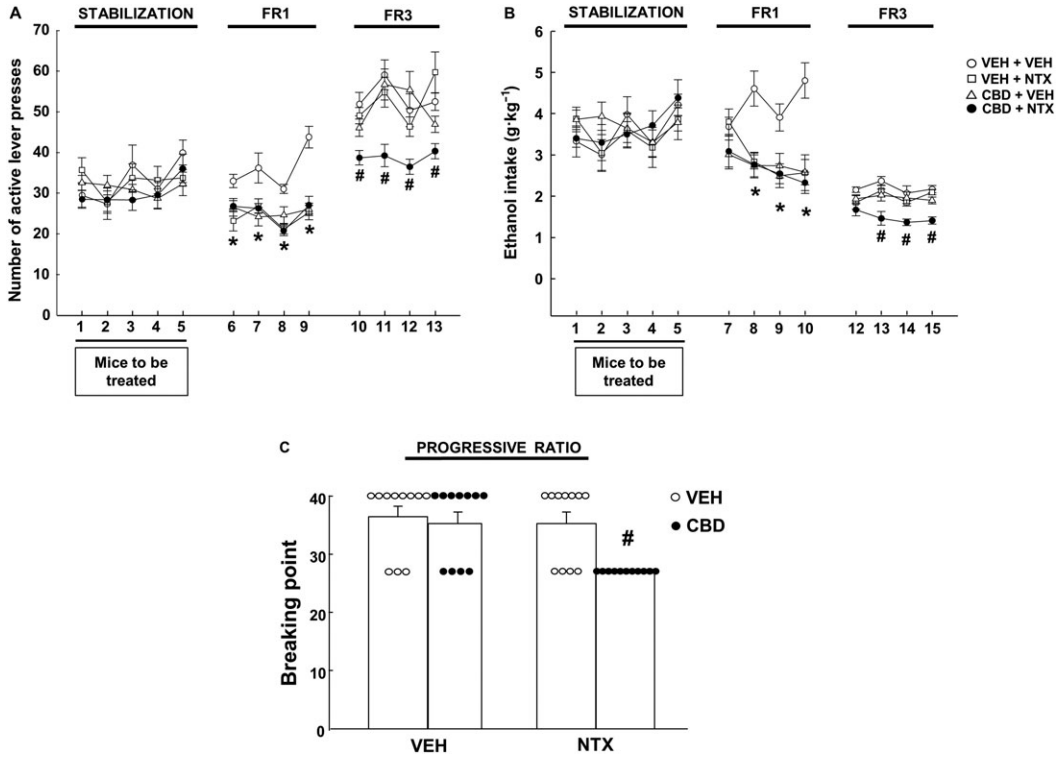
The administration of CBD controlled release microparticle s.c. formulation (20 mg·kg$^{-1}$, s.c.), naltrexone (0.7 mg·kg$^{-1}$, p.o.) or their combination significantly reduced the number of active lever presses during FR1 (Figure 2A) ($P < 0.05$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test). Both treatments and their combination successfully reduced ethanol intake on days 8, 9 and 10 during FR1 phase when compared with the control group VEH + VEH (Figure 2B) ($P < 0.001$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test).

The combination of CBD plus naltrexone was the only treatment successful in reducing the number of active lever presses during FR3 (Figure 2A) ($P < 0.05$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test). Furthermore, only the combination CBD + naltrexone was able to reduce ethanol intake on days 13, 14 and 15 during FR3 stage (Figure 2B) ($P < 0.05$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test).

Interestingly, during the PR phase, only the CBD + NTX group presented a lower breaking point compared to the VEH + VEH, VEH + NTX and CBD + VEH groups (Figure 2C) ($P < 0.05$, two-way ANOVA followed by Student–Newman–Keuls test).

### *Gene expression analyses*

The administration of CBD (20 mg·kg$^{-1}$, s.c.) reduced the relative gene expression of TH (−35%) in the VTA when compared to VEH + VEH. Interestingly, the combination of CBD + NTX reduced the gene expression of TH in a 50%




## Figure 2

Evaluation of the effects of the combination of CBD plus naltrexone (NTX) on ethanol oral self-administration in C57BL/6J mice. (A) Number of active responses (active levers presses that release ethanol solution) during the FR1 stabilization, FR1 + treatment and FR3 + treatment phases; (B) ethanol intake expressed as g·kg$^{-1}$ of all groups during the FR1 stabilization, FR1 + treatment and FR3 + treatment phases; (C) breaking point achieved during PR. The dots and the columns represent the means and vertical lines ± the SEM. * Represents the values from VEH + NTX, CBD- + VEH and CBD + NTX mice that are significantly different ($P < 0.05$) from VEH + VEH group. # Represents the values from CBD + NTX-treated group that are significantly different ($P < 0.05$) from VEH + VEH, NTX + VEH and CBD + VEH groups ($n = 11$ for each group).

when compared to VEH + VEH groups (Figure 3A) ($P < 0.05$, two-way ANOVA followed by Student–Newman–Keuls test). However, naltrexone failed to modify relative gene expression of TH.

In addition, only the combination of CBD + NTX significantly reduced *Oprm1* expression (−37%) in the NAc compared with its corresponding control group VEH + VEH ($P < 0.05$, two-way ANOVA followed by Student–Newman–Keuls test). Nevertheless, the administration of CBD or naltrexone did not induce any alteration (Figure 3B).

5-$HT_{1A}$ receptor gene expression was reduced in the DR of CBD + VEH (−22%) and CBD + NTX (−27%) groups compared with their corresponding control group VEH + VEH ($P < 0.05$, two-way ANOVA followed by Student–Newman–Keuls test). The administration of naltrexone failed to induce any modification (Figure 3C).

### *Experiment 2: to evaluate the role of 5-$HT_{1A}$ on the effects produced by the combination of CBD plus naltrexone on ethanol consumption and motivation to drink*

During the stabilization phase, no significant differences were observed in the number of active lever presses (Figure 4A) ($P > 0.05$, two-way ANOVA repeated measures) or in the ethanol intake ($P > 0.05$, two-way ANOVA repeated measures) (Figure 4B) between groups before being treated.

Again, the association of CBD plus naltrexone diminished the number of active lever presses during FR1 ($P < 0.05$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test) (Figure 4A) and the ethanol intake throughout this phase ($P < 0.05$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test) (Figure 4B).

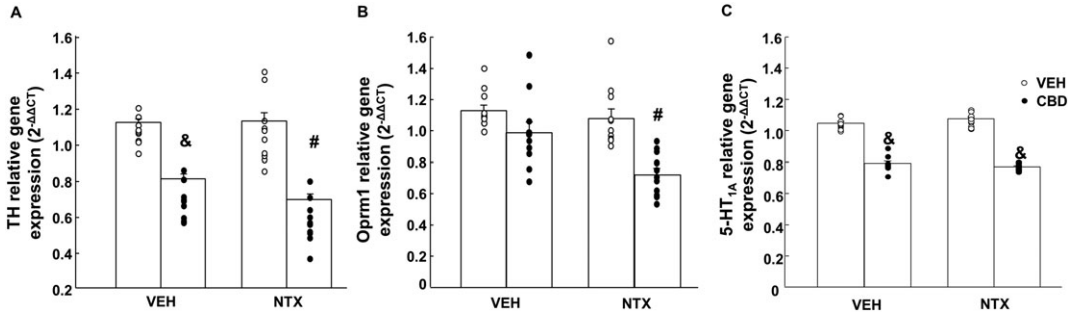
In the same direction, the combination of CBD plus naltrexone was successful in reducing the number of active lever presses ($P < 0.05$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test) (Figure 4A) and the ethanol intake on days 10, 11 and 13 during FR3 ($P < 0.05$, two-way ANOVA repeated measures followed by Student–Newman–Keuls test) (Figure 4B). Interestingly, the administration of WAY 100635 significantly blocked the effects induced by the combination of CBD + NTX on the number of active lever presses.

In agreement with the results obtained from the FR1 and FR3 phases, during the PR phase, WAY 100635 blocked the reduction in the breaking point induced by CBD + NTX (Figure 4C) ($P < 0.05$, two-way ANOVA). No effects were observed with the administration of WAY 100635 when it is given alone in any of the phases evaluated (FR1, FR3 and PR).

## Discussion

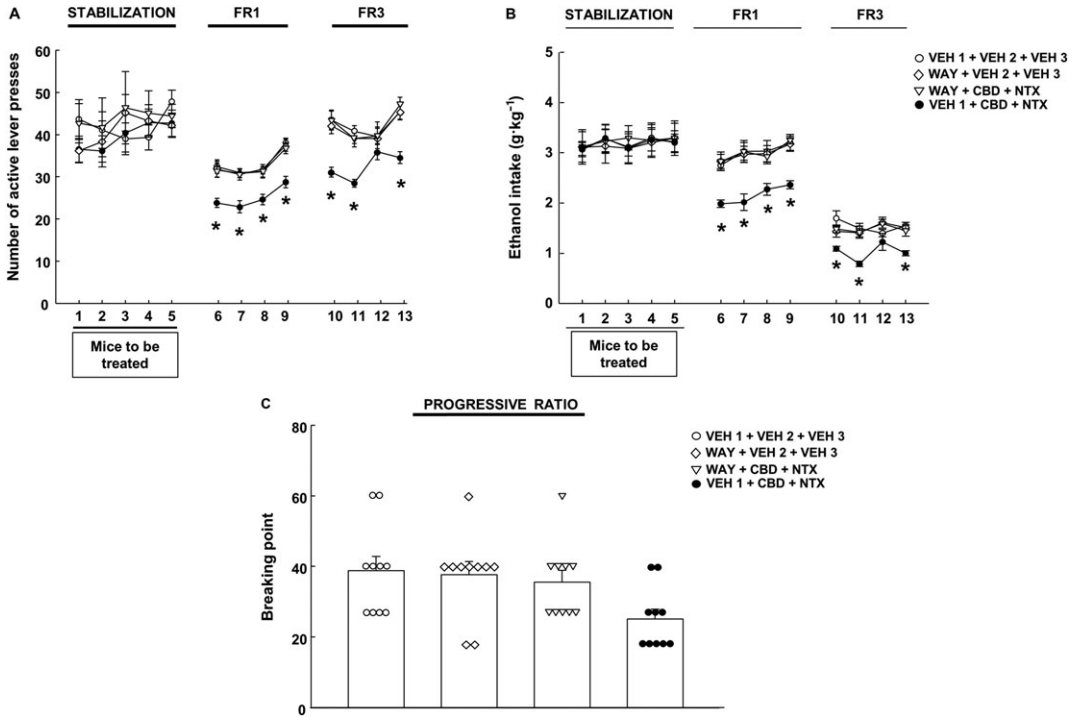
The results of the present study reveal the efficacy of combining low doses of CBD and naltrexone in regulating the reinforcing actions of ethanol consumption. This statement is supported by the following observations: (i) the administration of CBD (20 mg·kg$^{-1}$, s.c.) plus naltrexone (0.7 mg·kg$^{-1}$, p.o.) was the only treatment able to reduce motivation and ethanol intake in all the phases of OEA evaluated (FR1, FR3 and PR); (ii) the administration of CBD plus naltrexone produced a greater reduction of the TH gene expression (50%) and was the only treatment that reduced the expression of *Oprm1* (30%); (iii) the administration of CBD alone or in combination with naltrexone reduced the 5-$HT_{1A}$ receptor gene expression (22 and 27% respectively); and (iv) the administration of the 5-$HT_{1A}$ receptor antagonist, WAY 100635, blocked the reduction of motivation and ethanol intake induced by the combination of CBD plus naltrexone in the OEA paradigm.

Combination of drugs is a common clinical approach used to obtain a dual benefit: improve the clinical response to the pharmacological treatment and reduce the associated side effects. Regarding AUD, during the past few years, different studies have focused on the efficacy evaluation of the association of different drugs with naltrexone, the most effective current drug used for the treatment of alcohol dependence (Kim *et al.,* 2004; Lee and Leggio, 2014; Navarrete *et al.,* 2014; Nicholson *et al.,* 2018). Despite the efforts made,




## Figure 3

Gene expression studies of (A) TH in the VTA, (B) μ receptor (*Oprm1*) in the NAc and (C) 5-$HT_{1A}$ receptor in the DR of C57BL/6J mice treated with naltrexone (NTX; 0.7 mg·kg$^{-1}$, p.o.), CBD [a single administration of a microparticle formulation providing CBD continuous controlled release (20 mg·kg$^{-1}$·day$^{-1}$, s.c.)] or their combination. The columns represent the means and vertical lines ± the SEM. $^{\&}$ Represents the values from the groups that are significantly different ($P < 0.05$) from VEH + VEH and VEH + NTX groups. # Represents the values from the groups that are significantly different ($P < 0.005$) from CBD + VEH, VEH + NTX and VEH + VEH groups ($n = 11$ for each group).




## Figure 4

Evaluation of the effects of the 5-$HT_{1A}$ antagonist WAY 100635 on the actions exerted by the combination of CBD plus naltrexone (NTX) on ethanol self-administration in C57BL/6J mice. (A) Number of active responses during the FR1 stabilization, FR1 + treatment and FR3 + treatment phases; (B) ethanol intake expressed as g·$kg^{-1}$ of all groups during the FR1 stabilization, FR1 + treatment and FR3 + treatment; (C) breaking point achieved during PR. The dots and the columns represent the means and vertical lines ± the SEM. * Represents the values from CBD + NTX that are significantly different ($P < 0.05$) from VEH + VEH, WAY + VEH and CBD + NTX + WAY groups ($n = 10$ for each group).

no association has been proposed as superior due to the variability of results among studies.

Given the efficacy of CBD to reduce the reinforcing effects of ethanol and its neurodegenerative effects when administered at high doses in mice, in this study, we further explored if the combination of CBD plus naltrexone may present greater potential benefits for the treatment of AUD. For this purpose, a low dose of naltrexone, not always successful in reducing ethanol intake (Oliva and Manzanares, 2007), and a lower dose of CBD than the one we previously reported as effective for AUD (Viudez-Martinez *et al.,* 2018) were selected to evaluate potential either additive or synergistic effects achieved by combining both drugs.

The results of the present study revealed that the combination of lower doses of CBD plus naltrexone presented greater efficacy to reduce the reinforcing properties of ethanol in OEA compared with either of them when given alone. Despite CBD and naltrexone reducing ethanol consumption and motivation to drink (measured as active lever presses) in FR1 when given alone, only their combination reduced ethanol consumption and motivation to drink as the effort to get the reward increased (FR3 and PR). These results revealed that, as the effort required to obtain the drug increased, only the combination of both drugs was effective. The lack of effect of naltrexone at the dose selected (0.7 mg·$kg^{-1}$) to reduce ethanol consumption in the FR3 and PR phases differs from what was previously demonstrated by our group (Navarrete *et al.,* 2014). These discrepancies regarding the efficacy of naltrexone may be due, at least in part, to the strain of mice employed in each study (C57BL/6J from Charles River in the present study and C57BL/6 OlaHsd mice from Harlan in the previous one; Ramachandra *et al.,* 2007). Regarding CBD, it seems that lowering the dose (from 30 mg·$kg^{-1}$, s.c., used in the previous study to 20 mg·$kg^{-1}$, s.c.) may affect its ability to reduce ethanol intake and motivation in the OEA. Therefore, these results suggest that the association of low doses of CBD plus naltrexone may produce synergistic actions; nevertheless, further studies are needed in order to verify this conclusion.

To clarify the potential neurochemical mechanism underlying the effects of CBD plus naltrexone combination, the gene expressions of *Oprm1*, TH and 5-$HT_{1A}$ receptors were measured in the NAc, VTA and DR respectively. Rewarding and reinforcing properties of alcohol are mediated mainly by dopaminergic pathways from the VTA to the NAc (Nestler *et al.,* 1993; Koob *et al.,* 1998). In this respect, several authors described an up-regulation of TH gene expression in the VTA under acute (Oliva *et al.,* 2008) and chronic ethanol administration (Ortiz *et al.,* 1995; Lee *et al.,* 2005). Ethanol intake also promotes the release of endogenous opioids (Marinelli *et al.,* 2003). The exact mechanism by which ethanol interacts with the opioid circuitry remains unclear, although it seems that the μ receptor modulates dopamine transmission within the cortico-mesolimbic system (Thorsell, 2013) and mediates the rewarding properties of ethanol (Bilbao *et al.,* 2015).

Indeed, a recently published study showed that rewarding properties are also mediated by serotonergic pathways, which has cell bodies that project from the DR to the NAc (Liu *et al.,* 2014). Additionally, an increase in 5-$HT_{1A}$ receptor binding has been observed in the raphe nuclei of Rhesus monkeys exposed to chronic ethanol self-administration (Hillmer *et al.,* 2014). Likewise, exposure to chronic alcohol also seems to cause 5-$HT_{1A}$ receptor supersensitivity in mice, which in turn contributes to high levels of alcohol drinking (Kelai *et al.,* 2008). In the present study, only the combination of CBD and naltrexone modified the gene expression of *Oprm1* (−30%), TH (−50%) and 5-$HT_{1A}$ receptors (−27%) in the different brain regions analysed. Although CBD significantly reduced TH gene expression in the VTA, it is interesting to highlight that the association of CBD plus naltrexone induced a greater reduction of TH gene expression (−35 vs. −50%, respectively). Contrary to what our group previously reported (Navarrete *et al.,* 2014), naltrexone (0.7 $mg \cdot kg^{-1}$, p.o.) alone failed to reduce the relative gene expression of TH.

Besides, the administration of CBD alone or in combination with naltrexone induced a significant reduction of 5-$HT_{1A}$ receptor gene expression in the DR. However, no effect was induced by naltrexone when given alone. Therefore, the down-regulation of 5-$HT_{1A}$ receptor gene expression observed in the CBD + naltrexone group appears to be mediated by CBD. These results are in agreement with previous studies demonstrating that CBD can act as an allosteric modulator of 5-$HT_{1A}$ receptors (Russo *et al.,* 2005; Campos *et al.,* 2012). Since the chronic exposure to an agonist induces a reduction in the gene expression of its target (Salort *et al.,* 2017; Kim *et al.,* 2018), it seems feasible to hypothesize that a down-regulation of the 5-$HT_{1A}$ receptor gene expression would be expected when a 5-$HT_{1A}$ receptor agonist or allosteric modulator is administered.

Considering the role of the 5-$HT_{1A}$ receptor in alcohol consumption and the allosteric effects of CBD on these receptors, it is possible that the effects of the association of CBD plus naltrexone may be mediated, at least in part, by 5-$HT_{1A}$ receptors. To further investigate the relative specificity of the 5-$HT_{1A}$ receptor in the mechanism underlying the effects of CBD and naltrexone, mice were pretreated with the 5-$HT_{1A}$ antagonist WAY 100635 before receiving naltrexone and/or CBD in the OEA paradigm. Interestingly, pretreatment with WAY 100635 blocked the effects (reduction of ethanol intake and motivation) induced by CBD and naltrexone without having any effect by itself. Despite this finding, further studies are needed to clarify this mechanism, these results support the involvement of 5-$HT_{1A}$ receptors in the effects of CBD plus naltrexone.

In summary, the results indicate that combining low doses of CBD plus naltrexone has a greater efficacy at reducing ethanol consumption and the motivation to drink that either drug administered alone. These behavioural effects were accompanied by a more pronounced reduction of TH gene expression in the VTA and a reduction of *Oprm1* and 5-$HT_{1A}$ receptor gene expressions in the NAc and DR respectively. Interestingly, 5-$HT_{1A}$ receptors appear to play, at least in part, a relevant role in the effects mediated by the combination of CBD and naltrexone. Taken together, these results represent the first step regarding the potential therapeutic use of the combination of CBD plus naltrexone, serving as a reference point for future clinical research. The fact that CBD is currently marketed as a drug for the treatment of spasticity in multiple sclerosis (Sativex®) will further accelerate the development of clinical studies.

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## Author contributions

J.M. and M.S.G.G. conceived and designed the experiments; A.V.M performed the experiments; A.I.F.S. and A.I.T.S. elaborated the CBD s.c. controlled release formulation. A.V.M. analysed the data and drafted the relevant text; A.V.M., M.S.G.G. and J.M. wrote the manuscript. All authors have read and approved the final version of this manuscript.

## Conflict of interest

The authors declare no conflicts of interests.

## Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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**Data S1** 1. Information regarding the weight of mice for experiment 1.
2. Information regarding the weight of mice for experiment 2.