Addiction Biology

SSA SOCIETY FOR THE STUDY OF ADDICTION

ORIGINAL ARTICLE

doi:10.1111/adb.12495

Cannabidiol reduces ethanol consumption, motivation and relapse in mice

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ABSTRACT

This study evaluated the effects of cannabidiol (CBD) on ethanol reinforcement, motivation and relapse in C57BL/6 J mice. The effects of CBD (60 mg/kg, i.p.) on blood ethanol concentration, hypothermia and handling-induced convulsions associated to acute ethanol administration were evaluated. The two-bottle choice paradigm was performed to assess the effects of CBD (30, 60 and 120 mg/kg/day, i.p.) on ethanol intake and preference. In addition, an oral ethanol self-administration experiment was carried out to evaluate the effects of CBD [a single s.c. administration of a microparticle formulation providing CBD continuous controlled release (30 mg/kg/day)] on the reinforcement and motivation for ethanol. The effects of CBD (60 and 120 mg/kg/day, i.p.) on ethanol-induced relapse were also evaluated. Gene expression analyses of tyrosine hydroxylase in ventral tegmental area and μ-opioid (Oprm1), cannabinoid (CB_1r and CB_2r) and GPR55 receptors in nucleus accumbens (NAcc) were carried out by real-time polymerase chain reaction.
Cannabidiol reduced the ethanol-induced hypothermia and handling-induced convulsion but failed to modify blood ethanol concentration. CBD reduced ethanol consumption and preference in the two-bottle choice, significantly decreased ethanol intake and the number of effective responses in the oral ethanol self-administration, and reduced ethanol-induced relapse. Furthermore, the administration of CBD significantly reduced relative gene expression of tyrosine hydroxylase in the ventral tegmental area, Oprm1, CB_1r and GPR55 in the NAcc and significantly increased CB_2r in the NAcc.
Taken together, these results reveal that the administration of CBD reduced the reinforcing properties, motivation and relapse for ethanol. These findings strongly suggest that CBD may result useful for the treatment of alcohol use disorders.

Keywords AUD, cannabidiol, gene expression.

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INTRODUCTION

Alcohol use disorders (AUD), including alcohol dependence or alcohol abuse, represent a serious public health problem worldwide according to the World Health Organization (WHO 2011). Although patients receive pharmacological treatment and psychosocial therapies, the clinical outcome is poor, with up to 70 percent of patients resuming drinking within 1 year (Aronson 2015). Therefore, it is necessary to invest much effort and resources in identifying new therapeutic drugs to improve the efficacy of the treatment of alcoholism.

Cannabidiol (CBD) is one of the main compounds present in the plant *Cannabis sativa* that lacks psychoactive properties. Several studies showed that CBD presents anxiolytic (Guimaraes *et al.* 1990; Moreira *et al.* 2006; Resstel *et al.* 2006; Lemos *et al.* 2010; de Mello Schier *et al.* 2014), antidepressant (El-Alfy *et al.* 2010; Zanelati *et al.* 2010; Schiavon *et al.* 2016), antipsychotic (Zuardi *et al.* 1991; Moreira & Guimaraes 2005; Long *et al.* 2006; Leweke *et al.* 2012; Levin *et al.* 2014) and neuroprotective activity (Hamelink *et al.* 2005). Furthermore, the administration of CBD decreased defensive behaviors evoked by predator exposure, a proposed

model of panic attack and post-traumatic stress disorder (Campos *et al.* 2012; Uribe-Marino *et al.* 2012). In agreement with these findings, in humans, the administration of CBD reduced anxiety and the psychotic-like symptoms induced by Δ^9-THC (Zuardi *et al.* 1982; Morgan & Curran 2008).

In addition, the administration of CBD attenuated the neurodegeneration induced by a binge-drinking model of alcohol by reducing the number of Fluoro-Jade B positive cells in the entorhinal cortex, a common situation derived from ethanol intoxication (Liput *et al.* 2013).

Despite all the potential therapeutic uses suggested for CBD, its precise mechanism of action remains to be elucidated. Indeed, CBD may interact with more than 65 different targets, including voltage-gated sodium channel-1, voltage-gated calcium channels, cannabinoid receptors (CB_1r and CB_2r), G protein-coupled receptor 55 (GPR55r), vallinoid receptor 1 (TRPV), serotoninergic receptor 1A (5-HT_{1Ar}), μ and δ opioid receptors and peroxisome proliferator-activated receptor (PPARγ) (Bisogno *et al.* 2001; Russo *et al.* 2005; Kathmann *et al.* 2006; Ryberg *et al.* 2007; Thomas *et al.* 2007; Campos *et al.* 2012; Ibeas Bih *et al.* 2015).

It is also important to note that CBD presents low oral bioavailability (approximately 6 percent) and poor solubility in aqueous solutions (Agurell *et al.* 1981; Agurell *et al.* 1986). The topic administration of CBD provided more constant plasma levels of CBD because transdermal gels bypass the first pass phenomenon (Liput *et al.* 2013). In addition, i.p. administration of CBD presents a higher bioavailability when compared with oral administration (650 percent higher in plasma and 530 percent in brain) (Deiana *et al.* 2012). However, this option is limited in the daily practice. Therefore, further routes of administration for CBD deserve more exploration.

The aim of this study was to evaluate the potential efficacy of CBD in ethanol consumption, motivation and ethanol-induced relapse. To this purpose, the effects of CBD (60 and 120 mg/kg/day, i.p.) on ethanol consumption and preference for ethanol were evaluated in the two-bottle choice (TBC) paradigm. The oral ethanol self-administration (OEA) paradigm evaluated the effects of CBD (poly-ε-caprolactone spherical microparticles with small pores providing a continuous controlled release, a single administration lasted up to 2 weeks) (30 mg/kg/day, s.c.) on the reinforcement and motivation for ethanol. The effects of CBD (60 and 120 mg/kg/day, i.p.) on ethanol-induced relapse were also evaluated. Specific changes in the gene expression of tyrosine hydroxylase (TH) in the ventral tegmental area (VTA), μ-opioid (Oprm1), cannabinoid (CB_1r and CB_2r) and GPR55 receptors in the nucleus accumbens (NAcc) were measured by real-time polymerase chain reaction.

MATERIALS AND METHODS

Mice

Male C57BL/6 J mice from Charles River (Lille, France) 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 percent; 12-hour light/dark cycle, lights on from 8:00 AM to 8:00 PM). Behavioral 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 September 22, 2010 (2010/63/UE), regulating the care of experimental animals.

Drugs

Cannabidiol for i.p. administration was obtained from STI Pharmaceuticals (Essex, UK) and dissolved in ethanol : cremophor : saline (1:1:18) immediately before the use to obtain the required doses (30, 60 and 120 mg/kg).

Poly-ε-caprolactone spherical microparticles with small pores providing a CBD continuous controlled release (30 mg/kg/day) for s.c. administration was obtained from the Pharmaceutical Technology Department (Complutense University of Madrid, Madrid, Spain) (for more details, see Supporting Information).

For the oral self-administration procedures, absolute ethanol and saccharin sodium salt were dissolved in distilled water [8 percent (v/v) ethanol solution (EtOH)].

Handling-induced convulsions test

The effects of CBD (60 mg/kg, i.p.) or vehicle (VEH) on handling-induced convulsions (HICs) associated with the acute administration of ethanol (4 g/kg, p.o.) or its saline vehicle were evaluated. To this aim, mice were divided into three experimental groups: VEH + EtOH, CBD + EtOH and CBD + saline. CBD or VEH were administered 30 minutes before the administration of ethanol or saline. Briefly, each mouse was picked up gently by the tail and, if necessary, lightly rotated. The HIC was scored as follows: 0, no convulsion; 1, facial grimace only after a gentle spin; 2, no convulsion when lifted, but tonic convulsion elicited by a gentle spin; 3, tonic–clonic convulsion after a gentle spin; 4, tonic convulsion when lifted; 5, tonic–clonic convulsion when lifted; and 6, spontaneous tonic–clonic convulsions (Crabbe *et al.* 1990; Crabbe *et al.* 1991). The HIC score was measured hourly up to 6 hours after ethanol administration.

Sensitivity to acute ethanol-induced hypothermia

The effects of CBD on the hypothermia induced by the acute administration of ethanol were evaluated. To this aim, body temperature was determined in three different experimental groups (VEH + EtOH, CBD + saline and CBD + EtOH) by inserting a rectal probe of approximately 1 cm into the mice's rectum (Radcliffe *et al.* 2005). Briefly, CBD (60 mg/kg, i.p.) or VEH was administered 1 hour before the administration of ethanol (3 g/kg, p.o.) or saline. Body temperature was measured before and 30, 60, 120, 240 and 480 minutes after the administration of ethanol. The degree of hypothermia was expressed as the difference between the baseline temperature and the temperature measured at the times specified earlier. Only statistical differences higher than 1.1°C were considered significant.

Blood ethanol concentrations (BEC)

Mice were divided into three different groups (VEH + EtOH, CBD + saline and CBD + EtOH). CBD (60 mg/kg, i.p.) or VEH were administered 30 minutes before the administration of ethanol (3 g/kg, p.o.) or saline. One hour after the administration of ethanol, trunk blood was collected after rapid decapitation. Concentrations of EtOH were determined in plasma using an ethanol assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.

Voluntary ethanol consumption in a two-bottle choice paradigm

Mice were evaluated in a voluntary and chronic ethanol intake following a modified protocol (Hungund *et al.* 2003; Ortega-Alvaro *et al.* 2015). One week before the beginning of the experiment, all mice were individually housed in cages equipped with two feeding bottles containing only water to be acclimated and avoid possible stress. The two-bottle free choice paradigm with ethanol and water was performed as follows: one bottle always contained water, and the other contained gradually increasing ethanol concentrations (2, 4, 6 and 8 percent per 4 days). Once the ethanol intake/consumption was stabilized, mice were administered with different doses of CBD (30, 60 and 120 mg/kg, i.p.) each one of them for 5 days (6 days for the 120 mg/kg dose) until day 16. Bottles were alternated in order to prevent any place preference bias. Food was available *ad libitum*, and mice were weighed every 4 days. The volume of ethanol and water consumed was carefully measured every day. The amount of ethanol consumed was calculated individually for each mouse, and the values were expressed as g/kg/day. The ratio of ethanol preference was also determined [ethanol preference: ethanol intake/(ethanol intake + water intake)].

Oral ethanol self-administration

Experiment 1: effects of cannabidiol on the reinforcement and motivation for ethanol

The OEA evaluation was carried out in 12 modular operant chambers (Panlab) 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 stimulus and fluid delivery and recorded operant responses. Pressing one lever did not have any consequence (inactive lever), whereas pressing the other lever delivered 36 µl of fluid combined with a 0.5-second stimulus light and a 0.5-second, 2850-Hz, 85-dB buzzer beep (active lever), followed by a 6-second timeout period. The experiment was divided into four phases: training, saccharin substitution and basal 8 percent ethanol consumption as previously described (Navarrete *et al.* 2014) (for more details, see Supporting Information).

Once the animals underwent fixed ratio 1 (FR1), fixed ratio 3 (FR3) and progressive ratio (PR) stages, they were selected according to the following learning criteria: (1) reaching ≥70 percent of preference for the active lever; (2) ≥10 reinforced trials by session in FR1 and FR3, and ≥5 reinforced trials in PR; (3) ≤30 percent deviation in the number of reinforced trials, all during three consecutive days (FR1 and FR3); (4) mean 8 percent ethanol consumption ≥500 µl (1.5 g/kg) in FR1, ≥300 µl (0.9 g/kg) in FR3 and ≥117.5 µl (0.35 g/kg) in PR; and (5) a breaking point ≥12 in PR. Mice reaching these criteria were randomly distributed into two treatment groups: CBD or VEH. Once the ethanol intake was normalized again, mice received a single administration of CBD (microparticle formulation providing a constant release of CBD, 30 mg/kg/day, s.c.) or VEH. Subsequently, the selected mice underwent FR1 (5 days), FR3 (5 days) and PR (1 day).

Experiment 2: effects of cannabidiol on the reinforcement and motivation for water

In order to discard that the pharmacological treatment with CBD could also modify the motivation of mice for natural stimulus, an oral self-administration with water was performed as previously described (Navarrete *et al.* 2014) (for more details, see Supporting Information).

Experiment 3: effects of cannabidiol on ethanol-induced relapse

In order to evaluate the effects of CBD on ethanol-induced relapse, another OEA was carried out following

the previously mentioned protocol with the following exception: after training, substitution and intake stabilization (8 percent ethanol), the OEA paradigm used in the present experiment included the following consecutive experimental sessions: deprivation period 1—reintroduction 1—deprivation period 2—reintroduction 2-extinction–relapse (Supporting Information Fig. S1). The periods of deprivation consisted in 2-day suspension of ethanol self-administration. Mice remained in their home cages and received water and food *ad libitum*. After this period, mice were exposed again to the operant chambers, and their responses were assessed for 5 days (re-introduction sessions). Once stable responses for ethanol after the second re-introduction were reached (5 days), an extinction period, in which responding on the active lever delivered water, was introduced. Test for reinstatement began after 5 days of extinction, once the mice reached the extinction criteria: a significant reduction in the number of active lever presses compared with the number of active lever presses during the last day of FR1. During the days of reinstatement evaluation, responding on the active lever delivered EtOH 8 percent (v/v). During the last 2 days of reinstatement evaluation, mice received CBD (60 mg/kg, i.p.) (first day) and CBD (120 mg/kg, i.p.) (second day) 90 minutes before the session.

Gene expression studies by real-time polymerase chain reaction

Relative gene expression analyses of TH in the VTA and μ-opioid (Oprm1), cannabinoid (CB_1r and CB_2r) and GPR55 receptors in the NAcc were carried out in mice exposed to the TBC and OEA-experiment 1. Briefly, brain sections were cut (500 μm) in a cryostat (−10°C), containing the regions of interest according to Paxinos and Franklin (Paxinos & Franklin 2001), mounted onto slides and stored at −80°C. Sections were microdissected following the method described by Palkovits (Palkovits 1983; Garcia-Gutierrez *et al.* 2010).Total RNA was obtained from brain micropunches with TRI Reagent extraction reagent (Applied Biosystems, Madrid, Spain). After DNAse digestion, 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), CB_1r (Mm00432621_s1), CB_2r (Mm00438286_m1) and GPR55 (Mm02621622_s1) gene expressions was performed on the StepOne Sequence Detector System (Applied Biosystems, Madrid, Spain). All reagents were obtained from Life Technologies, and the manufacturer's protocols were followed. The reference gene used was 18S rRNA (Mm03928990_g1), detected using Taqman ribosomal RNA control reagents. All primer-probe combinations were optimized and validated for relative quantification of gene expression. 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 & Schmittgen 2001).

Statistical analyses

Statistical analyses were performed using two-way analysis of variance (ANOVA) with repeated measures followed by the Student–Newman–Keuls test to compare the treatment and control groups at different time points on the TBC and OEA paradigms. HIC, BEC and the sensitivity to acute ethanol-induced hypothermia test were also analyzed using two-way analysis of variance (ANOVA) with repeated measures followed by the Student–Newman–Keuls test. Statistical analyses of gene expression studies and progressive ratio phase in OEA were performed using the Student's *t*-test. Statistical analyses were performed with SIGMASTAT (Systat Software Inc., Chicago, IL, USA) software. Differences were considered significant if the probability of error was less than 5 percent.

RESULTS

Handling induced convulsions test

Handling-induced convulsion score was measured every hour, up to 6 hours after ethanol administration. A single dose of ethanol (4 g/kg, i.p.) produced significantly more signs of withdrawal in VEH + EtOH group (n = 10) than in CBD + EtOH (n = 10) and CBD + saline (n = 10) groups during the first and second hours of withdrawal (Supporting Information Fig. S2a) [two-way repeated measures (RM) ANOVA, treatment $F_{(2,209)} = 27.630$; $P < 0.001$, time course $F_{(6,209)} = 29.246$; $P < 0.001$, treatment × time course $F_{(12,209)} = 25.408$; $P < 0.001$].

Ethanol-induced hypothermia

After an acute administration of ethanol (3 g/kg, p.o.), VEH + EtOH (n = 10) mice reached their greatest hypothermia at 30 minutes, while CBD + saline (n = 10) and CBD + EtOH (n = 10) mice did not alter their basal temperature. The temperature in VEH + EtOH mice decreased notably at 30 minutes and then gradually increased to reach almost baseline temperature at 480 minutes (Supporting Information Fig. S2b) (two-way RM ANOVA, treatment $F_{(2,149)} = 0.803$; $P = 0.459$, temperature $F_{(4,149)} = 10.352$; $P < 0.001$, treatment × temperature $F_{(8,149)} = 5.548$; $P < 0.001$).

Blood ethanol concentration

No significant differences were found in BEC between CBD + EtOH ($n = 10$) and VEH + EtOH groups ($n = 10$) (Supporting Information Fig. S2c) (Student's *t*-test, $t = -0.00977$, 16 d.f., $P = 0.992$).

Voluntary ethanol consumption in a two-bottle choice paradigm

A significant reduction of ethanol consumption (Fig. 1b) (two-way RM ANOVA, treatment $F_{(1,446)} = 41.104$; $P < 0.001$, day $F_{(15,446)} = 8.386$; $P < 0.001$, treatment × day $F_{(15,446)} = 3.583$; $P < 0.001$) and preference (Fig. 1c) (two-way RM ANOVA; treatment $F_{(1,438)} = 56.554$, $P < 0.001$; day $F_{(15,438)} = 3.085$, $P < 0.001$; treatment × day $F_{(15,446)} = 2.796$, $P < 0.001$) were observed in the CBD-treated group ($n = 10$) compared with VEH group ($n = 10$).

Gene expression alterations induced by cannabidiol i.p. administration during two-bottle choice

The results showed that the administration with CBD decreased TH gene expression in the VTA (Fig. 2a) (Student's *t*-test: $t = 9.865$, 28 d.f., $P < 0.001$) and Oprm1 (Fig. 2b) (Student's *t*-test: $t = 3.859$, 15 d.f., $P = 0.002$), CB_1r (Fig. 2c) (Student's *t*-test: $t = 7.235$, 28 d.f., P = <0.001) and GPR55 gene expression (Fig. 2e) (Student's *t*-test: $t = 5.691$, 15 d.f., $P < 0.001$) in the NAcc. On the other hand, a significant increase of CB_2r gene expression in the NAcc was observed in CBD-treated group compared with VEH-treated group (Fig. 2d) (Student's *t*-test: $t = -5.313$, 28 d.f., $P < 0.001$).

Oral ethanol self-administration

Experiment 1: effects of cannabidiol on the reinforcement and motivation for ethanol

During the stabilization phase, no significant difference was observed in the number of active lever presses (Fig. 3b) (two-way RM ANOVA; group $F_{(1,99)} = 0.05$, $P = 0.826$; day $F_{(4,99)} = 12.269$, $P < 0.001$; group × day $F_{(4,99)} = 0.528$, $P = 0.715$) nor in the ethanol intake (Fig. 3c) (two-way RM ANOVA; group $F_{(1,99)} = 0.254$, $P = 0.620$; day $F_{(4,99)} = 12.434$, $P < 0.001$;

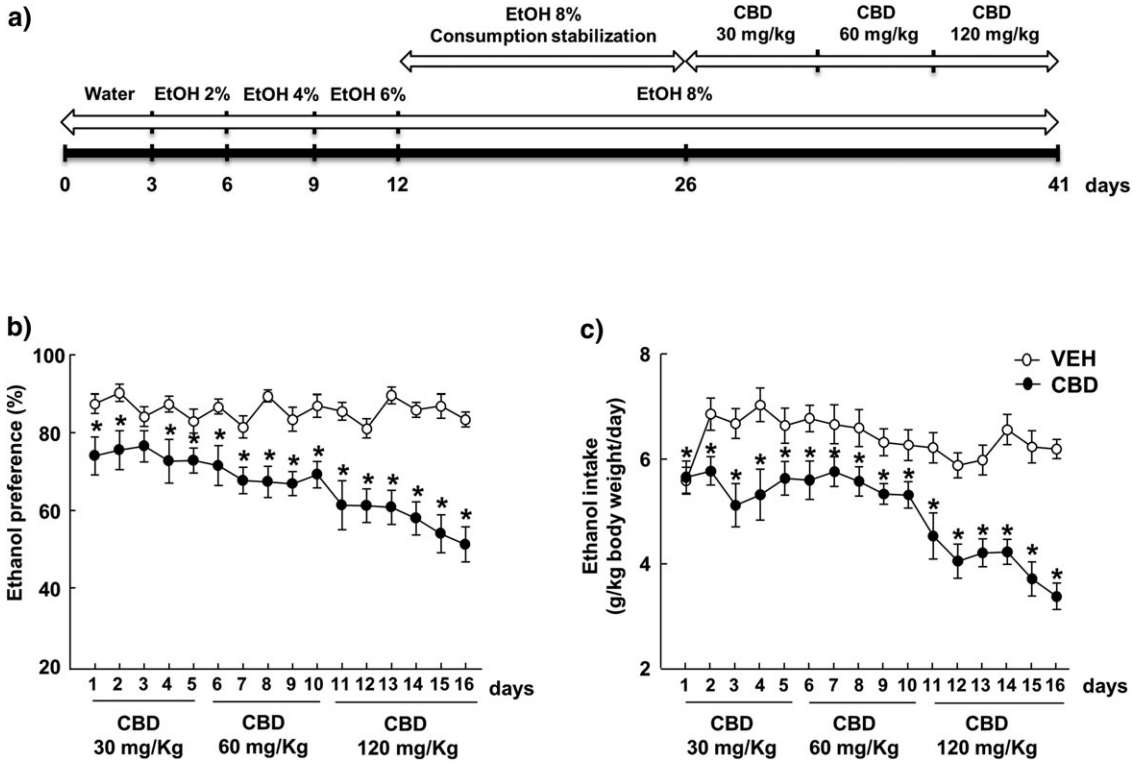

Figure 1 Evaluation of two-bottle choice experiment in C57BL/6 J mice treated with VEH or cannabidiol (CBD). (a) Schematic diagram of the two-bottle choice paradigm. The ethanol concentration was gradually increased (2, 4, 6 and 8 percent v/v) every 4 days until ethanol 8 percent v/v was stabilized. After that, mice underwent treatment with CBD (starting dose was 30 mg/kg, and it was increased every 3 days until reaching 120 mg/kg). (b) Preference for ethanol consumption expressed as the ratio of the preference for ethanol consumption [ethanol preference = ethanol consumption/(ethanol consumption + water consumption)]. (c) The measures were taken from volume of ethanol consumed every 24 hours and expressed as g/kg/day. The dots represent the means and vertical lines ± the standard error of the mean. *Represents values from CBD-treated mice that are significantly different (two-way repeated measures ANOVA, $P < 0.005$) (Student's *t*-test, $P < 0.005$) from vehicle-treated group (VEH)

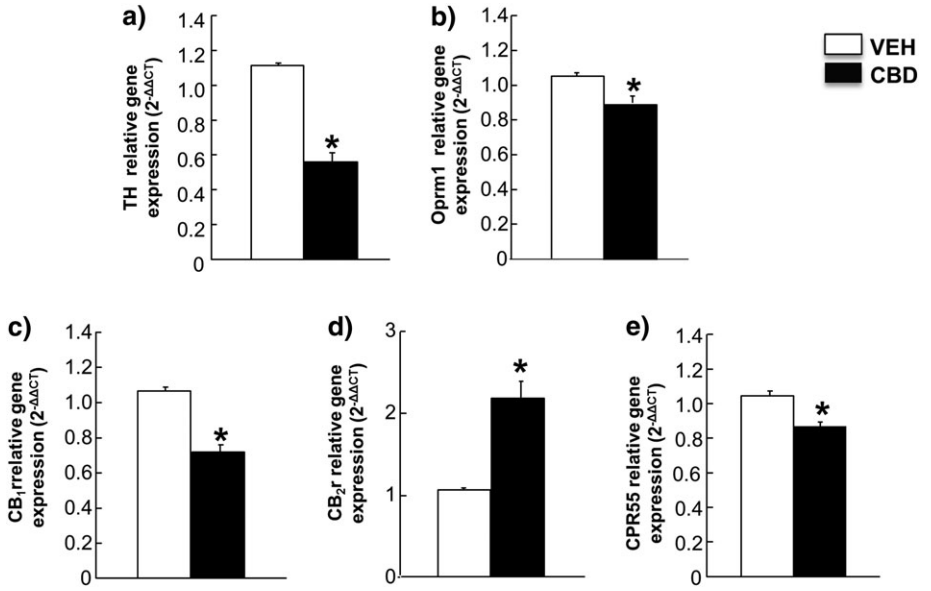

Figure 2 Real-time polymerase chain reaction studies of tyrosine hydroxylase (TH) in the VTA and Oprm1, CB_1r, CB_2r and GPR55 in the nucleus accumbens and of C57BL/6 J mice treated with increasing doses of cannabidiol (CBD) (30, 60 and 120 mg/kg) during the two-bottle choice paradigm. $2^{-\Delta\Delta CT}$ relative gene expression of (a) TH, (b) Oprm1, (c) CB_1 receptor, (d) CB_2 receptor and (e) GPR55. Columns represent the means and vertical lines ± the standard error of the mean. *Represents values from CBD-treated mice that are significantly different (Student's *t*-test, $P < 0.005$) from vehicle-treated group (VEH)

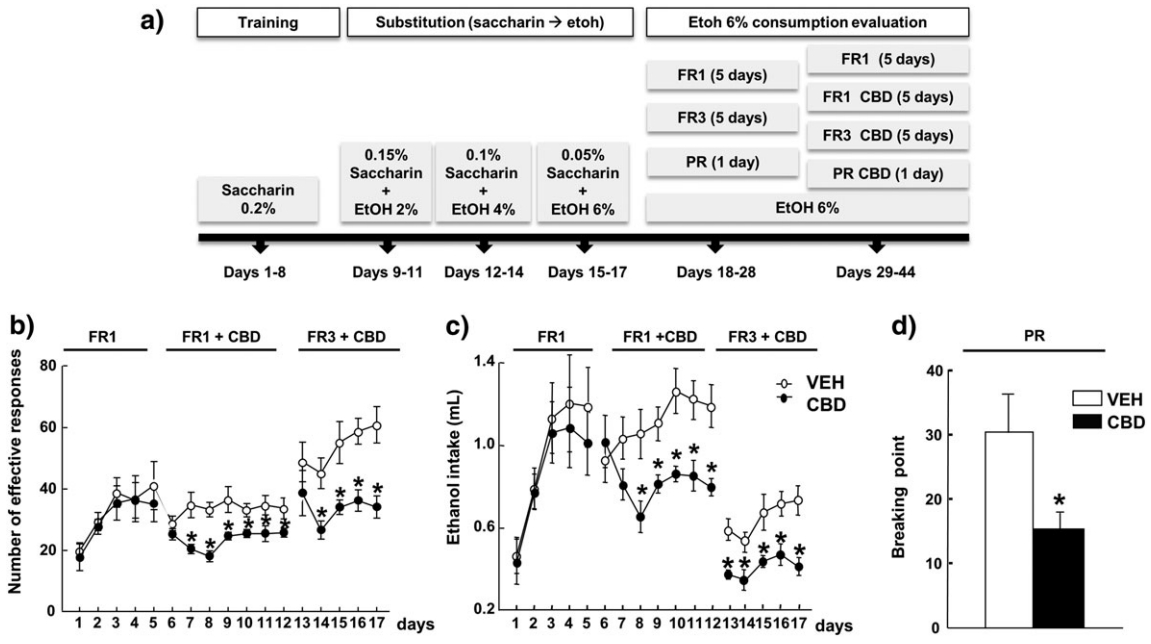

Figure 3 Evaluation of oral ethanol self-administration in C57BL/6 J mice treated with vehicle or cannabidiol (CBD). (a) Schematic diagram including the different experimental phases of ethanol self-administration FR1, fixed ratio 1; FR3, fixed ratio 3; and PR, progressive ratio. (b) Number of effective responses of both groups (VEH and CBD) during the FR1 stabilization and FR1 + treatment and FR3 + treatment stages; (c) ethanol intake expressed as ml of both groups (VEH and CBD) during the FR1 stabilization, FR1 + treatment and FR3 + treatment; (d) breaking point achieved during progressive ratio. The dots represent the means and vertical lines ± the standard error of the mean (SEM) and the columns represent the means and vertical lines ± SEM of. *Represents values from CBD-treated mice that are significantly different (Figs 3b& 3c, two-way RM ANOVA, $P < 0.005$) (Fig. 3d, Student's *t*-test, $P < 0.005$) from vehicle-treated group (VEH)

group × day $F_{(4,99)} = 0.147$, $P = 0.964$) between mice that will be treated with CBD and those that will be treated with VEH.

The administration of the CBD-controlled release microparticle s.c. formulation (30 mg/kg/day, s.c.) significantly reduced the number of active lever presses (Fig. 3b)

(two-way RM ANOVA; treatment $F_{(1,139)} = 21.911$, $P < 0.001$; day $F_{(6,139)} = 1.659$, $P = 0.138$; treatment × day $F_{(6,139)} = 3.236$, $P = 0.006$) and ethanol intake (Fig. 3c) (two-way RM ANOVA; treatment $F_{(1,139)} = 11.851$, $P = 0.003$; day $F_{(6,139)} = 1.861$, $P = 0.094$; treatment × day $F_{(6,139)} = 3.238$, $P = 0.006$) in FR1. Also, CBD reduced the number of active lever pressers (Fig. 3b) (two-way RM ANOVA; treatment $F_{(1,99)} = 70.971$, $P < 0.001$, day $F_{(4,99)} = 6.129$, $P < 0.001$; treatment × day $F_{(4,99)} = 2.745$, $P = 0.035$) and ethanol intake (Fig. 3c) (two-way RM ANOVA; treatment $F_{(1,99)} = 156.687$, $P < 0.001$; day $F_{(4,99)} = 12.765$, $P < 0.001$; treatment × day $F_{(4,99)} = 2.551$, $P = 0.046$) in FR3. Interestingly, CBD-treated mice achieved a lower breaking point compared with the VEH group (Fig. 3d) (Student's *t*-test, $t = 2.368$, 18 d.f., $P = 0.029$).

Experiment 2: effects of cannabidiol on the reinforcement and motivation for water

The administration of the CBD-controlled release microparticle s.c. formulation (30 mg/kg/day, s.c.) did not reduce the number of active lever presses in FR1 (two-way RM ANOVA; group $F_{(1,104)} = 0.0477$, $P = 0.829$; day $F_{(4,104)} = 6.958$, $P < 0.001$; group × day $F_{(4,104)} = 0.877$, $P = 0.482$) nor in FR3 (two-way RM ANOVA; group $F_{(1,104)} = 0.0451$, $P = 0.510$; day $F_{(4,104)} = 1.988$, $P = 0.105$; group × day $F_{(4,104)} = 0.611$, $P = 0.656$) (Supporting Information Fig. S3a). No reduction in the water intake was seen after the administration of CBD in FR1 (two-way RM ANOVA; group $F_{(1,104)} = 0.0081$, $P = 0.929$; day $F_{(4,104)} = 6.996$, $P < 0.001$; group × day $F_{(4,104)} = 0.876$, $P = 0.483$) nor in FR3 (two-way RM ANOVA; group $F_{(1,104)} = 0.213$, $P = 0.65$; day $F_{(4,104)} = 1.219$, $P = 0.310$; group × day $F_{(4,104)} = 0.747$, $P = 0.563$) (Supporting Information Fig. S3b). No difference was found between the breaking point achieved in the PR stage by the group treated with CBD and the one treated with VEH (Student's *t*-test, $t = 0.257$, 19 d.f., $P = 0.800$) (Supporting Information Fig. S3c).

Gene expression alterations induced by cannabidiol controlled release microparticle subcutaneous formulation (30 mg/kg, s.c.)

The administration of CBD significantly decreased TH gene expression (Fig. 4a) (Student *t*-test: $t = 3.859$, 15 d.f., $P = 0.002$) in the VTA and Oprm1 (Fig. 4b) (Student *t*-test: $t = 3.173$, 18 d.f., $P = 0.005$), CB_1r (Fig. 4c) (Student *t*-test: $t = 2.722$, 16 d.f., $P < 0.05$) and GPR55 gene expression (Fig. 4e) (Student *t*-test: $t = 5.691$, 15 d.f., $P = <0.001$) in the NAcc. Interestingly, CBD significantly increased CB_2r gene expression in NAcc (Fig. 4d) (Student *t*-test: $t = -15.700$, 14 d.f., $P < 0.001$) compared with VEH group.

Experiment 3: effects of cannabidiol on ethanol relapse

During the normalization FR1 phase, no significant difference in the number of active lever presses (Fig. 5a) (two-way RM ANOVA; group $F_{(1,543)} = 0.227$, $P = 0.637$; day $F_{(15,543)} = 3.037$, $P < 0.001$; group × day $F_{(15,543)} = 0.849$, $P = 0.622$) nor in the ethanol intake (Fig. 5b) (two-way RM ANOVA group $F(1,543) = 0.005$; $P = 0.943$, day $F(15,543) = 2.924$; $P < 0.001$ and group × day $F(1,543) = 1.207$; $P = 0.262$) were observed between mice that will be treated with CBD and those that will be treated with VEH.

During the extinction period, a significant reduction in the number of active presses was observed in both groups compared with the last day of FR1 before extinction. The degree of reduction was similar in both groups: 66.29 percent in mice that will be treated with VEH and 57.94 percent in those that will be treated with CBD (Fig. 5a) (two-way RM ANOVA; group $F_{(1,169)} = 0.392$, $P = 0.536$; day $F_{(4,169)} = 0.855$, $P = 0.493$; group × day $F_{(4,169)} = 1.850$, $P = 0.123$).

The administration of CBD (60 mg/kg, i.p.) did not induce any modification in the number of active lever presses nor in ethanol intake. However, the administration of CBD (120 mg/kg, i.p.) significantly reduced the number of active lever presses (Fig. 5a) (two-way RM ANOVA; group $F_{(1,101)} = 2.472$, $P = 0.126$; day $F_{(2,101)} = 4.794$, $P = 0.011$; group × day $F_{(2,101)} = 5.528$, $P = 0.006$) and ethanol intake (Fig. 5b) (two-way RM ANOVA; group $F_{(1,101)} = 3.860$, $P = 0.058$; day $F_{(2,101)} = 1.336$, $P = 0.270$; group × day $F_{(2,101)} = 6.927$, $P = 0.002$) compared with its corresponding control group.

DISCUSSION

The present study clearly demonstrated that the administration of CBD reduced the reinforcing properties, motivation and relapse for ethanol. This statement is supported by the following observations: (1) The administration of CBD (60 mg/kg, i.p.) reduced hypothermia and HICs associated with high acute doses of ethanol. Indeed, CBD did not modify BEC. (2) CBD (30, 60 and 120 mg/kg, i.p.) significantly decreased ethanol consumption and preference in the TBC. (3) A single s.c. administration of a microparticle formulation providing CBD continuous controlled release (30 mg/kg/day) significantly reduced ethanol intake and motivation to drink ethanol in the OEA; nevertheless, CBD administration had no effect over natural stimulus such as water. (4) Furthermore, the administration of CBD (60 and 120 mg/kg, i.p.) reduced alcohol-induced relapse in the OEA. (5) These behavioral

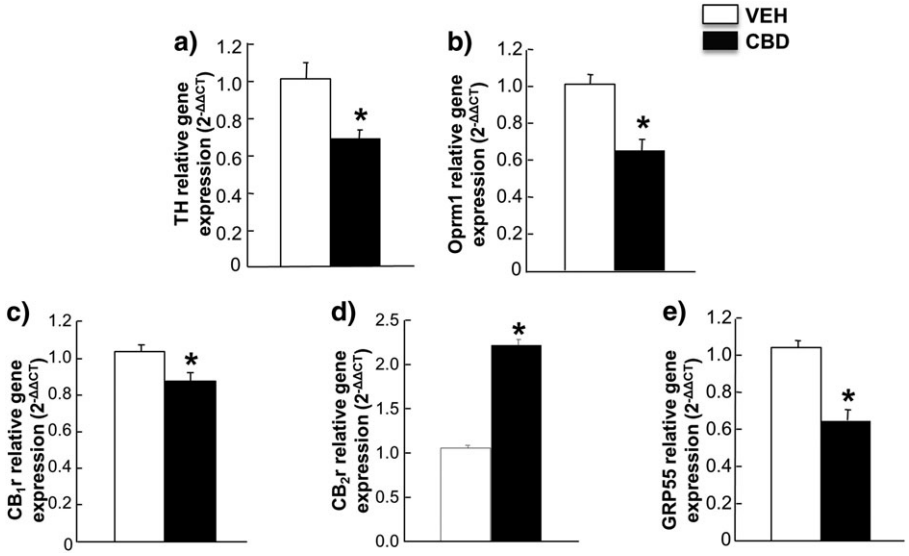

Figure 4 Real-time polymerase chain reaction studies of Oprm1, GPR55, CB_1r and CB_2r in the nucleus accumbens and tyrosine hydroxylase (TH) in the VTA of C57BL/6 J mice treated with cannabidiol (CBD) (a single administration of a microparticle formulation providing CBD continuous controlled release (30 mg/kg/day, s.c.) during the oral ethanol self-administration. $2^{-\Delta\Delta CT}$ relative gene expression of (a) TH, (b) Oprm1, (c) CB_1 receptor, (d) CB_2 receptor and (e) GPR55. Columns represent the means and vertical lines ± the standard error of the mean (SEM). *Represents values from CBD treated mice that are significantly different (Student's *t*-test, $P < 0.005$) from vehicle-treated group (VEH)

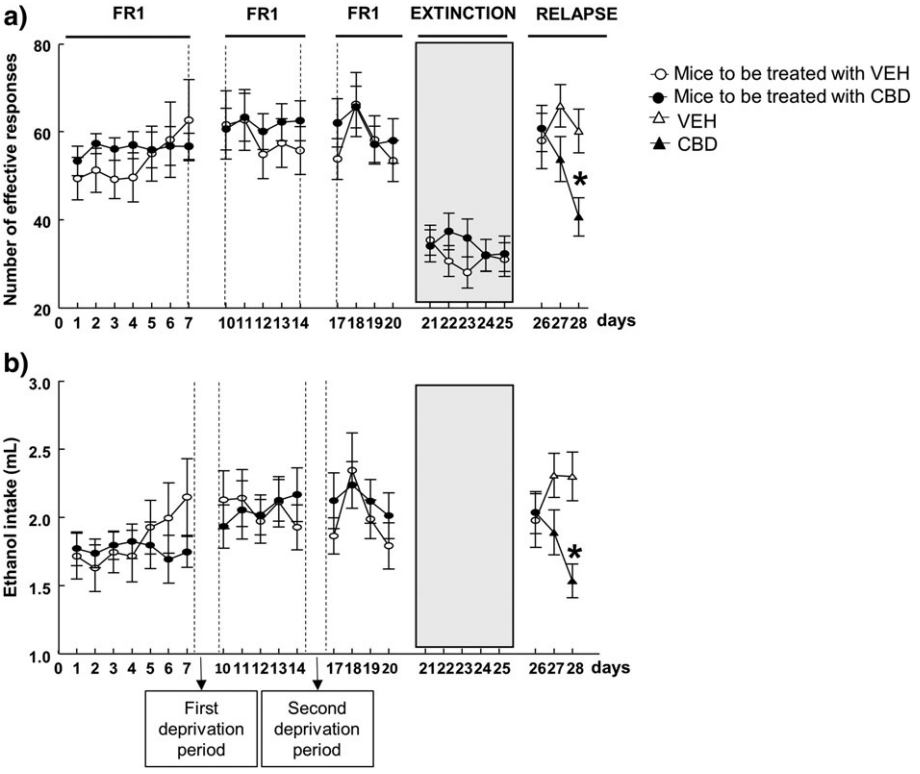

Figure 5 Effects of cannabidiol (CBD) (60 and 120 mg/kg/day, i.p.) on ethanol relapse. (a) The number of effective responses of both groups (VEH and CBD) during the fixed ratio 1 (FR1), extinction and relapse phases; (b) the ethanol intake (EtOH 8 percent v/v) during FR1 and relapse phases. The dots represent the means and the vertical lines ± the standard error of the mean. *****Represents values from CBD treated mice that are significantly different (two-way RM ANOVA, $P < 0.005$)

alterations were accompanied by gene expression alterations in cannabinoid (CB_1r and CB_2r) and GPR55 receptors and Oprm1 in the NAcc and TH in the VTA.

Recently, CBD has emerged as a promising therapeutic option for the treatment of AUD based on its anxiolytic, antidepressant, antipsychotic and neuroprotective

properties (Guimaraes *et al.* 1990; Zuardi *et al.* 1991; Hamelink *et al.* 2005; Long *et al.* 2006; Moreira *et al.* 2006; El-Alfy *et al.* 2010; Zanelati *et al.* 2010; Leweke *et al.* 2012; Levin *et al.* 2014). In our study, several paradigms were used to evaluate the effects of CBD on ethanol reinforcement, motivation and relapse in C57BL/6 J mice.

Initially, we evaluated the effects of CBD (60 mg/kg, i.p.) on BEC, hypothermia and HICs associated to acute ethanol administration. The administration of CBD reduced the hypothermia and HICs associated with the acute administration of high doses of ethanol. Interestingly, the results demonstrated that CBD did not modify BEC. Despite further studies are needed to elucidate the exact mechanism underling these effects, it is tempting to speculate that a pharmacodynamic interaction between CBD and ethanol may be involved.

To elucidate if CBD may modulate the reinforcing properties of ethanol, the effects of CBD were evaluated in the TBC. The results demonstrated that CBD (30, 60 and 120 mg/kg, i.p.) significantly reduced ethanol consumption and ethanol preference. These results suggest that CBD reduces the motivational properties of ethanol.

Furthermore, to evaluate the effects of CBD on motivation for ethanol consumption, an oral self-administration study was performed. As previously mentioned, one of the main limitations to study the effects of CBD is the low bioavailability associated to certain patterns of administration, such as the oral and oral-mucosal/sublingual administrations (Hawksworth & McArdle 2004). For this reason, other routes of administration, such as inhaled route, were examined. However, the specialized equipment and patient cooperation needed hampered its potential therapeutic use (Ohlsson *et al.* 1986). In our study, we evaluated an s.c. formulation (poly-ε-caprolactone spherical microparticles with small pores formulation) providing a continuous controlled release of CBD (30 mg/kg, s.c.) during 14 days (de la Ossa Hernán Pérez *et al.* 2012). Data analysis demonstrated that one s.c. administration of this formulation significantly reduced the motivation (number of active lever pressers) and ethanol intake during FR1 and FR3 and PR 11 days after its administration, but had no effect on the water oral self-administration paradigm.

In addition, we also evaluated if CBD may modulate the ethanol-induced relapse. Interestingly, the administration of CBD (60 and 120 mg/kg, i.p.) significantly reduced ethanol-induced relapse in the OEA. Taken together, these results demonstrated that CBD reduced the reinforcing and motivational properties of ethanol and the relapse to ethanol. These studies are in agreement with preliminary results suggesting the potential utility of CBD for the treatment of drug addiction; however, further studies of the effects of CBD in stress-induced or context-induced relapse are needed. Animal studies revealed that CBD reduced reward-facilitating effect of heroin (Katsidoni *et al.*, 2013), withdrawal signs associated with naloxone (Bhargava, 1976) and cue-induced heroin-seeking behavior in rodents (Ren *et al.* 2009). In humans, a case report study revealed that CBD induced a rapid decrease of withdrawal symptoms of cannabis (Crippa *et al.*, 2013). In addition, preliminary clinical studies suggest the potential therapeutic use of CBD for nicotine (Morgan *et al.*, 2010) and heroin-related use disorders (Hurd *et al.*, 2015).

Despite CBD was proposed to activate or modify the function of several receptors in the central nervous system, including CB_1r, CB_2r, GPR55r, TRPV, 5-HT_{1A}, μ and δ receptors (Bisogno *et al.* 2001; Campos *et al.*, 2012; Kathmann *et al.* 2006; Russo *et al.* 2005; Ryberg *et al.* 2007; Thomas *et al.* 2007), the exact mechanisms underlying CBD's actions remain unclear. In this study, we investigated potential alterations in key targets closely related with alcohol addiction, including Oprm-1, TH, CB_1r, CB_2r and GPR55 receptors by real-time polymerase chain reaction in mice exposed to TBC and OEA (Manzanares *et al.* 2005; Erdozain & Callado 2014; Ortega-Alvaro *et al.* 2015). These neurobiological studies were measured in the NAcc and VTA, critical regions for reward, goal-directed behavior and habit formation (Ikemoto & Bonci 2014; Navarrete *et al.* 2014). The results demonstrated that CBD significantly reduced Oprm-1, CB_1r, GPR55 and TH and significantly increased CB_2r gene expression in the VTA in both patterns of administration (i.p. in TBC and s.c. in OEA). Despite few data are available, these results are in agreement with some preliminary studies demonstrating a reduction CB_1r in the NAcc after the administration of CBD (Ren *et al.* 2009). Previous studies suggested that CBD acts as a non-competitive allosteric modulator of CB_1r through the alteration of anandamide hydrolysis by inhibiting its catabolic enzyme, fatty acid amide hydrolase (Bisogno *et al.* 2001; Laprairie *et al.* 2015). Based on that, it is possible to speculate that the modification of the endocannabinoid levels may be responsible (at least in part) of the neurochemical changes induced by CBD. Further studies are needed to elucidate the exact mechanism underlying these neurochemical effects.

In conclusion, the present results clearly demonstrated that CBD reduced the reinforcing and motivational properties of ethanol and prevent ethanol-induced relapse. These behavioral alterations are associated with alterations in key targets closely related with alcohol addiction (Oprm-1, TH, CB_1r, CB_2r and GPR55). Besides, this study points out the CBD poly-ε-caprolactone microspheres formulation as an ideal type of administration that deserves further exploration and CBD as a potential therapeutic drug for the treatment of AUD.

Acknowledgements

This research was supported by 'Instituto de Salud Carlos III' (RETICS, RD12/0028/0019), 'Plan Nacional Sobre Drogas' (PNSD 2016/016) and 'Ministerio de Economía y Competitividad' (FIS, PI14/00438). Adrián Viudez-Martínez is a predoctoral fellow supported by 'Plan Nacional Sobre Drogas' (PNSD 2016/016).

CONFLICT OF INTEREST

All authors state that they have no biochemical financial interest or potential conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Schematic diagram of the oral ethanol self-administration followed in experiment 2 to evaluate the effects of CBD on relapse

Figure S2: Evaluation of physiological effects of ethanol. The dots represent the means and vertical lines ± the standard error of the mean (SEM) of: (A) the hourly measured HICs score of CBD + VEH, CBD + EtOH or VEH + EtOH treated mice (n = 10 per group) after the administration of ethanol (4 g/kg i.p.); (B) results of ethanol (3 g/kg p.o.) induced hypothermia in CBD + VEH, CBD + EtOH or VEH + EtOH treated mice (n = 10 per group). Columns represent the means and vertical lines ± SEM of (C) BEC (mg/dl) 1 h after the administration of ethanol (3 g/kg p.o.). *Represents values from CBD treated mice that are significantly different (Student's t-test, $P < 0.005$)

Figure S3: Effects of CBD on the reinforcement and motivation for water. (A) Schematic diagram including the different experimental phases of oral water self-administration: training; FR1 = fixed ratio 1; FR3 = fixed ratio 3; PR = progressive ratio. (B) Number of effective responses of both groups (VEH and CBD) during FR1, FR3 and PR stages; (C) water intake expressed as mL of both groups (VEH and CBD) during FR1, FR· and PR stages; (D) breaking point achieved during progressive ratio. The dots represent the means and vertical lines ± the standard error of the mean (SEM) and the columns represent the means and vertical lines ± SEM of. *Represents values from CBD-treated mice that are significantly different (Figures 3B and 3C, two-way RM ANOVA, $P < 0.005$) (Figure 3D, Student's t-test, $P < 0.005$) from vehicle-treated group (VEH)