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RESEARCH PAPER

Cannabidiol regulates behavioural alterations and gene expression changes induced by spontaneous cannabinoid withdrawal

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BACKGROUND AND PURPOSE

Cannabidiol (CBD) represents a promising therapeutic tool for treating cannabis use disorder (CUD). This study aimed to evaluate the effects of CBD on the behavioural and gene expression alterations induced by spontaneous cannabinoid withdrawal.

EXPERIMENTAL APPROACH

Spontaneous cannabinoid withdrawal was evaluated 12 h after cessation of CP-55,940 treatment (0.5 mg·kg^{-1} every 12 h, i.p.; 7 days) in C57BL/6J mice. The effects of CBD (5, 10 and 20 mg·kg^{-1}, i.p.) on withdrawal-related behavioural signs were evaluated by measuring motor activity, somatic signs and anxiety-like behaviour. Furthermore, gene expression changes in TH in the ventral tegmental area, and in the opioid μ receptor (*Oprm1*), cannabinoid CB_1 receptor (*Cnr1*) and CB_2 receptor (*Cnr2*) in the nucleus accumbens, were also evaluated using the real-time PCR technique.

KEY RESULTS

The administration of CBD significantly blocked the increase in motor activity and the increased number of rearings, rubbings and jumpings associated with cannabinoid withdrawal, and it normalized the decrease in the number of groomings. However, CBD did not change somatic signs in vehicle-treated animals. In addition, the anxiogenic-like effect observed in abstinent mice disappeared with CBD administration, whereas CBD induced an anxiolytic-like effect in non-abstinent animals. Moreover, CBD normalized gene expression changes induced by CP-55,940-mediated spontaneous withdrawal.

CONCLUSIONS AND IMPLICATIONS

The results suggest that CBD alleviates spontaneous cannabinoid withdrawal and normalizes associated gene expression changes. Future studies are needed to determine the relevance of CBD as a potential therapeutic tool for treating CUD.

Abbreviations

AEA, anandamide; CBD, cannabidiol; CB receptor, cannabinoid receptor; CUD, cannabis use disorder; FAAH, fatty acid amide hydrolase; NAcc, nucleus accumbens; SMART, Spontaneous Motor Activity Recording and Tracking; THC, Δ^9-tetrahydrocannabinol; VTA, ventral tegmental area

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Introduction

Cannabis preparations, such as hashish and marijuana, are the most commonly used illicit drugs worldwide. The data available suggest that their consumption will continue to rise in coming years, representing a serious public health problem (Volkow *et al*., 2014). Approximately 24% of patients initiating treatment for substance abuse are diagnosed with cannabis use disorder (CUD) (Danovitch and Gorelick, 2012). According to the latest *World Drug Report* (United Nations Office on Drugs and Crime, 2016), around 182.5 million people used cannabis in 2016.

To date, neither the European Medicine Agency nor the US Food and Drug Administration have approved any medications for treating CUD; however, different pharmacological approaches have been developed. These fall into two main categories: medications that attenuate symptoms of cannabis withdrawal and/or those that reduce subjective and reinforcing effects of cannabis (for a recent review, see Copeland and Pokorski, 2016). About half the patients treated for CUD report symptoms of a withdrawal syndrome. As these symptoms can serve as a negative reinforcement for relapse to cannabis use in individuals trying to abstain (Levin *et al*., 2010), cannabis withdrawal should be a focus of treatment. Previous clinical trials have evaluated the therapeutic usefulness of different pharmacological approaches for managing cannabis withdrawal and modulating the reinforcing effects and craving for cannabis, with inconsistent results (Danovitch and Gorelick, 2012). However, the overall clinical outcome in the treatment arms of randomized trials is poor, and fewer than 20% of participants achieve long-term abstinence (Stephens and Roffman, 2006). In spite of the animal models developed to analyse cannabis abuse liability (Justinova *et al*., 2005), limited knowledge of the neurochemical mechanisms underlying CUD may contribute, at least in part, to the low efficacy of the medications evaluated to date. Therefore, it is necessary to invest effort and resources into identifying new drugs that, alone or in combination, may improve the efficacy of CUD treatment.

Recent clinical data suggest that **cannabidiol** (CBD), one of the main constituents of the *Cannabis sativa* plant, may hold promise as a therapeutic tool for managing CUD. Our group has recently shown that unlike **Δ^9-tetrahydrocannabinol** (THC), CBD is devoid of rewarding psychotropic properties (Manzanares *et al*., 2016). Studies in animal models have shown that CBD presents anxiolytic (for a recent review, see Blessing *et al*., 2015), antidepressant (Zanelati *et al*., 2010; Schiavon *et al*., 2016) and antipsychotic properties (Zuardi *et al*., 1995; Leweke *et al*., 2012). Although the exact mechanisms underlying these effects remain unclear (Campos *et al*., 2012b), some authors have posited that CBD modulates the function of more than 65 targets in the CNS (Ibeas Bih *et al*., 2015), including cannabinoid receptors (**CB_1** and **CB_2**) and **GPR55**, the **vanilloid receptor TRPV1**, the **$5\text{-}HT_{1A}$ receptor** (Bisogno *et al*., 2001; Russo *et al*., 2005; Ryberg *et al*., 2007; Thomas *et al*., 2007; Campos *et al*., 2012a), the **anandamide** (AEA)-hydrolysing enzyme [**fatty acid amide hydrolase (FAAH)**] and the **adenosine transporter** (Carrier *et al*., 2006; Massi *et al*., 2008). However, additional studies are needed to fully determine CBD's target engagement profile.

Previous clinical results point to the therapeutic usefulness of combining CBD with THC as a 'cannabinoid replacement therapy' to modulate cannabinoid withdrawal (Allsop *et al*., 2015). Combination therapy with THC plus CBD in an oromucosal spray (nabiximols or Sativex in the United States of America or European Union, respectively) shows some interesting therapeutic benefits. Indeed, a recent clinical trial demonstrated that nabiximols suppressed cannabis withdrawal symptoms and achieved successful retention in treatment (Allsop *et al*., 2014). Two additional studies showed that Sativex produces a significant reduction in cannabis withdrawal score, craving and cannabis consumption levels (Trigo *et al*., 2016a; Trigo *et al*., 2016b). However, the presence of THC in nabiximols/Sativex preparations could be problematic, especially in (still unexplored) long-term treatment, since it may be associated with THC-related negative psychoactive effects. Thus, recent interest has turned to clinically evaluating CBD alone for managing CUD-related problems. Crippa *et al*. (2013) found that CBD monotherapy led to a rapid decrease in cannabis withdrawal symptoms. Another clinical study, comparing p.o. CBD alone *versus* placebo, found no difference between groups for cannabis-induced subjective effects (Haney *et al*., 2016). These reports join a raft of ongoing clinical trials evaluating the effects of CBD alone for CUD (Mclean Hospital, NCT03102918), cannabis dependence (University College, London, NCT02044809), cannabis withdrawal (The University of New South Wales, NCT02083874) or smoked marijuana's (5.6% THC) subjective, reinforcing, cognitive and cardiovascular effects (National Institute on Drug Abuse, NCT01844687). In the context of this increasing interest in CBD for managing CUD, studies using animal models are crucial to providing information about the therapeutic potential of CBD and the underlying neurobiological mechanisms involved.

The objective of this study is to evaluate the effects of CBD on the spontaneous withdrawal induced by the repeated administration of the potent synthetic cannabinoid receptor agonist **CP-55,940** (Devane *et al*., 1988; Wiley *et al*., 1995; Oliva *et al*., 2003; 2004; Aracil-Fernandez *et al*., 2013) in C57BL/6J mice. Motor activity (distance travelled in the open field test), withdrawal-related somatic signs (number of rearings, groomings, rubbings and jumpings evaluated in the open field test) and the anxiety-like response (light–dark box test) were assessed 12 h after the last administration of CP-55,940. Furthermore, gene expression analyses by real-time PCR were carried out to evaluate the changes induced by cannabinoid withdrawal in specific key targets involved in cannabinoid addiction and withdrawal (Romero *et al*., 1998; Corchero *et al*., 1999; Manzanares *et al*., 1999; Oliva *et al*., 2003, 2004; Lupica *et al*., 2004; Corchero *et al*., 2004a, b; Fattore *et al*., 2008; Aracil-Fernandez *et al*., 2013), namely, **TH** in the ventral tegmental area (VTA), and the opioid **μ receptor** (*Oprm1*), CB_1 receptor (*Cnr1*) and CB_2 receptor (*Cnr2*) in the nucleus accumbens (NAcc). Briefly, TH is the rate-limiting enzyme for dopamine synthesis in the VTA, playing a pivotal role in the rewarding effects of cannabinoids. Similarly, the μ receptor in the NAcc plays a crucial role in the reinforcing actions of cannabinoid drugs, increasing the release of endogenous opioid peptides that in turn enhance the dopamine tone. Finally, CB_1 and CB_2 receptors directly

modulate the neurobiological actions produced by the administration of cannabinoid compounds.

Methods

Mice

A total of 180 male C57BL/6J mice from Charles River (Lille, France) weighing 20–25 g were employed in this study, and were housed in groups of five per cage (40 × 25 × 22 cm) under controlled conditions (temperature, 23 ± 2°C; relative humidity, 60 ± 10%; and 12 h light/dark cycle, lights on from 08:00 to 20:00 h). One week after acclimatization to the animal room, behavioural analyses were initiated and performed by placing the home cage in the operant-task room during the development of conditioning experiments. All studies complied with the Spanish Royal Decree 53/2013, 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 were approved by the ethics committee of Miguel Hernandez University. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015).

Drugs

Cannabinoid CB_1 receptor agonist CP-55,940 ((−)-*cis*-3-(2-hydroxy-4-(1,1,dimethyl-heptyl)-phenyl)-*trans*-4(3-hydroxypropyl)cyclohexanol) obtained from Biogen (Madrid, Spain) was dissolved in ethanol : cremophor : saline (1:1:18) immediately before use and administered i.p. (0.5 mg·kg^{-1} every 12 h, for 7 days) as described elsewhere (Aracil-Fernandez *et al.*, 2013). CBD, obtained from STI Pharmaceuticals (Essex, UK), was dissolved in ethanol : cremophor : saline (1:1:18) immediately before use to obtain the required doses (5, 10 and 20 mg·kg^{-1}) and administered i.p. during spontaneous cannabinoid withdrawal 90 min before behavioural evaluation according to plasma and brain pharmacokinetic profiles (Deiana *et al.*, 2012).

Experimental design

Evaluation of CBD effects on CP-55,940-induced spontaneous cannabinoid withdrawal. In order to evaluate cannabinoid tolerance and withdrawal, the synthetic cannabinoid agonist CP-55,940 was employed as described previously (Oliva *et al.*, 2003; 2004; Aracil-Fernandez *et al.*, 2013). Forty C57BL/6J male mice were injected with CP-55,940 (0.5 mg·kg^{-1} every 12 h, i.p.) and 10 mice with vehicle (ethanol : cremophor : saline; 1:1:18) for 7 days (schematic diagram displayed in Figure 1A). Changes in rectal temperature and motor activity were measured during tolerance development (days 1, 3 and 7). On the day of withdrawal and following the last cannabinoid administration, CP-55,940-treated mice were randomly allocated to four experimental groups to be administered CBD (5, 10 and 20 mg·kg^{-1}, i.p., 10 mice per dose) or its corresponding vehicle, 90 min before the evaluation of motor activity and behavioural signs associated with abstinence (number of rearings, groomings, rubbings and jumpings) in a 15 min session. At 150 min after administration of the study drug (CBD or vehicle), the brains of these mice were collected for the analysis of alterations in gene transcription. An additional set of 50 C57BL/6J male mice was evaluated during the abstinence period to study the effects of CBD treatment on anxiety-like behaviour induced by cannabinoid withdrawal, following the same randomization procedure to assign animals to the different treatment groups. All the behavioural paradigms (open field test to evaluate motor activity and somatic signs and light–dark box test to evaluate anxiety-like level) were made under blind conditions.

Evaluation of CBD effects on vehicle-treated animals. Forty C57BL/6J male mice were injected with vehicle (ethanol : cremophor : saline; 1:1:18; twice a day every 12 h, i.p.) for 7 days (schematic diagram displayed in Figure 1B) in order to study the effects of CBD alone. Changes in rectal temperature and motor activity were measured during

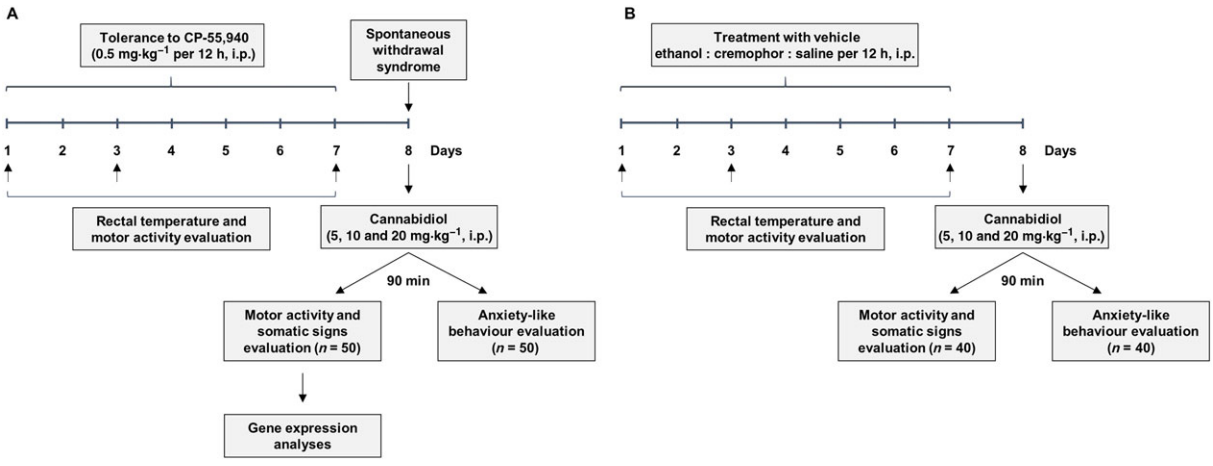

Figure 1

Experimental designs to induce and evaluate the spontaneous cannabinoid withdrawal (A) and analyse the effects of CBD administration in vehicle-treated animals (B).

treatment with vehicle (days 1, 3 and 7). On the day after the last vehicle administration, mice were randomly allocated to four experimental groups to be administered CBD (5, 10 and 20 mg·kg^{-1}, i.p., 10 mice per dose) or its corresponding vehicle, 90 min before the evaluation of motor activity and behavioural signs associated with abstinence (number of rearings, groomings, rubbings and jumpings) in a 15 min session. An additional set of 40 C57BL/6J male mice was evaluated after the last vehicle administration to study the effects of CBD treatment on anxiety-like behaviour, following the same randomization procedure to assign animals to the different treatment groups. All the behavioural paradigms (open field test to evaluate motor activity and somatic signs and light–dark box test to evaluate anxiety-like level) were made under blind conditions.

Assessment of tolerance to cannabinoid administration

Motor behaviour – open field test. On day 0, under baseline conditions and 60 min after the administration of CP-55,940 or vehicle on days 1, 3 and 7, the mice were placed into individual methacrylate boxes (25 × 25 × 25 cm), and their motor responses were evaluated according to distance travelled for 15 min. The assessment of motor activity was carried out by using the Spontaneous Motor Activity Recording and Tracking (SMART) programme by Panlab (Barcelona, Spain).

Rectal temperature. Rectal temperature was measured in each mouse using a lubricated digital thermistor probe (Panlab), inserted 1 cm into the rectum for 30 s. Rectal temperature was determined 30 min before and after each morning injection of CP-55,940 or vehicle during the tolerance period on days 1, 3 and 7.

Behavioural assessment after cannabinoid withdrawal

Behaviour in CP-55,940-induced spontaneous cannabinoid withdrawal and vehicle-treated animals was assessed by measuring motor activity; the number of rearings, groomings, rubbings, jumpings; and the anxiety-like behaviour on the day after the cessation of cannabinoid or vehicle treatment.

Motor activity and somatic expression. Twelve hours after cessation of treatment with CP-55,940 or vehicle, mice were placed into individual methacrylate boxes (25 × 25 × 25 cm). Ninety minutes after CBD (5, 10 and 20 mg·kg^{-1}) or vehicle administration, mice behaviour was videotaped for 15 min, and the somatic signs associated with abstinence (number of rearings, groomings, rubbings and jumpings) were subsequently analysed from the recording. At the same time, motor responses were also evaluated by measuring the distance travelled by the mice for 15 min with the SMART programme (Panlab).

Light–dark box test. To evaluate anxiety-like behaviour associated with cannabinoid withdrawal, 12 h after cessation of treatment with CP-55,940 or vehicle, mice were individually tested for 5 min in the light–dark box paradigm, 90 min after the administration of CBD (5, 10 and 20 mg·kg^{-1}) or its corresponding vehicle. At the beginning of the session, the mice were placed in the tunnel facing the dark side. The number of transitions and the time spent on the light side were measured in each session.

Gene expression studies by real-time PCR

Relative gene expression analyses of TH in the VTA, and *Oprm1*, *Cnr1* and *Cnr2* in the NAcc, were carried out in vehicle-treated and CP-55,940-treated C57BL/6J mice employed to evaluate the effects of CBD on motor activity and somatic signs during CP-55,940-induced spontaneous cannabinoid withdrawal (see Figure 1A). Briefly, mice were killed by cervical dislocation 150 min after CBD administration on day 8. Brains were removed from the skull and frozen over dry ice. Coronal sections (500 μm) containing the regions of interest were cut in a cryostat (−10°C) according to the Paxinos and Franklin atlas (Paxinos and Franklin, 2001), mounted onto slides and stored at −80°C. Sections were microdissected following the method described by Palkovits (Palkovits, 1983). Total RNA was obtained from brain micropunches by treatment with TRI Extraction Reagent (Applied Biosystems, Madrid, Spain). Reverse transcription to cDNA was carried out following the manufacturer's instructions (Applied Biosystems). The relative abundance of TH (Mm00447546_m1), *Oprm1* (Mm01188089_m1), *Cnr1* (Mm00432621_s1) and *Cnr2* (Mm00438286_m1) gene expression was quantified in a StepOne Plus Sequence Detector System (Life Technologies, Madrid, Spain). Each assay was undertaken in technical duplicate to ensure the reliability of single values and the average calculated for data analyses. All reagents were obtained from Life Technologies according to the manufacturer's protocols. The reference gene used was 18S rRNA (Mm03928990_g1). 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 and Schmittgen, 2001).

Statistical analyses

Statistical analyses were performed using one- and two-way ANOVA with repeated measures (RM) when several time points are present, followed by the Student–Newman–Keul's test when comparing different experimental groups. Differences were considered significant if the probability of error was less than 5%. Data are presented as mean ± SEM. Sigmaplot 11 software (Systat software Inc., Chicago, IL, USA) was used for all statistical analyses. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Table 1 presents all the statistical data included in the results section. P values < 0.05 were considered to indicate statistical significance.

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,c,d).

Table 1

Details of the statistical results from the different experimental procedures performed in the present study

Experiment	Statistical test	Parameter	Factors	d.f.	*F*	*P*-value
Evaluation of tolerance development to CP-55,940 administration	Two-way RM ANOVA	Motor activity	Treatment	1, 149	21.443	<0.001
			Day	2, 149	4.231	<0.016
			Treatment × day	2, 149	25.210	<0.001
		Rectal temperature	Treatment	1, 149	175.968	<0.001
			Day	2, 149	75.524	<0.001
			Treatment × day	2, 149	70.372	<0.001
Evaluation of CBD effects on spontaneous cannabinoid withdrawal behaviour	One-way ANOVA	Motor activity	Distance travelled (cm)	4, 49	8.116	<0.001
		Somatic signs	Number of rearings	4, 49	6.623	<0.001
			Number of groomings	4, 49	10.055	<0.001
			Number of rubbings	4, 49	5.684	<0.001
			Number of jumpings	4, 49	10.313	<0.001
		Anxiety-like behaviour	Time spent in light box (s)	4, 49	16.157	<0.001
			Number of transitions	4, 49	11.982	<0.001
Evaluation of CBD effects on gene expression changes induced by spontaneous cannabinoid withdrawal	One-way ANOVA	Relative gene expression (real-time PCR)	TH	4, 49	8.797	<0.001
			Oprm1	4, 49	22.859	<0.001
			Cnr1	4, 49	9.298	<0.001
			Cnr2	4, 49	4.141	0.007
Evaluation of motor activity and rectal temperature in vehicle-treated animals	One-way ANOVA	Motor activity	Distance travelled (cm)	2, 119	18.844	<0.001
		Rectal temperature	Temperature change (°C)	2, 119	1.586	0.209
Evaluation of CBD effects on motor activity and somatic signs in vehicle-treated animals	One-way ANOVA	Motor activity	Distance travelled (cm)	3, 39	0.716	0.551
		Somatic signs	Number of rearings	3, 39	0.253	0.859
			Number of groomings	3, 39	0.232	0.874
			Number of rubbings	3, 39	0.148	0.930
		Anxiety-like behaviour	Time spent in light box (s)	3, 39	4.988	0.007
			Number of transitions	3, 39	1.484	0.240

Results

Development of tolerance induced by the administration of CP-55,940

The development of tolerance to cannabinoid administration was evaluated by measuring motor behaviour and rectal temperature. Two-way RM ANOVA revealed decreased motor activity in the CP-55,940-treated group on day 1 compared with the other days and with the vehicle-treated group only on day 1 [Figure 2A; treatment ($P < 0.001$), day ($P = 0.016$) and treatment × day interaction ($P < 0.001$)]. Two-way ANOVA showed that CP-55,940 administration significantly decreased rectal temperature in comparison with the vehicle-treated group on days 1 and 3, reaching similar temperatures to control mice on day 7 [Figure 2B; treatment ($P < 0.001$), day ($P < 0.001$) and treatment × day interaction ($P < 0.001$)].

Effects of CBD on increased motor activity and somatic signs induced by spontaneous cannabinoid withdrawal

The one-way ANOVA showed that CP-55,940 treatment cessation significantly increased motor activity and the number of rearings, rubbings and jumpings compared with vehicle-treated mice [Figure 3A, motor activity ($P < 0.001$); Figure 3B, number of rearings ($P < 0.001$); Figure 3D, number of rubbings ($P < 0.001$); and Figure 3E, number of jumpings ($P < 0.001$)]. The administration of CBD fully blocked these parameters, which reached values similar to those found in the control group. On the other hand, the number of groomings after cannabinoid cessation decreased significantly compared with the control group. One-way ANOVA indicated that the CP-55,940 + CBD-treated group showed a normalization effect reaching a significant difference compared with the CP-55,940 + vehicle-treated group with the highest CBD dose (Figure 3C, number of groomings, $P < 0.001$).

Effects of CBD on anxiogenic-like behaviour induced by spontaneous cannabinoid withdrawal

After CP-55,940 treatment, the one-way ANOVA revealed a significant decrease in the time spent in the light box (Figure 4A, $P < 0.001$) and the number of transitions (Figure 4B, $P < 0.001$) in the CP-55,940 + vehicle-treated group compared with the control group. The administration

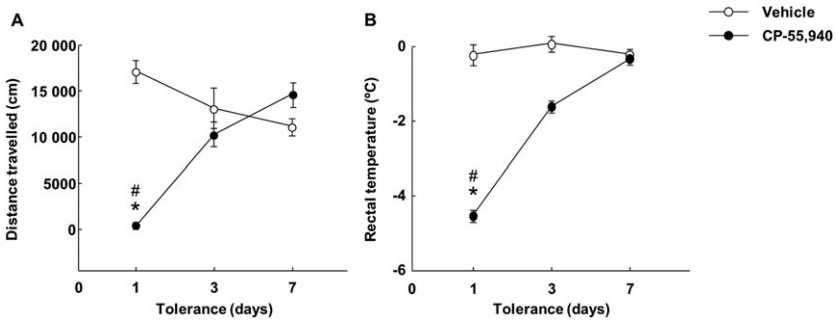

Figure 2

Development of tolerance after repeated administration of CP-55,940. The mice were injected with CP-55,940 (0.5 mg·kg^{-1} every 12 h, i.p.; for 7 days) or with its vehicle. (A) The effects of CP-55,940 administration on motor behaviour. On days 1, 3 and 7, motor activity was measured for 15 min (60 min after the morning CP-55,940 administration) to evaluate tolerance due to cannabinoid treatment. Symbols represent the means and vertical lines ±SEM for distance travelled by mice in the open field test. *Values from CP-55,940-treated mice that are significantly different ($P < 0.05$) from vehicle-treated mice. #Values from CP-55,940-treated mice on day 1 that are significantly different ($P < 0.05$) from CP-55,940-treated mice treated on days 3 and 7 (two-way RM ANOVA followed by Student–Newman–Keul's test). (B) The changes in rectal temperature induced by CP-55,940 treatment. On days 1, 3 and 7, rectal temperature was measured (30 min before and after the morning CP-55,940 administration) to evaluate tolerance. Symbols represent the means and vertical lines ±SEM of differences in rectal temperature. *Values from CP-55,940-treated mice that are significantly different ($P < 0.05$) from vehicle-treated mice. #Values from CP-55,940-treated mice on day 1 that are significantly different ($P < 0.05$) from CP-55,940-treated mice on days 3 and 7 (two-way RM ANOVA followed by Student–Newman–Keul's test).

of CBD blocked, at all doses, the decrease in the time spent in the lighted area, which reached values similar to those found in the control group, without affecting the number of transitions compared with the CP-55,940 + vehicle-treated group.

Effects of CBD on changes in the TH, Oprm1, Cnr1 and Cnr2 gene expression induced by spontaneous cannabinoid withdrawal

Changes in TH gene expression, and in *Oprm1*, *Cnr1* and *Cnr2* gene expressions, were measured in the VTA and in the NAcc

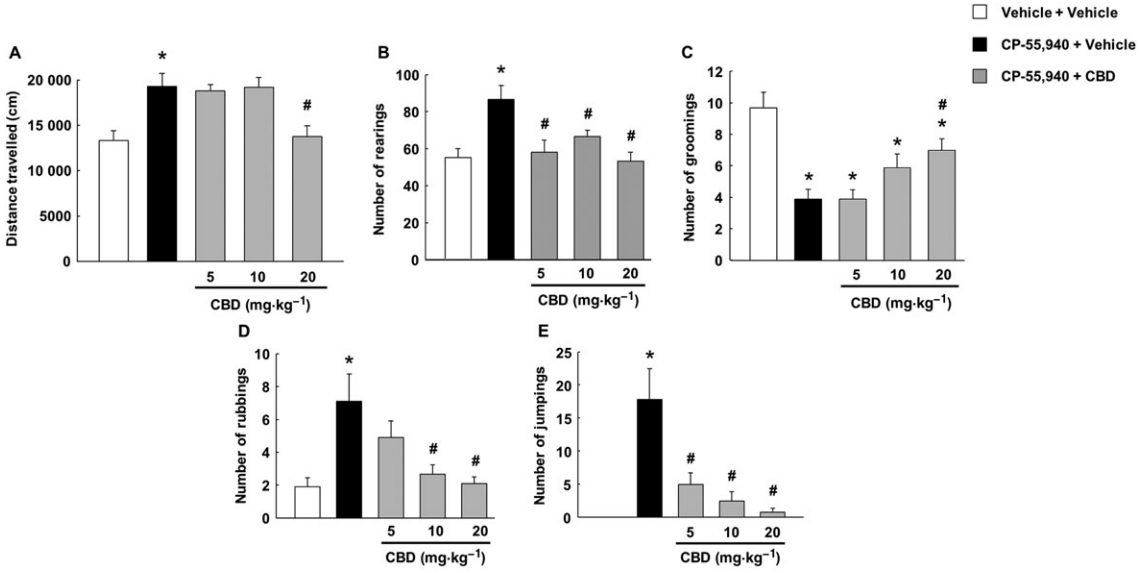

Figure 3

Assessment of spontaneous cannabinoid withdrawal and CBD actions on motor activity and behavioural signs of abstinence (number of rearings, groomings, rubbings and jumpings). Mice were injected with CP-55,940 (0.5 mg·kg^{-1} every 12 h, i.p.) or vehicle (ethanol : cremophor : saline; 1:1:18) for 7 days. CBD (5, 10 and 20 mg·kg^{-1}, i.p.) or vehicle (ethanol : cremophor : saline; 1:1:18) was administered approximately 12 h after the last CP-55,940 administration. Mice received the CBD dose, and 90 min later, motor activity and behavioural signs were evaluated in 15 min sessions. Columns represent the means and vertical lines ±SEM of distance travelled (cm) by mice in the open field test (A) and the number of rearings (B), groomings (C), rubbings (D) and jumpings (E). *Values from CP-55,940-treated mice that are significantly different ($P < 0.05$) from vehicle + vehicle-treated mice. #Values from mice treated with CP-55,940 + CBD that are significantly different ($P < 0.05$) from CP-55,940 + vehicle-treated mice (one-way ANOVA followed by Student–Newman–Keul's test).

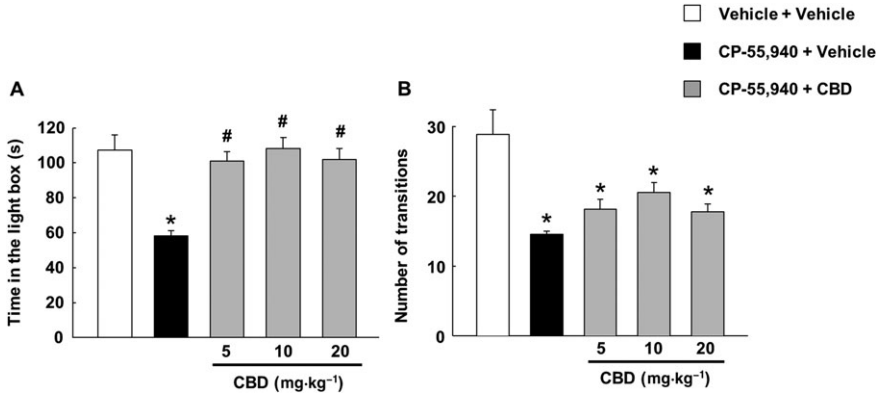

Figure 4

Assessment of anxiety-like behaviour associated with spontaneous cannabinoid withdrawal and subsequent treatment with CBD. Mice were injected with CP-55,940 (0.5 mg·kg^{-1} every12 h, i.p.) or vehicle (ethanol : cremophor : saline; 1:1:18) for 7 days. CBD (5, 10 and 20 mg·kg^{-1}, i.p.) or its vehicle (ethanol : cremophor : saline; 1:1:18) was administered 12 h after the last administration of CP-55,940. Mice received the CBD dose, and 90 min later, they were exposed to the light–dark box paradigm for 5 min. (A) The evaluation of the time in the light box and (B) the evaluation of the number of transitions. Columns represent the means and vertical lines ±SEM of time (s) on the light side and number of transitions. *Values from CP-55,940-treated mice that are significantly different ($P < 0.05$) from the control group. #Values from CP-55,940 + CBD-treated mice that are significantly different ($P < 0.05$) from the CP-55,940 + vehicle-treated mice (one-way ANOVA followed by Student–Newman–Keul's test).

respectively. The results showed that cessation of CP-55,940 administration reduced TH (Figure 5A, one-way ANOVA, $P < 0.001$) and *Cnr2* gene expression (Figure 5D, one-way ANOVA, $P = 0.007$), whereas it increased both *Oprm1* (Figure 5B, one-way ANOVA, $P < 0.001$) and *Cnr1* gene expressions (Figure 5C, one-way ANOVA, $P < 0.001$). The administration of CBD blocked the effects of cannabinoid withdrawal on TH, *Oprm1*, *Cnr1* and *Cnr2* gene expression.

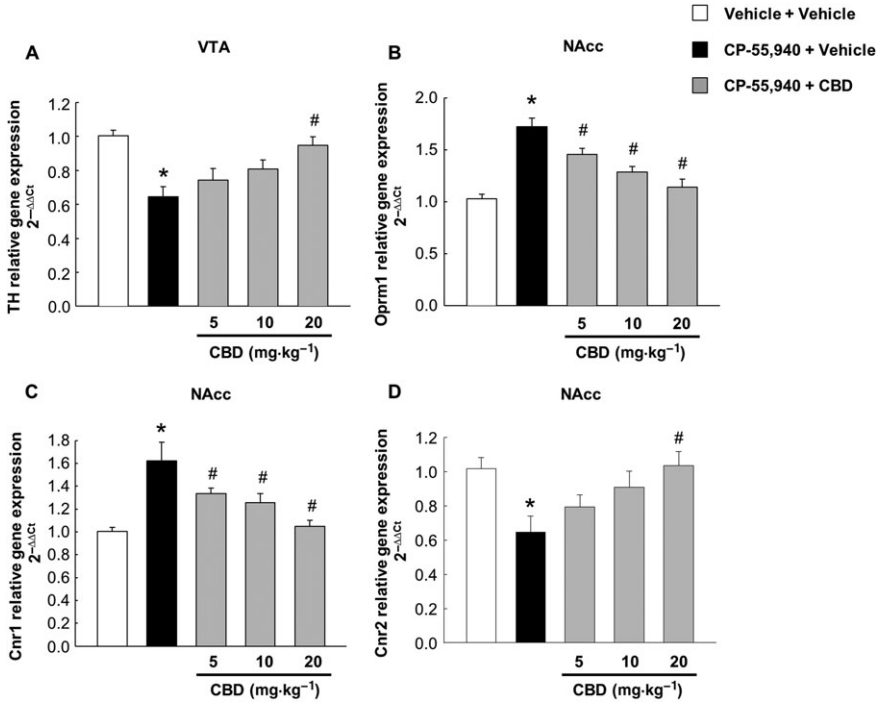

Figure 5

Gene expression changes induced by spontaneous cannabinoid withdrawal and subsequent CBD treatment. Evaluation of TH gene expression in the VTA (A) and of *Oprm1* (B), *Cnr1* (C) and *Cnr2* (D) in the NAcc. Columns represent the means and vertical lines ±SEM of $2^{-\Delta\Delta Ct}$. *Values from CP-55,940-treated mice that are significantly different ($P < 0.05$) from vehicle-treated mice. #Values from CP-55,940 + CBD-treated mice that are significantly different ($P < 0.05$) from CP-55,940 + vehicle-treated mice (one-way ANOVA followed by Student–Newman–Keul's test).

Evaluation of motor activity and rectal temperature during 7 days of vehicle administration

Distance travelled and rectal temperature variation (30 min before and after administration) was evaluated in the animals treated with vehicle. Motor activity progressively decreased from day 1 to day 7 in the open field (Figure 6A, one-way ANOVA, $P < 0.001$) reflecting a habituation effect similar to that observed in the vehicle-treated animals represented in Figure 2. With regard to rectal temperature, the vehicle group did not show any change between the evaluation days (Figure 6B, one-way ANOVA, $P = 0.209$).

Effects of CBD on motor activity and somatic signs in vehicle-treated animals

CBD administration did not induce changes in the somatic signs displayed by the vehicle-treated animals (as assessed by one-way ANOVA) suggesting that the normalization effect achieved in CP-55,940-treated animals was specific [Figure 7A, motor activity ($P = 0.551$); Figure 7B, number of rearings ($P = 0.859$); Figure 7C, number of groomings ($P = 0.874$); and Figure 7D, number of rubbings ($P = 0.930$)]. Figure 7 does not present the number of jumpings as vehicle-treated animals did not display this behaviour.

Effects of CBD on anxiety-like behaviour in vehicle-treated animals

Anxiety-like behaviour in vehicle-treated animals was reduced significantly after the administration of CBD (10 and 20 $mg{\cdot}kg^{-1}$), increasing the latency time in the light box (Figure 8A, $P = 0.007$), whereas the number of transitions was similar between the different treatment groups (Figure 8B, $P = 0.240$).

Discussion

The results of this study suggest that CBD is useful for alleviating the motor symptoms and anxiety-like behaviours associated with spontaneous withdrawal of the cannabinoid receptor agonist CP-55,940, with several observations supporting this assumption: (i) after cessation of treatment with CP-55,940, CBD normalized the increase in the distance travelled and the number of rearings, rubbings and jumpings, as well as the reduction in the number of groomings; (ii) mice receiving CBD showed greatly reduced anxiety-like behaviour associated with the cannabinoid withdrawal compared with controls; (iii) the administration of CBD also blocked the reduction in TH and *Cnr2* gene expression in the VTA and NAcc, respectively, and the increase in *Oprm1* and *Cnr1* gene expression in the NAcc, induced by spontaneous cannabinoid withdrawal; and (iv) CBD did not modify the motor activity or the somatic signs (number of rearings, rubbings and groomings) displayed by non-abstinent mice but induced an anxiolytic-like effect in vehicle-treated animals.

The reduction in motor activity and rectal temperature found on day 1 after CP-55,940 administration returned to normal values on days 3 and 7 of treatment, respectively, indicating the development of tolerance. Subsequently, treatment with CP-55,940 was abruptly discontinued to generate a spontaneous withdrawal (without cannabinoid antagonist administration). This was characterized by a variety of behavioural manifestations, such as an increase in distance travelled, number of rearings, rubbings and jumpings, and anxiety-like behaviour, together with a decrease in the number of groomings. These results are in agreement with previous findings from our laboratory employing the same model of spontaneous cannabinoid withdrawal (Oliva *et al.*, 2003; 2004; Aracil-Fernandez *et al.*, 2013).

The administration of CBD significantly affected the motor activity observed in mice during the spontaneous cannabinoid withdrawal, although this effect was only reached at the highest dose (20 $mg{\cdot}kg^{-1}$). In addition, all CBD doses tested (5, 10 and 20 $mg{\cdot}kg^{-1}$) blocked the increase in the number of rearings and reduced the significant increase in the number of rubbings and jumpings. The number of groomings decreased considerably during cannabinoid withdrawal, as described elsewhere (Cook *et al.*, 1998), but the highest dose (20 $mg{\cdot}kg^{-1}$) of CBD administered led to a significant increase in this behaviour. Interestingly, the effects

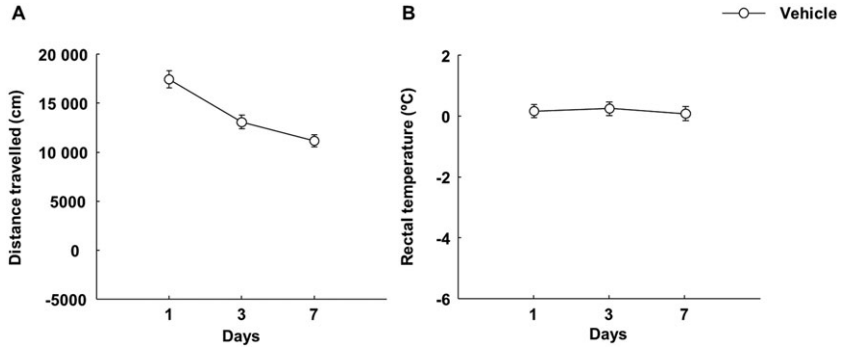

Figure 6

Assessment of motor activity and rectal temperature variations in mice injected i.p. with vehicle (ethanol : cremophor : saline; 1:1:18; every 12 h) for 7 days. (A) The effects of vehicle administration on motor behaviour. On days 1, 3 and 7, motor activity was measured for 15 min (60 min after the morning vehicle administration). Symbols represent the means and vertical lines ±SEM of distance travelled by mice in the open field test. (B) The changes in rectal temperature induced by vehicle treatment. On days 1, 3 and 7, rectal temperature was measured (30 min before and after the morning vehicle administration). Symbols represent the means and vertical lines ±SEM of differences in rectal temperature.

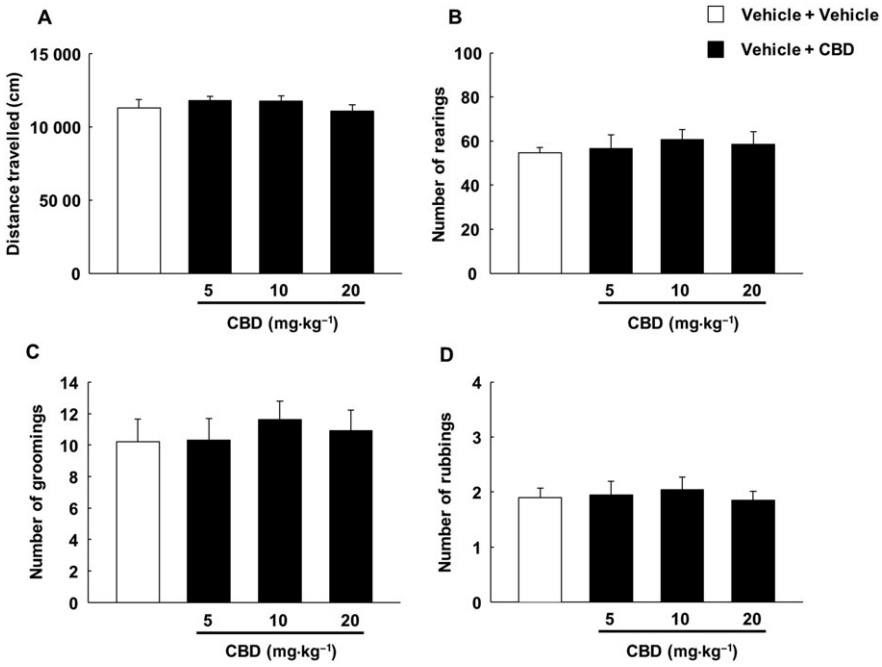

Figure 7

Assessment of effects of CBD on motor activity and behavioural signs of abstinence (number of rearings, groomings and rubbings) in mice injected i.p. with vehicle (ethanol : cremophor : saline; 1:1:18; every 12 h) for 7 days. CBD (5, 10 and 20 mg·kg^{-1}, i.p.) or vehicle (ethanol : cremophor : saline; 1:1:18) was administered approximately 12 h after the last vehicle administration. Mice received the CBD dose, and 90 min later, motor activity and behavioural signs were evaluated in 15 min sessions. Columns represent the means and vertical lines ±SEM of distance travelled (cm) by mice in the open field test (A) and the number of rearings (B), groomings (C) and rubbings (D). The number of jumpings is not displayed as the vehicle-treated animals did not show this behavioural sign.

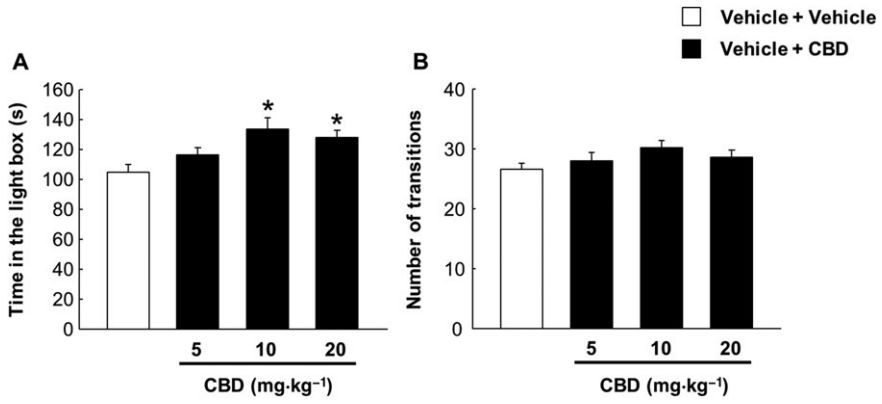

Figure 8

Effects of CBD on anxiety-like behaviour in mice injected i.p. with vehicle (ethanol : cremophor : saline; 1:1:18; every 12 h) for 7 days. CBD (5, 10 and 20 mg·kg^{-1}, i.p.) or its vehicle (ethanol : cremophor : saline; 1:1:18) was administered approximately 12 h after the last vehicle administration. Mice received the CBD dose, and 90 min later, they were exposed to the light–dark box paradigm for 5 min. (A) The evaluation of the time in the light box and (B) the evaluation of the number of transitions. Columns represent the means and vertical lines ±SEM of time (s) in the light side and number of transitions. *Values from vehicle + CBD-treated mice that are significantly different ($P < 0.05$) from the control group (vehicle + vehicle) (one-way ANOVA followed by Student–Newman–Keul's test).

of CBD on motor activity and somatic signs were specific to the CP.55,940-induced spontaneous withdrawal since its administration to vehicle-treated mice (animals not developing spontaneous cannabinoid withdrawal) did not induce any significant behavioural change. Moreover, mice experiencing cannabinoid withdrawal presented a high degree of anxiety

with a significant reduction in the time spent in the lighted box. All doses of CBD completely abolished this anxiogenic-like effect without affecting the number of transitions compared with the CP-55,940 + vehicle-treatment group. In addition, CBD also induced an anxiolytic-like effect in vehicle-treated animals without modifying the number of transitions between the different treatment groups. The literature contains numerous reports supporting an anxiolytic effect for CBD; for a recent review, see Blessing *et al*. (2015).

The results obtained in the present study suggest that under these experimental conditions, CBD significantly alleviates the most prominent behavioural symptoms associated with CP-55,940-induced spontaneous cannabinoid withdrawal. To our knowledge, this is the first study that has evaluated the effect of CBD on spontaneous cannabinoid withdrawal in an animal model and explored some of the underlying neurobiological mechanisms potentially involved. Although there is currently little clinical evidence for this treatment approach and especially for the effects of CBD alone, our results are consistent with some existing findings, such as those reported by Crippa *et al*. (2013). Therefore, additional translational studies combining both clinical and basic perspectives are warranted to clarify the therapeutic potential of CBD for cannabinoid withdrawal symptoms. This experimental approximation will be crucial to the development of future pharmacological strategies for managing CUD at the clinical level.

Nowadays, it is well known that cannabis withdrawal – similar to withdrawal from other drugs of abuse – decreases mesolimbic dopamine neuronal activity (Oleson and Cheer, 2012). Indeed, cessation of THC treatment produced a reduction in the spontaneous firing rate of dopamine neurons in VTA similar to that observed with other addictive drugs (Diana *et al*., 1998). Likewise, our group's present and past research clearly demonstrates that abrupt cessation of CP-55,940 treatment significantly decreases TH gene expression in VTA neurons (Oliva *et al*., 2003; Aracil-Fernandez *et al*., 2013). Interestingly, CBD administration normalized this alteration, increasing the mRNA levels of TH in the VTA. In line with this result, a recent study from our laboratory also revealed that CBD modulates TH gene expression in animals exposed to EtOH under different behavioural paradigms (Viudez-Martinez *et al*., 2018). The mechanisms that could be involved in these phenomena are still unknown, although several hypotheses have recently emerged regarding the effects of CBD on the mesolimbic dopamine system (Renard *et al*., 2017). Indeed, one of the well-described CBD's mechanisms of action is the inhibition of FAAH, which in turn has been associated with TH modulation (Bosier *et al*., 2013). Therefore, the supposed regulation of dopamine synthesis by means of CBD-mediated effects on TH gene expression could be involved in the alleviation of the effects of cannabinoid withdrawal.

There is evidence supporting the interaction between the endogenous opioid and cannabinoid systems (Corchero *et al*., 2004a). Previous results demonstrated that cannabinoid administration increases the release of endogenous opioid peptides and opioid-related gene expression, mechanisms closely involved in the regulation of cannabinoid reinforcing actions (Manzanares *et al*., 1999; Corchero *et al*., 2004b). Indeed, our results indicate that spontaneous withdrawal induced by interruption of cannabinoid receptor agonist administration significantly increased *Oprm1* gene expression in the NAcc, and this effect was completely normalized by the administration of CBD. Interestingly, Kathmann *et al*. (2006) described the CBD-mediated allosteric modulation of the μ receptor by means of kinetic binding studies with [^{3}H]-DAMGO, although the observed effect only occurred at very high concentrations and cannot be expected to contribute to its *in vivo* action. In addition, CBD significantly down-regulated *Oprm1* in the NAcc, with this effect being accompanied by a reduction in EtOH consumption (Viudez-Martinez *et al*., 2018). Thus, it is reasonable to suggest that the CBD-mediated regulation of the μ receptor function could be involved in alleviating cannabinoid withdrawal symptoms, as well as those of opioid withdrawal, as proposed elsewhere (Hine *et al*., 1975a,b; Bhargava, 1976).

In our study, as in previous reports (Oliva *et al*., 2003; 2004; Aracil-Fernandez *et al*., 2013), *Cnr1* gene expression was significantly up-regulated in the NAcc. Different authors have attributed this increase to a compensatory neuroadaptative response to down-regulation of cannabinoid receptors found after repeated treatment with a cannabinoid receptor agonist (Sim *et al*., 1996; Romero *et al*., 1998). CBD reduced the *Cnr1* up-regulation induced by spontaneous cannabinoid withdrawal. This result is also in agreement with CBD-induced reduction associated with a decrease in EtOH intake in C57BL/6J mice (Viudez-Martinez *et al*., 2018). Previous studies suggested that CBD acts as a non-competitive allosteric modulator of the cannabinoid CB_1 receptor through the alteration of AEA hydrolysis by inhibiting its catabolic enzyme, FAAH (Bisogno *et al*., 2001; Laprairie *et al*., 2015). Based on this assumption, it is tempting to hypothesize that the modification of the AEA concentrations could be related, at least in part, to the neurochemical changes induced by CBD and the modulation of the cannabinoid withdrawal syndrome. However, additional experiments are necessary to shed light on this matter.

Finally, previous studies have shown that the cannabinoid CB_2 receptor is involved in regulating addictive behaviours, suggesting that this target plays a pivotal role in modulating the reinforcing effects of cocaine, nicotine and alcohol (Aracil-Fernandez *et al*., 2012; Navarrete *et al*., 2013; Ortega-Alvaro *et al*., 2015). However, there is very little information regarding the involvement of CB_2 receptors in cannabis addiction, or specifically in cannabis withdrawal. The real-time PCR analyses from the present study show for the first time that spontaneous cannabinoid withdrawal is linked to a significant *Cnr2* down-regulation in the NAcc, although the underlying mechanisms have yet to be elucidated. As CBD up-regulates *Cnr2* gene expression (Viudez-Martinez *et al*., 2018), this normalization effect could be related to the improvement in cannabinoid withdrawal behavioural disturbances induced by the administration of CBD. There is controversy with regard to the pharmacological effect of CBD on CB_2 receptors, but a recent report points out that CBD acts as an allosteric modulator (Martinez-Pinilla *et al*., 2017). Future studies are needed to better understand the interaction between CBD and CB_2 receptors.

In conclusion, the results of the present study provide unequivocal evidence for the efficacy of CBD to reduce the behavioural disturbances associated with CP-55,940-induced

spontaneous cannabinoid withdrawal. Furthermore, the gene expression analyses of targets closely involved in the cannabinoid-related addictive actions and withdrawal (TH, *Oprm1*, *Cnr1* and *Cnr2*) provide valuable information about the neurochemical processes that could be involved, at least in part, in the CBD-mediated regulation of cannabinoid withdrawal. Further studies are needed to evaluate the potential therapeutic actions of CBD for the clinical management of CUD and to elucidate the precise underlying neurobiological mechanisms involved.

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Author contributions

All named authors made an active contribution to the conception, design, performance and statistical analysis of the results. A.A.-F. and F.N. carried out the behavioural analyses. A.A.-F. and F.N. performed the real-time PCR analyses. F.N. wrote the first draft of the manuscript. F.N. and J.M. critically reviewed the content, validated the accuracy of the data and approved the final version for publication.

Conflict of interest

The authors declare no conflicts of interest.

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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