Received: 25 October 2021 | Revised: 12 December 2021 | Accepted: 11 January 2022

DOI: 10.1111/adb.13150

ORIGINAL ARTICLE

Addiction Biology SSA WILEY

CBD-mediated regulation of heroin withdrawal-induced behavioural and molecular changes in mice

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Funding information
Instituto de Salud Carlos III, Grant/Award Number: Red de Trastornos Adictivos - RD16/0017/0014; Ministerio de Sanidad, Consumo y Bienestar Social, Grant/Award Number: Plan Nacional Sobre Drogas - 2019I012

Abstract

Cannabidiol (CBD) may represent a promising therapeutic tool for treating opioid use disorder (OUD). This study was aimed to evaluate the effects of CBD on the behavioural and gene expression alterations induced by spontaneous heroin withdrawal. Thirty hours after cessation of 8-day heroin treatment (5, 10, 20 and 40 mg·kg^{-1}/12 h; s.c.), spontaneous heroin withdrawal was evaluated in CD1 male mice. The effects of CBD (5, 10 and 20 mg·kg^{-1}; i.p.) on withdrawal-related behaviour were evaluated by measuring anxiety-like behaviour, motor activity and somatic signs. Furthermore, gene expression changes of mu-opioid receptor (Oprm1), pro-opiomelanocortin (Pomc), cannabinoid CB1 (Cnr1) and CB2 (Cnr2) receptors in the nucleus accumbens (NAcc) and tyrosine hydroxylase (TH) and Pomc in the ventral tegmental area (VTA) were also evaluated by real-time PCR. Anxiety-like behaviour, motor activity and withdrawal-related somatic signs were significantly increased in heroin-treated mice compared to the control group. Interestingly, CBD treatment significantly reduced these behavioural impairments and normalized gene expression of Cnr1 and Pomc in the NAcc and TH in the VTA of mice exposed to spontaneous heroin withdrawal. Also, CBD induced an up-regulation of Cnr2, whereas it did not change the increased gene expression of Oprm1 in the NAcc of abstinent animals. The results suggest that CBD alleviates spontaneous heroin withdrawal and normalizes the associated gene expression changes. Future studies are needed to determine the relevance of CBD as a potential therapeutic tool for the treatment of heroin withdrawal.

KEYWORDS
cannabidiol, gene expression, heroin, mice, withdrawal

1 | INTRODUCTION

Nowadays, opioid addiction is the most harmful substance use disorder (SUD), accounting for 76% of worldwide drug-related deaths.[1] In the United States, it is considered an epidemic issue mainly because of opioid analgesics misuse and overprescription, which according to recent data exceeds the clinical needs of the entire American adult population.[2] It is worth highlighting that approximately 80% of heroin consumers began misusing opioid prescription analgesics first.[3] Importantly, opioid use disorder (OUD) is linked to high mortality rates. In 2016, 63 632 persons in the US died from drug overdoses, of which 42 249 deaths involved opioids (66%).[1]

The alternation of drug consumption (intoxication) and abstinence periods is typical of most opioid addicts. Hence, patients suffering from OUD are characterized by three main therapeutic goals: (1) to control acute opioid intoxication, (2) to attenuate opioid withdrawal syndrome, and (3) to maintain abstinence avoiding craving and relapse. Accordingly, the US Food and Drug Administration (FDA) and the European

Medicine Agency (EMA) authorize the marketing of three classes of medications: (1) Naloxone, a short-acting opioid antagonist employed to reverse the life-threatening effects of opioid overdose; (2) Lofexidine, an alpha 2-adrenergic agonist that reduces the release of norepinephrine, which actions in the autonomic nervous system are believed to play a role in many of the symptoms of opioid withdrawal, and (3) oral long-acting mu-opioid receptor agonists such as methadone and buprenorphine, highly employed for opioid maintenance programmes to achieve abstinence and avoid relapse.

Despite the direct socio-economic impact of OUD, current options for treatment are insufficient, with limited efficacy motivating the exploration of new pharmacological avenues. In this sense, cannabidiol (CBD) has recently attracted much attention. In contrast to tetrahydrocannabinol (THC), CBD lacks abuse liability.[4] Interestingly, CBD has shown a promising therapeutic profile in Nabiximols (Sativex®, GW Pharmaceuticals) and the recently FDA-approved drug Epidiolex® (GW Pharmaceuticals). A large body of information regarding its safety shows no significant side effects in preclinical and clinical studies with CBD in children and adults.[5] Besides, recent rodent studies revealed that CBD presents significant anxiolytic-,[6] antidepressant-[7] and antipsychotic-like properties.[8,9] The mechanisms underlying the effects of CBD remain unclear. Evidence suggests that CBD activates or modifies the function of different targets within the central nervous system,[10] including cannabinoid receptors 1 (CB1r) and 2 (CB2r),[11] non-cannabinoid receptor GPR55,[10] vanilloid receptor 1 (TRPV1),[12] serotonin 5-HT_{1A} receptor,[13] adenosine A1 and A2a receptors[14] and opioid receptors (μ- and δ-opioid receptors).[15] Moreover, CBD inhibits the enzyme fatty acid amide hydrolase (FAAH) and the adenosine transporter, indirectly increasing the levels of endocannabinoids and adenosine, respectively.[14,16]

More recently, CBD has shown potential therapeutic suitability for SUD treatment.[17] Several reports suggest that CBD modulates the reinforcing and motivational effects and withdrawal-related actions of alcohol,[18,19] cannabis,[20,21] cocaine,[22,23] amphetamine,[24,25] nicotine,[26,27] and opioids.[28] In a small pilot study, CBD reduced cue-induced and general craving in opioid-dependent individuals.[29] Interestingly, a recent double-blind, randomized placebo-controlled clinical trial showed that CBD significantly reduced both craving and anxiety in drug-abstinent patients with heroin use disorder.[30] Subsequently, other studies revealed that treatment with CBD successfully disrupts the reconsolidation of contextual morphine-related memories in Wistar rats[31] and prevents opioid-induced conditioned place preference in C57BL/6J mice.[32] Altogether, these results support the therapeutic potential of CBD in the treatment of OUD, including opioid withdrawal syndrome.[33]

Thus, this study aimed to evaluate the effects of CBD on the spontaneous withdrawal induced by the repeated administration of heroin in CD1 male mice. Anxiety-like response (light-dark box test), motor activity (travelled distance in the open field test) and withdrawal-related somatic signs (rearings, grooming, rubbings, jumpings and diggings evaluated in the open field test) were assessed 30 h after the last administration of heroin. Furthermore, gene expression analyses were carried out by real-time PCR to evaluate the changes induced by spontaneous heroin withdrawal in specific key targets, namely, tyrosine hydroxylase (TH) and proopiomelanocortin (Pomc) in the ventral tegmental area (VTA) and mu-opioid receptor (Oprm1), Pomc and cannabinoid receptor 1 (Cnr1) and 2 (Cnr2) in the nucleus accumbens (NAcc). These targets were selected according to their involvement in regulating heroin effects on dopaminergic, opioidergic and cannabinoid systems, as well as considering previous data from our group showing CBD-mediated changes in their relative gene expression.

2 | MATERIALS AND METHODS

2.1 | Mice

A total of 90 CD1 male mice from Charles River (Lille, France) weighing 20–25 g were employed in this study, housed in groups of 5 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). Behavioural analyses were initiated 1 week after acclimatization to the animal room and were performed by placing the home cage in the behavioural room during the light cycle. All animal experiments were conducted in compliance 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/U.E.) regulating the care of experimental animals and were approved by the Ethical Committee of the Miguel Hernandez University.

2.2 | Drugs

Heroin was obtained from the Spanish Drugs Agency (AEMPS, Madrid, Spain) and was dissolved in tap water to get the required doses for its subcutaneous administration (s.c.). Cannabidiol (CBD) obtained from STI Pharmaceuticals (Essex, United Kingdom) was dissolved in ethanol:cremophor: saline (1:1:18) immediately before its use to get the required doses (5, 10 and 20 $mg{\cdot}kg^{-1}$) and administered i.p. during the spontaneous heroin withdrawal 1 h and 30 min before behavioural evaluation according to its plasma and brain pharmacokinetic profile after i.p. administration.[34] CBD doses were selected according to previous studies evaluating its effects on spontaneous withdrawal to CP-55,940[21] and cocaine.[23]

2.3 | Experimental design

2.3.1 | Animal model of heroin-induced spontaneous withdrawal

First, CD1 male mice were injected with increasing doses of heroin starting with 5 $mg{\cdot}kg^{-1}$/12 h (s.c.) at day 1 and rising to 40 $mg{\cdot}kg^{-1}$/12 h (s.c.) at day 8, as displayed in Figure 1. The progressive increase in heroin dosage was done to generate a higher degree of tolerance, allowing the characterization of the spontaneous withdrawal-

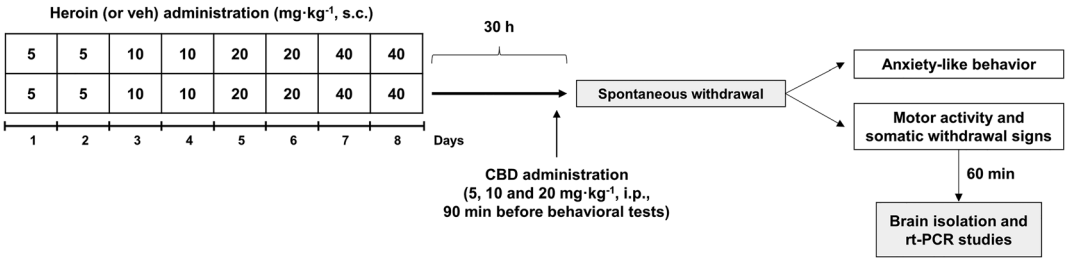


FIGURE 1 Experimental designs to induce and evaluate spontaneous heroin withdrawal in mice. Heroin administration (5–40 mg·kg^{-1}/12 h) was performed during eight consecutive days in CD1 male mice. Thirty hours after the last heroin administration, the effects of CBD (5, 10 and 20 mg·kg^{-1}) on spontaneous withdrawal syndrome were evaluated by the light-dark box and open field paradigms

induced behavioural traits evaluated 30 h after the last heroin administration. Mice were administered with CBD (5, 10 and 20 mg·kg^{-1}, i.p., 9 mice per dose) or its corresponding vehicle, 1 h and 30 min before the evaluation of anxiety-like behaviour (light-dark box), motor activity and behavioural signs related with abstinence (open field).

Light-dark box test

A set of 45 CD1 male mice ($n = 9$ by experimental group) were used to evaluate anxiety-like behavioural changes induced by heroin abstinence. Mice were individually tested for 5 min in the light-dark box paradigm, 1 h and 30 min after CBD administration (5, 10 and 20 mg·kg^{-1}) or its corresponding vehicle. This test uses the natural aversion of rodents to bright areas compared with darker ones. The apparatus consisted of two methacrylate boxes (20 × 20 × 15 cm), one transparent and one black and opaque, separated by an opaque tunnel (4 cm). Light form 60 W desk lamp placed 25 cm above the transparent box provided room illumination. At the beginning of the session, mice were placed in the light box facing the tunnel that connects to the dark box. The time spent in the light box and the number of transitions between the two compartments were measured during the sessions. A mouse whose four paws were in the new box was considered to have changed boxes.

Open field test

Another set of 45 CD1 male mice ($n = 9$ by experimental group) was used to evaluate the motor activity and somatic withdrawal signs placing mice into individual methacrylate boxes (25 × 25 × 25 cm). One hour and 30 min after CBD (5, 10 and 20 mg·kg^{-1}) or vehicle administration, mice behaviour was videotaped for 15 min, and the somatic signs related to abstinence (rearings, rubbings, groomings, jumpings and diggings) were subsequently analysed from the recording. At the same time, motor responses were also evaluated, measuring the distance travelled by the mice for 15 min with the SMART program (Panlab). The brains of these mice were collected 150 min after the CBD or vehicle administration to analyse gene transcription alterations.

2.3.2 | Gene expression studies by real-time PCR

Relative gene expression analyses of TH and Pomc in the VTA, and Cnr1, Cnr2, Oprm1 and Pomc in the NAcc were carried out in vehicle-treated and in heroin-treated CD1 male mice employed to evaluate the effects of CBD on motor activity and somatic signs under heroin-induced spontaneous withdrawal (see Figure 1). Briefly, mice were sacrificed by dislocation 150 min after the CBD administration on day 9. 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 Paxinos and Franklin atlas,[35] mounted onto slides and stored at −80°C. Sections were microdissected following the method described by Palkovits.[36] Total RNA was obtained from brain micropunches using TRI extraction reagent (Applied Biosystems, Madrid, Spain). Reverse transcription to complementary DNA (cDNA) was carried out following the instructions of the manufacturer (Applied Biosystems). The relative abundances of TH (Mm00447546_m1), Oprm1 (Mm01188089_m1), Cnr1 (Mm00432621_s1), Cnr2 (Mm00438286_m1) and Pomc (Mm00435874_m1) gene expressions were 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, followed by 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.[37]

2.3.3 | Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) 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 ± standard error of the mean (SEM). Sigmaplot 11 software (Systat Software Inc., Chicago, IL, USA) was used for all statistical analyses. In addition, effect sizes were calculated with the 'effect size calculator' of public domain (https://www.cem.org/effect-size-calculator), provided by the 'Centre for Evaluation and Monitoring' (part of Cambridge University). Tables 1 and 2 gather all the statistical data from the behavioural and gene expression studies, respectively, in Section 3.

TABLE 1 Detail of the statistical results from the behavioural studies

Experiment	Statistical test	Parameter	Factors	d.f.	F	*p* value	Comparison	Effect size	S.E.	95% C.I.
Evaluation of CBD effects on behavioural alterations induced by spontaneous heroin withdrawal	One-way ANOVA	Motor activity	Travelled distance (cm)	4,44	8.167	<0.001	V-V vs V-H	2.17	0.58	0.92 to 3.21
							V-H vs CBD5-H	0.01	0.47	−0.91 to 0.93
							V-H vs CBD10-H	−1.08	0.50	−2.02 to −0.05
							V-H vs CBD20-H	−1.66	0.54	−2.64 to −0.52
		Somatic signs	Rearings	4,44	7.149	<0.001	V-V vs V-H	2.10	0.58	0.87 to 3.13
							V-H vs CBD5-H	−1,59	0.54	−2.57 to −0.47
							V-H vs CBD10-H	−1.83	0.55	−2.83 to −0.66
							V-H vs CBD20-H	−2.25	0.59	−3.30 to −0.98
			Rubbings	4,44	5.028	0.002	V-V vs V-H	1.29	0.51	0.22 to 2.23
							V-H vs CBD5-H	0.06	0.47	−0.86 to 0.98
							V-H vs CBD10-H	−0.94	0.49	−1.87 to 0.07
							V-H vs CBD20-H	−0.99	0.50	−1.92 to 0.03
			Groomings	4,44	5.340	0.002	V-V vs V-H	1.67	0.54	0.53 to 2.65
							V-H vs CBD5-H	−0.20	0.47	−1.12 to 0.74
							V-H vs CBD10-H	−0.99	0.50	−1.92 to 0.03
							V-H vs CBD20-H	−1.19	0.51	−2.12 to −0.13
			Jumpings	4,44	6.430	<0.001	V-V vs V-H	3.03	0.67	1.56 to 4.20
							V-H vs CBD5-H	−1.46	0.53	−2.42 to −0.36
							V-H vs CBD10-H	−1.94	0.56	−2.42 to 0.74
							V-H vs CBD20-H	−2.56	0.62	−3.66 to −1.22
			Diggings	4,44	7.728	<0.001	V-V vs V-H	1.47	0.53	0.37 to 2.43
							V-H vs CBD5-H	−0.28	0.47	−1.20 to 0.66
							V-H vs CBD10-H	−1.67	0.54	−2.65 to -0.53
							V-H vs CBD20-H	−1.92	0.56	−2.92 to -0.72
		Anxiety-like behaviour	Time spent in light box (s)	4,44	7.960	<0.001	V-V vs V-H	−1.86	0.56	−2.87 to −0.68
							V-H vs CBD5-H	0.82	0.49	−0.18 to 1.74
							V-H vs CBD10-H	3.13	0.69	1.64 to 4.33
							V-H vs CBD20-H	2.24	0.59	0.97 to 3.29
			Number of transitions	4,44	1.848	0.139	V-V vs V-H	−0.58	0.48	−1.50 to 0.39
							V-H vs CBD5-H	−0.19	0.47	−1.10 to 0.75
							V-H vs CBD10-H	0.74	0.49	−0.25 to 1.66
							V-H vs CBD20-H	0.99	0.50	−0.03 to 1.91

Note: CBD-H, cannabidiol + heroin; C.I.: confidence interval; d.f., degrees of freedom; S.E., standard error; V-H, vehicle + heroin; V-V, vehicle + vehicle.

TABLE 2 Detail of the statistical results from the gene expression studies

Experiment	Statistical test	Parameter	Brain region	Genes	d.f.	F	*p* value	Comparison	Effect size	S.E.	95% C.I.
Evaluation of CBD effects on gene expression changes induced by spontaneous heroin withdrawal	One-way ANOVA	Relative gene expression (real time-PCR)	VTA	TH	4,44	7.153	<0.001	V-V vs V-H	2.48	0.61	1.15 to 3.56
								V-H vs CBD5-H	−2.56	0.62	−3.66 to −1.22
								V-H vs CBD10-H	−2.27	0.59	−3.32 to −1.00
								V-H vs CBD20-H	−1.71	0.54	−2.69 to −0.56
				Pomc	4,44	3.047	0.028	V-V vs V-H	1.42	0.52	0.33 to 2.38
								V-H vs CBD5-H	−0.54	0.48	−1.46 to 0.42
								V-H vs CBD10-H	−0.50	0.48	−1.42 to 0.46
								V-H vs CBD20-H	−1.16	0.51	−2.10 to −0.11
			NAcc	Cnr1	4,44	10.090	<0.001	V-V vs V-H	−2.49	0.61	−1.16 to −2.21
								V-H vs CBD5-H	−0.11	0.47	−1.03 to 0.82
								V-H vs CBD10-H	0.21	0.47	−0.73 to 1.13
								V-H vs CBD20-H	2.33	0.60	1.04 to 3.39
				Cnr2	4,44	69.137	<0.001	V-V vs V-H	2.85	0.65	1.43 to 3.99
								V-H vs CBD5-H	2.07	0.57	0.85 to 3.10
								V-H vs CBD10-H	1.93	0.56	0.74 to 2.94
								V-H vs CBD20-H	7.42	1.27	4.58 to 9.55
				Oprm1	4,44	9.381	<0.001	V-V vs V-H	1.88	0.56	0.69 to 2.88
								V-H vs CBD5-H	−0.04	0.47	−0.96 to 0.89
								V-H vs CBD10-H	0.39	0.48	−0.56 to 1.30
								V-H vs CBD20-H	0.92	0.49	−0.09 to 1.85
				Pomc	4,44	6.217	<0.001	V-V vs V-H	−1.15	0.51	−2.09 to −0.11
								V-H vs CBD5-H	−0.26	0.47	−1.18 to 0.68
								V-H vs CBD10-H	0.26	0.47	−0.68 to 1.18
								V-H vs CBD20-H	1.56	0.53	0.44 to 2.53

Note: CBD-H, cannabidiol + heroin; C.I., confidence interval; d.f., degrees of freedom; Cnr1, cannabinoid receptor 1, Cnr2, cannabinoid receptor 2; Oprm1, mu-opioid receptor; Pomc, proopiomelanocortin; S.E., standard error; TH, tyrosine hydroxylase; V-H, vehicle + heroin; V-V, vehicle + vehicle.

3 | RESULTS

3.1 | Effects of CBD on anxiogenic-like behaviour induced by spontaneous heroin withdrawal

After cessation of heroin administration, vehicle-treated mice significantly decreased the time spent in the lighted box. The administration of CBD normalized these anxiety-like behaviours at a dose of 10 mg·kg^{-1}, inducing an additional anxiolytic effect at a dose of 20 mg·kg^{-1} (Figure 2A; one-way ANOVA, $F_{(4,\ 44)} = 7.960$, $P < 0.001$). Furthermore, no differences were observed in the number of transitions between all groups (Figure 2B; one-way ANOVA, $F_{(4,\ 44)} = 1.848$, $P = 0.139$).

3.2 | Effects of CBD on increased motor activity and somatic signs induced by spontaneous heroin withdrawal

3.2.1 | Motor activity

Mice exposed to the spontaneous heroin withdrawal model showed significantly increased total distance recorded compared to control mice. CBD administration modulated this increase at 10 and 20 mg·kg^{-1} doses (Figure 3F; one-way ANOVA, $F_{(4,\ 44)} = 8.167$, $P < 0.001$).

3.2.2 | Somatic withdrawal signs

The one-way ANOVA showed an increase in the number of rearings, rubbings, groomings, jumpings and diggings compared with controls. Interestingly, CBD completely blocked the enhanced number of rearings (Figure 3A; one-way ANOVA, $F_{(4,\ 44)} = 7.149$, $P < 0.001$) and jumpings (Figure 3D; one-way ANOVA; $F_{(4,\ 44)} = 6.430$, $P < 0.001$) at all three evaluated doses. Besides, the number of rubbings, groomings and diggings was normalized only with the intermediate and high doses of CBD (10 and 20 mg·kg^{-1}) (rubbings: Figure 3B; one-way ANOVA, $F_{(4,\ 44)} = 5.028$, $P = 0.002$; groomings: Figure 3C; one-way ANOVA, $F_{(4,\ 44)} = 5.340$, $P = 0.002$; diggings: Figure 3E; one-way ANOVA, $F_{(4,\ 44)} = 7.728$, $P < 0.001$).

3.3 | Effects of CBD on changes in the TH, Oprm1, Cnr1, Cnr2 and Pomc gene expression induced by spontaneous heroin withdrawal

3.3.1 | Gene expression changes in the VTA

Mice exposed to the animal model of spontaneous heroin withdrawal showed significantly increased relative gene expression of TH compared to controls. Interestingly, all three doses of CBD achieved the complete normalization of these alterations (Figure 4A; one-way ANOVA, $F_{(4,\ 44)} = 7.153$, $P < 0.001$). Significative changes in the same direction were observed in the POMC gene expression in this brain region, with an increase in heroin withdrawal-exposed mice and a normalization with the highest dose of CBD (20 mg·kg^{-1}) (Figure 4B; one-way ANOVA, $F_{(4,\ 44)} = 3.047$, $P = 0.028$).

3.3.2 | Gene expression changes in the NAcc

One-way ANOVA showed a decreased gene expression of cannabinoid receptor *Cnr1* in mice exposed to the spontaneous withdrawal model, which was normalized with the highest dose of CBD (20 mg·kg^{-1}) (Figure 5A; one-way ANOVA, $F_{(4,\ 44)} = 10.090$, $P < 0.001$). Similar interesting effects were observed when analysing Pomc gene expression, with a significant decrease in abstinent mice

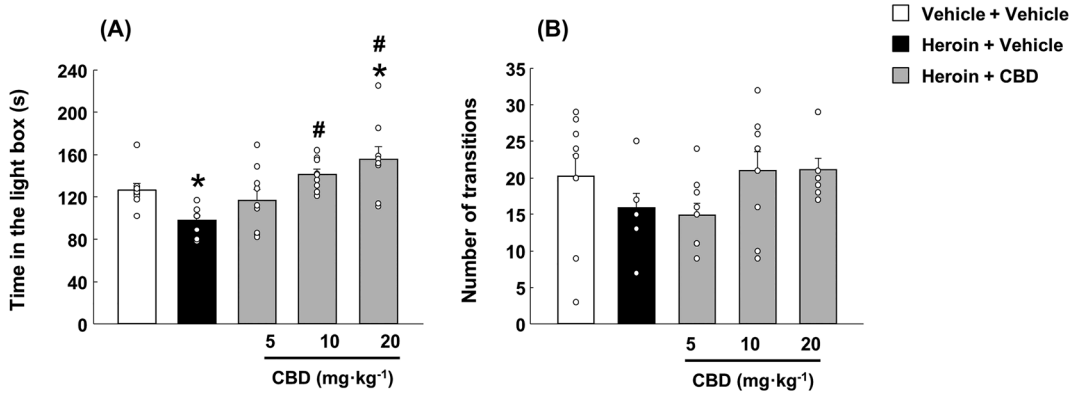


FIGURE 2 Evaluation of CBD (5, 10 and 20 mg·kg^{-1}) effects on increased anxiety-like behaviour induced by the spontaneous heroin withdrawal model in the light-dark box paradigm. Columns represent the means and vertical lines ± SEM of the time spent in the lighted box (s) (A) and the number of transitions (B). *, values from HER-VEH and HER-CBD20 treated mice significantly different from VEH-VEH treated mice (one-way ANOVA, $P < 0.05$). #, values from HER-CBD10 and HER-CBD20 treated mice significantly different from HER-VEH treated mice (one-way ANOVA, $P < 0.01$)

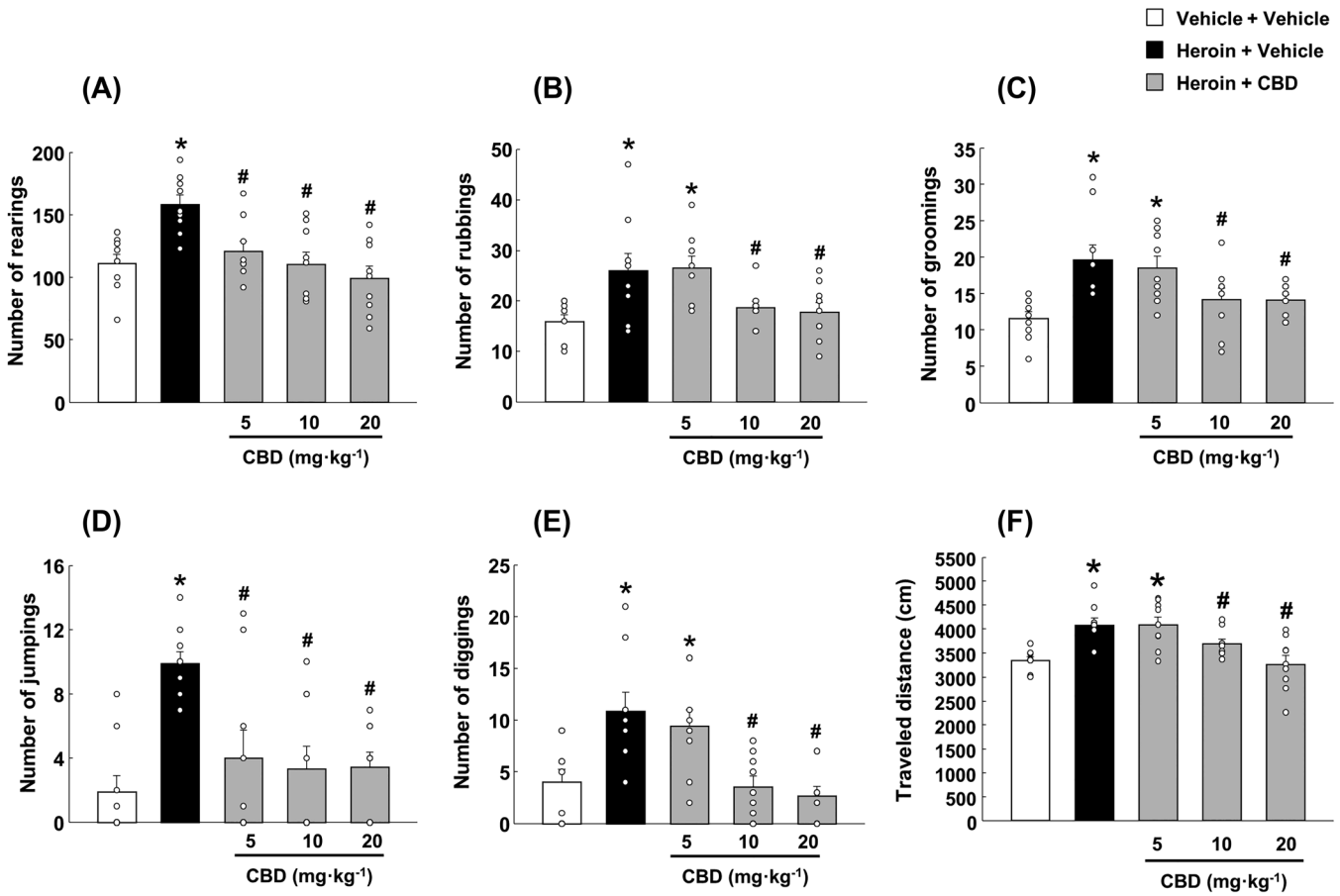
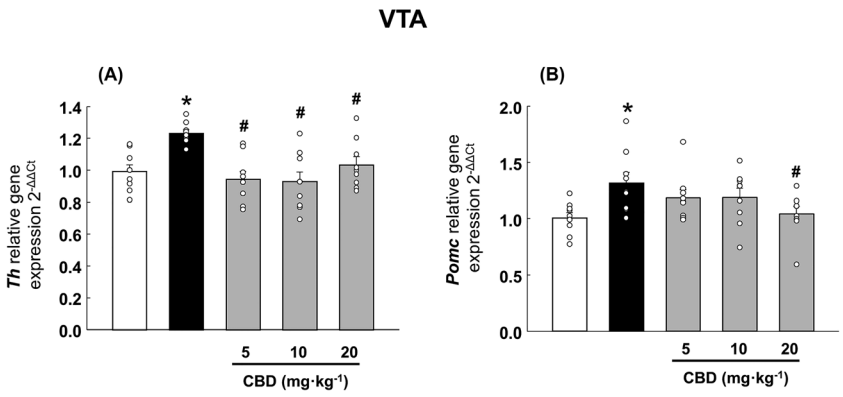


FIGURE 3 Evaluation of CBD (5, 10 and 20 mg·kg^{-1}) effects on somatic signs alterations and increased motor activity induced by the spontaneous heroin withdrawal model in the open field paradigm. Columns represent the means and vertical lines ± SEM of the number of rearings (A), rubbings (B), groomings (C), jumpings (D), diggings (E) and the travelled distance (F). *, values from HER-VEH and HER-CBD5 treated mice significantly different from VEH-VEH treated mice (one-way ANOVA, $P < 0.05$). #, values from HER-CBD10 and HER-CBD20 treated mice significantly different from HER-VEH treated mice (one-way ANOVA, $P < 0.05$)

FIGURE 4 Evaluation of CBD (5, 10 and 20 mg·kg^{-1}) effects on tyrosine hydroxylase (TH) and proopiomelanocortin (Pomc) relative gene expression changes by real-time PCR in the ventral tegmental area (VTA). Columns represent the means and vertical lines ± SEM of $2^{-\Delta\Delta Ct}$. *, values from HER-VEH treated mice significantly different from VEH-VEH treated mice (one-way ANOVA, $P < 0.05$). #, values from HER-CBD treated mice significantly different from HER-VEH treated mice (one-way ANOVA, $P < 0.05$)



and a normalization with a CBD dose of 20 mg·kg^{-1} (Figure 5D; one-way ANOVA, $F_{(4, 44)} = 6.217$, $P < 0.001$).

In addition, abstinent animals exhibited increased relative gene expression of the Cnr2 cannabinoid receptor compared to controls. Interestingly, CBD administration at a dose of 5 and 10 mg·kg^{-1} induces an additional up-regulation, achieving a more pronounced increase with a dose of 20 mg·kg^{-1} (Figure 5B; one-way ANOVA, $F_{(4, 44)} = 69.137$, $P < 0.001$).

The exposure to the spontaneous heroin withdrawal increased Opmr1 gene expression in this brain region, but CBD administration did not induce any significant change (Figure 5C; one-way ANOVA, $F_{(4, 44)} = 9.381$, $P < 0.001$).

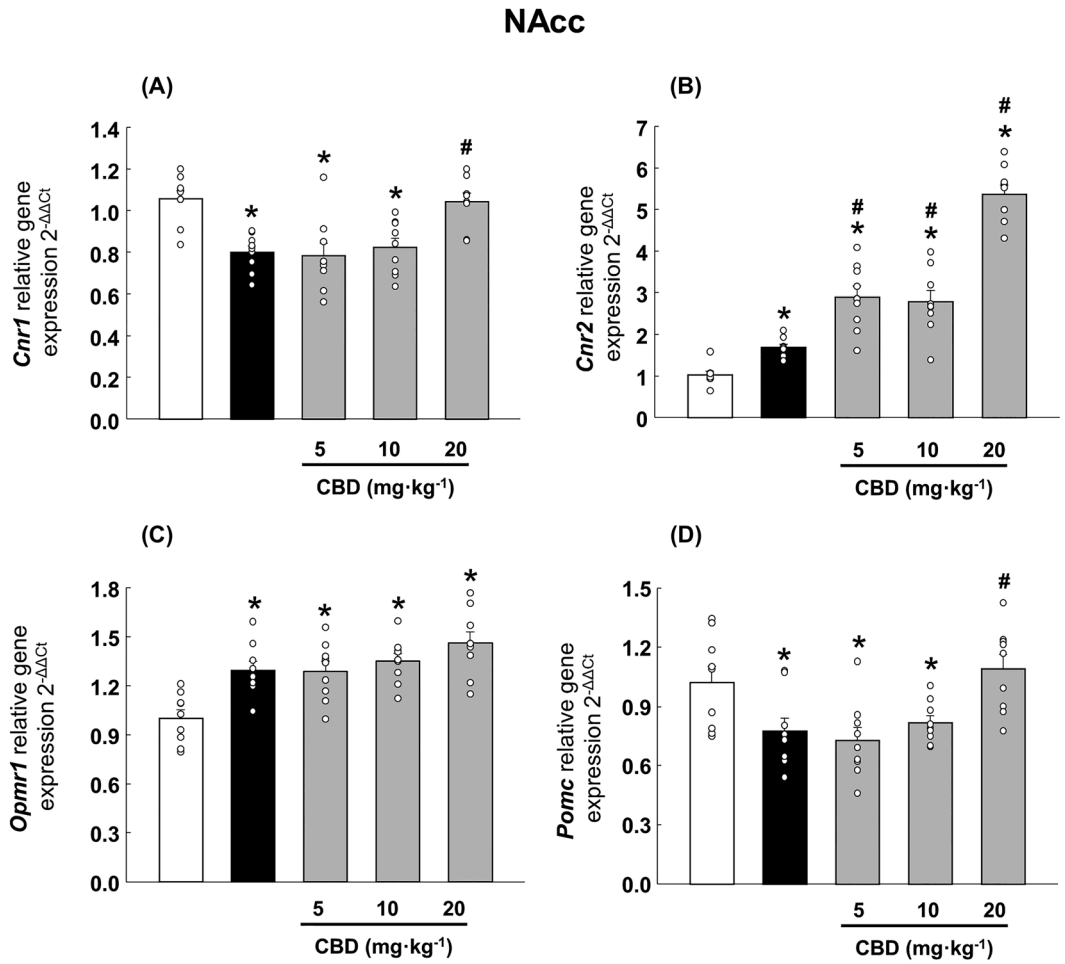


FIGURE 5 Evaluation of CBD (5, 10 and 20 mg·kg^{-1}) effects on cannabinoid receptors 1 (Cnr1) and 2 (Cnr2), mu-opioid receptor (Opmr1) and Pomc relative gene expression changes by real-time PCR in the nucleus accumbens (NAcc). *, values from HER-VEH or HER-CBD treated mice significantly different from VEH-VEH treated mice (one-way ANOVA, $P < 0.05$). #, values from HER-CBD treated mice significantly different from HER-VEH treated mice (one-way ANOVA, $P < 0.05$)

4 | DISCUSSION

The present work reveals that CBD administration may be helpful to alleviate the anxiety-like behaviours and the somatic symptoms associated with the spontaneous withdrawal induced by the administration of heroin in CD1 male mice. The following observations support this assumption: (1) The administration of CBD significantly reduced the high anxiety-like behaviour associated with the spontaneous heroin withdrawal; (2) after cessation of treatment with heroin, CBD normalized the increase in the travelled distance and the number of rearings, rubbings, groomings, jumpings and diggings; (3) the administration of CBD blocked the increase of TH and Pomc gene expression in the VTA; and (4) CBD up-regulated Cnr2 and normalized the decrease in Cnr1 and Pomc gene expression in the NAcc induced by the spontaneous heroin withdrawal.

Animal models recapitulating behavioural disturbances associated with opioid withdrawal syndrome (i.e., heroin or morphine) have commonly employed the administration of the mu-opioid antagonist naloxone to pharmacologically precipitate the typical somatic signs, including emotional alterations such as increased anxiety-like behaviour.[38] However, this experimental approach lacks adequate face validity with poor translational value since it does not simulate the natural conditions under which opioid withdrawal occurs from a clinical perspective. For these reasons, spontaneous withdrawal was evaluated 30 h after the last heroin administration. This time point corresponds to the highest expression of heroin abstinence signs according to previous preliminary studies performed by our group.

Once the model of spontaneous heroin withdrawal was established, showing an anxiogenic phenotype with hyperactivity and increased somatic signs, the objective was to evaluate whether CBD would normalize the observed behavioural alterations. Interestingly, CBD administration increased the time spent in the light box, which was significantly higher than the control group at the dose of 20 mg·kg^{-1}, thus demonstrating the marked anxiolytic effect of CBD according to preliminary evidence.[6] Also, the hyperactivity observed in abstinent animals was blocked entirely with the highest CBD dose. Regarding the withdrawal somatic signs, it is relevant to note that

CBD abolished the increase in the number of rearings, rubbings, groomings, jumpings and diggings, especially at 10 and 20 mg·kg^{-1}. Importantly, these results demonstrate that CBD ameliorates the behavioural impairments induced by spontaneous heroin withdrawal in mice. These data add novel and relevant information to previous evidence showing that CBD has therapeutic potential to alleviate behavioural disturbances associated with abstinence from other drugs of abuse such as cannabinoids,[21] cocaine[23] and nicotine.[27] Similarly, these studies revealed that CBD significantly reduces the occurrence of specific drug withdrawal somatic signs (e.g., hyperactivity, jumping, rearing, grooming and rubbing) in CD1 mice[21,23] and Wistar rats.[27]

It is relevant to note that one limitation of the present study is the lack of CBD treatment in non-abstinent animals. However, a previous report of our group using a very similar experimental design demonstrated that CBD (acute administration, 90 min before behavioural evaluation) induced an anxiolytic effect and did not change either motor activity or somatic signs in vehicle-treated animals (7 days; twice a day every 12 h; i.p.).[21] In addition, another recent study also showed that the repeated administration of CBD (30 mg·kg^{-1}) did not alter the somatic signs of withdrawal in the group treated with saline.[27] Thus, it is tempting to hypothesize that in the context of the present model, vehicle-treated mice might show a similar behavioural profile accounting for the CBD-mediated specific actions under spontaneous heroin withdrawal conditions.

The opioidergic system plays a crucial role in developing opioid addiction and the onset of withdrawal syndrome. Binding to μ-opioid receptors (MOR) by endogenous peptides (i.e., β-endorphin and enkephalins) and exogenous opioids (i.e., morphine and heroin) regulates the mesolimbic dopaminergic pathway critically involved in drug reward.[39] As part of the tolerance phenomenon, prolonged exposure to exogenous opioids down-regulates MOr through phosphorylation and arrestin-driven receptor internalization and degradation.[40,41] Conversely, under opioid withdrawal conditions, an opposite compensatory effect has been described. Indeed, acute morphine withdrawal was associated with enhanced Oprm1 gene expression levels in the lateral hypothalamus, the caudate-putamen and the NAcc.[42] Accordingly, real-time PCR studies revealed an up-regulation of Oprm1 in the NAcc of mice with spontaneous abstinence to heroin. However, the treatment with CBD did not modify the gene expression of Oprm1 at any of the doses used. The interaction between CBD and opioidergic system components has been scarcely explored. Few studies evaluated changes in the main targets of the opioidergic system after CBD administration. The CBD-mediated allosteric modulation of mu- and delta-opioid receptors was first described using kinetic binding in vitro studies.[15]

Interestingly, CBD-induced reduction of voluntary ethanol consumption, ethanol self-administration and binge-drinking was associated with a down-regulation of Oprm1 in the NAcc.[18,19,43] Similarly, CBD normalized increased Oprm1 gene expression in the NAcc of mice experiencing spontaneous cannabinoid withdrawal.[21] However, no changes were observed in these experimental conditions. One hypothesis explaining this lack of effect by CBD could be based on the marked agonist properties of heroin on MOr. Perhaps, the modulation of increased Oprm1 gene expression in spontaneous heroin withdrawal may require a higher dose of CBD.

To further explore whether CBD could regulate other components of the opioidergic system under heroin withdrawal conditions, Pomc gene expression was analysed. Pomc gene encodes the precursor of the endogenous opioid peptide β-endorphin. Pomc gene expression is primarily restricted to specific nuclei of the hypothalamus (i.e., arcuate and solitary tract). Previous studies have also detected mRNA in some extra-hypothalamic regions such as VTA and NAcc, suggesting its role in regulating the reward system.[44,45] Interestingly, in the present study, Pomc was significantly elevated in the VTA while reduced in the NAcc of abstinent animals. The available literature on extra-hypothalamic changes in Pomc gene expression in spontaneous heroin withdrawal is scarce. Only one article showed an up-and-down-regulation in the amygdala and hippocampus, respectively.[46] Notably, the highest dose of CBD (i.e., 20 mg·kg^{-1}) significantly normalized these Pomc gene expression alterations. Accordingly, a previous study of our laboratory demonstrated that CBD blocked the effects induced by acute stress on Pomc gene expression.[47] Thus, it is highly relevant to note that the CBD-mediated normalization effect on Pomc gene expression could underlie, at least in part, the improvement of withdrawal-related behavioural traits.

Opioid withdrawal is associated with functional modifications in the dopaminergic mesolimbic system, leading to a hypodopaminergic state.[48] Of particular interest are the changes in the gene expression of TH, the rate-limiting enzyme of dopamine, in the body cells of VTA dopaminergic projecting neurons. Here, spontaneous heroin withdrawal induced an up-regulation of TH in the VTA. However, two previous studies did not find differences during spontaneous[49] or induced[50] morphine withdrawal, whereas a marked TH up-regulation was shown in the locus coeruleus. These apparent discrepancies may occur because of differences in the opioid employed (morphine vs. heroin) or the protocol to evaluate spontaneous withdrawal (administration route, opioid treatment duration and time point of withdrawal evaluation). Despite that, it is relevant to note that CBD completely blocked the increase of TH gene expression at all the tested doses, as previously described in the spontaneous cocaine withdrawal.[23]

Finally, there is strong evidence regarding the opioid-cannabinoid crosstalk.[51] The endogenous cannabinoid system, particularly CB1r and CB2r, has an essential role in the modulation of opioid-induced dopamine release in the NAcc. Real-time PCR studies performed in this brain region revealed a significant down-regulation of Cnr1 while an up-regulation of Cnr2, pointing out the involvement of both receptors in spontaneous heroin withdrawal. Notably, CBD normalized Cnr1 (20 mg·kg^{-1}) and induced a much higher increase of Cnr2 (all tested doses). There is much controversy regarding the direct or indirect interaction of CBD with both cannabinoid receptors. Recent results suggested that CBD could act as a non-competitive negative allosteric modulator of CB1r.[11,52] Also, previous evidence points out that CBD inhibits the reuptake and degradation of the endogenous cannabinoid anandamide (AEA).[12,52] In addition, it was proposed that CBD could

act as a partial agonist,[11] inverse agonist or antagonist,[53] or as an allosteric modulator of CB2r.[54] Therefore, additional experiments are needed to shed light on the actions of CBD on CB1r and CB2r.

In conclusion, the results clearly show CBD-mediated regulation of anxiogenic response, hyperactivity and somatic signs induced by spontaneous heroin withdrawal in mice. Gene expression analyses provide relevant information regarding the underlying neurobiological mechanisms involving opioidergic, dopaminergic and cannabinoid systems. However, some limitations of the present study should be addressed in the future, such as the evaluation of female mice to explore sex-dependent effects, the performance of additional dose-response experiments or the assessment of pharmacological target engagement by CBD. Therefore, additional studies are warranted to explore further the therapeutic potential of CBD for managing opioid withdrawal.

ACKNOWLEDGEMENTS

This research was supported by Grants from the 'Instituto de Salud Carlos III' (Red de Trastornos Adictivos - RD16/0017/0014, Fondos FEDER) and Ministerio de Sanidad, Consumo y Bienestar Social (Plan Nacional Sobre Drogas - 2019I012) to JM.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

All named authors contributed to the conception, design, performance and statistical analysis of the results. FN and AG carried out the behavioural studies and performed the real-time PCR analyses. FN wrote the first draft of the manuscript. FN, AG and JM critically reviewed the content, validated the accuracy of the data and approved the final version for publication.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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How to cite this article: Navarrete F, Gasparyan A, Manzanares J. CBD-mediated regulation of heroin withdrawal-induced behavioural and molecular changes in mice. *Addiction Biology*. 2022;27(2):e13150. doi:10.1111/adb.13150