Effects of cannabidiol on behavioural and gene expression changes induced by spontaneous cocaine withdrawal

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April 28, 2020

Abstract

Background and purpose. The purpose of this study was to evaluate the effects of CBD on the behavioural and gene expression changes induced by a new animal model of spontaneous cocaine withdrawal. Experimental approach. Six hours after cessation of progressive increase of cocaine administration for 12 days (15 mg·kg-1·day-1 to 60 mg·kg-1·day-1, i.p.), spontaneous cocaine withdrawal was evaluated in male mice. The effects of CBD (10, 20 and 40 mg·kg-1, i.p.) were evaluated on cocaine withdrawal-induced alterations in motor activity, somatic signs and anxiety-like behaviour. Furthermore, gene expression changes in dopamine transporter (DAT) and tyrosine hydroxylase (TH) in the ventral tegmental area, and in cannabinoid receptors 1 (CNR1) and 2 (CNR2) in the nucleus accumbens were analysed by real-time PCR. Key results. Mice exposed to the spontaneous cocaine withdrawal model showed increased motor activity, somatic withdrawal signs and high anxiety-like behaviour. Interestingly, the administration of CBD normalized motor and somatic signs disturbances and induced an anxiolytic effect. Moreover, the administration of CBD blocked the increase of DAT and TH gene expression in mice exposed to the cocaine withdrawal. In addition, the administration of CBD modulated the cocaine withdrawal-induced decrease of CNR1 and induced an additional up-regulation of CNR2 gene expression. Conclusions and implications. These results show behavioural and gene expression alterations in mice exposed to a new model of spontaneous cocaine withdrawal. Interestingly, CBD alleviates cocaine withdrawal-induced behavioural and gene expression alterations suggesting potential for the management of cocaine withdrawal.

Introduction

Cocaine use disorder (CUD) is a chronic and relapsing disorder characterized by compulsive drug seeking and drug use despite its negative consequences. The most recent data suggests that CUD remains a major public health problem with an estimated 20 million users, being this drug of abuse the most predominant psychostimulant used in the world (Richards, Garber et al., 2016). Recent epidemiological reports indicate that the number of cocaine users increased, associated with socio-economic and legal complications as well as with the number of hospitalisations caused by cocaine dependence (Mena, Giraudon et al., 2013).

*Cocaine*is a psychostimulant drug with high potential for addiction because of its short half-life and dopaminergic mechanism of action. Up to 5% to 6% of cocaine users will develop cocaine dependence within the first year of use (Ryan, 2019) which involves a recurring cycle of intoxication, bingeing, withdrawal and craving resulting in an excessive drug use (Agurto, Norel et al., 2019). Cardiac symptoms are the most commonly reported by cocaine users, being cocaine addiction the leading cause of death among adults using illicit substances. In addition, cocaine use was reported as a risk factor in 25% of nonfatal myocardial infarction in younger people (Ryan, 2019). Given the absence of specific tools for the CUD management, the controlling of cocaine withdrawal could be an effective way to break the cycle of addiction and to prevent relapse. The

development of appropriate animal models results an essential tool to improve the knowledge and understanding of the behavioural neurobiological mechanisms involved in cocaine withdrawal. Unfortunately, only few animal models of cocaine withdrawal were reported in the recent years. Some studies simulated cocaine abstinence by administering the same dose of cocaine for several days (Li, Xu et al., 2017; Valzachi, Teodorov et al., 2013) or by increasing cocaine doses once a day combined with periods of abstinence (Aguilar, Ledesma et al., 2017; Ledesma, Aguilar et al., 2017). Nevertheless, in humans the cocaine is taken several times a day at increasing doses, and the abstinence syndrome is of quick appearance once drug consumption stops. Thus, these animal models reproduce only partially the principal clinical features of human cocaine addiction and withdrawal (Benuck, Lajtha et al., 1987). Consequently, new animal models of cocaine withdrawal are needed to simulate closely CUD. The development of an adequate animal model of cocaine withdrawal will allow to identify more precisely the neurobiological mechanisms underlying this complex phase of cocaine addiction and to suggest new therapeutic targets for the development of specific pharmacological strategies.

Currently, there are not specific drugs approved by principal regulatory agencies with established efficacy for the treatment of cocaine withdrawal. In the last years, several preclinical and clinical studies showed that*cannabidiol*(CBD), a main constituent of *Cannabis sativa* plant that lacks activity as drug of abuse (Viudez-Martinez, Garcia-Gutierrez et al., 2019), may result a promising therapeutic tool for CUD management. CBD can interact with more than 65 different targets, producing anxiolytic, antidepressant, antipsychotic and neuroprotective effects (Elsaid & Le Foll, 2019). In addition, CBD has been proposed as an interesting pharmacological candidate to treat substance use disorders (Navarrete, Aracil-Fernandez et al., 2018; Prud'homme, Cata et al., 2015; Viudez-Martinez, Garcia-Gutierrez et al., 2018c). Furthermore, several preclinical findings indicate that CBD attenuated cue-induced cocaine seeking in rats after withdrawal (Gonzalez-Cuevas, Martin-Fardon et al., 2018) and cocaine-induced conditioned place preference (Lujan, Castro-Zavala et al., 2018; Parker, Burton et al., 2004). Additionally, CBD usefulness to reduce cocaine consumption and the progressive ratio in a cocaine self-administration paradigm was proposed recently (Lujan, Castro-Zavala et al., 2018). Taken together, more studies are needed to evaluate the use of CBD as a possible pharmacological tool for the treatment of CUD.

The main goals of this study were to develop a new model of spontaneous cocaine withdrawal in mice induced by repeated administration of increasing doses of cocaine for 12 days and to evaluate the effects of CBD on behavioural and gene expression changes associated with cocaine withdrawal. Anxiety-like behaviour (light-dark box), motor activity (distance travelled in the open field test) and somatic withdrawal signs (number of rearings, rubbings, groomings and diggings) were assessed 6 hours after the last cocaine administration. Furthermore, gene expression analyses by real-time PCR were carried out to evaluate changes induced by cocaine withdrawal in specific targets involved in cocaine addiction and withdrawal. Relative gene expression analyses of*dopamine transporter* (DAT) and*tyrosine hydroxylase* (TH) in the ventral tegmental area (VTA), and cannabinoid receptors 1 (*CNR1*) and 2 (*CNR2*) in the nucleus accumbens (NAcc) were carried out.

Methods

Mice

A total of 100 CD1 male mice were purchased from Charles River laboratories (Lille, France). Mice, weighing 20-25 g were housed in individual cages (40 x 25 x 22 cm) under controlled environmental conditions (temperature, 21 ± 2 ºC, relative humidity, 60 ± 10 %, and 12 h light-dark cycle, lights on from 08:00 to 20:00). One week after mice acclimatization to the animal room, behavioural analyses were initiated. 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, Browne et al., 2010; McGrath & Lilley, 2015).

Drugs

Cocaine (cocaine hydrochloride) was obtained from Spanish Agency of Drugs (AEMPS, Madrid, Spain) and dissolved in saline (0.9 % sodium chloride) to prepare required doses immediately before its intraperitoneal

(i.p.) administration 3 times a day (every 5 hours during mice light cycle) for 12 consecutive days. Cannabidiol (CBD) was obtained from STI Pharmaceuticals (Essex, UK) and was dissolved in ethanol:cremophor:saline (1:1:18) immediately before its use to obtain the required doses of 10, 20 and 40 $mg{\cdot}kg^{-1}$ (i.p.).

Animal model of spontaneous cocaine withdrawal

Spontaneous cocaine withdrawal was induced by treating mice with increased doses of cocaine (i.p.) starting with 15 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ at day 1 up to 60 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$ at day 11 (Figure 1). Cocaine administrations were carried out every 5 h during mice light cycle. Mice spontaneous cocaine withdrawal-induced behavioural and gene expression alterations were evaluated 6 hours after the last cocaine administration at day 12 (20 $mg{\cdot}kg^{-1}$) (Figure 1).

Experimental design

Motor activity and somatic withdrawal signs . A set of 50 CD1 mice were used to evaluate motor activity and somatic withdrawal signs in the open field paradigm. For this purpose, 6 hours after the last cocaine administration (day 12, 20 $mg{\cdot}kg^{-1}$) mice were placed into individual methacrylate boxes (25 x 25 x 25 cm) and were videotaped for 15 minutes to evaluate somatic signs associated with abstinence (number of rearings, rubbings, groomings and diggings). Simultaneously, motor responses were also evaluated by measuring the total distance travelled for 15 minutes with the SMART programme (Panlab). Ninety minutes before the behavioural evaluation, CBD (10, 20 and 40 $mg{\cdot}kg^{-1}$) or its corresponding vehicle were administered (i.p.) to evaluate the effects on the modulation of spontaneous cocaine withdrawal signs and motor activity.

Light-dark box test. An additional set of 50 CD1 mice were used to evaluate anxiety-like behaviour changes induced by cocaine abstinence. Six hours after cocaine last administration (Day 12, 20 $mg{\cdot}kg^{-1}$) mice were individually tested for 5 minutes in the light-dark box paradigm. Ninety minutes before this evaluation, CBD (10, 20 and 40 $mg{\cdot}kg^{-1}$) or its corresponding vehicle were administered. The time spend in the lighted box and the number of transitions were recorded for each session. After this behavioural evaluation, mice were sacrificed by cervical dislocation and brain samples were removed to analyse relative gene expression of targets of interest.

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.

Relative gene expression analyses by real-time PCR

Relative gene expression analyses of DAT and TH in the VTA, and CNR1 and CNR2 in the NAcc were carried out in vehicle-treated and cocaine-treated mice used to analyse the effects of spontaneous cocaine withdrawal on anxiety-like behaviour and its pharmacological modulation by CBD administration. Briefly, mice were sacrificed 150 minutes after CBD or its corresponding vehicle administration and brain samples were removed from the skull and frozen at -80 ^{o}C. These samples were used to obtain coronal sections (500 μm) of regions of interest in a cryostat (-10 ^{o}C) according to Paxinos and Franklin atlas (Paxinos, 2001). Brain nucleus of interest were microdissected following the method described by Palkovits and performed by our group (Navarrete, Perez-Ortiz et al., 2012; Palkovits, 1983). Total RNA was extracted from brain micropunches with TRI Reagent (Applied Biosystem, Madrid, Spain) and reverse transcription was carried out (Applied Biosystem, Madrid, Spain). Quantitative analyses of the relative expression of DAT (Mm00438388_m1), TH (Mm00447546_m1), CNR1 (Mm00432621_s1) and CNR2 (Mm00438286_m1) genes were performed on the StepOne Sequence Detector System (Applied Biosystem, Madrid, Spain). All reagents used in this study were obtained from Life Technologies and manufacturer instructions were followed. The reference gene used was 18S rRNA (Mm03928990_g1), and data for each target was normalized to the endogenous reference gene. The fold change in target gene expression was calculated using the $2^{\Delta\Delta\text{-}\tau}$ method (Livak & Schmittgen, 2001).

Data and statistical analyses

Statistical analyses were performed using One-way ANOVA followed by Student-Newman-Keul's test when comparing different experimental groups. Differences were considered significant if the probability of error

was less than 5%. 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, Alexander et al., 2018).

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, Sharman et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017; Alexander, Kelly et al., 2017; Alexander, Striessnig et al., 2017).

Results

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

Open field paradigm . Mice exposed to the spontaneous cocaine withdrawal model showed significantly increased total distance values compared with vehicle-treated mice. CBD administration fully blocked this increase at all three administered doses (Figure 2A, One-way ANOVA, $F_{(4,48)} = 4.173$, $P = 0.006$).

Withdrawal somatic signs . The one-way ANOVA showed an increase of the number of rearings and diggings after cocaine treatment cessation in comparison with the vehicle-treated group. All the three doses of CBD (10, 20 and 40 mg·kg^{-1}) completely normalized the increased number of rearings (Figure 2B, One-way ANOVA, $F_{(4,48)} = 4.589$, $P = 0.003$), whereas only 20 and 40 mg·kg^{-1} doses of CBD significantly reduced the number of diggings (Figure 2E, One-way ANOVA, $F_{(4,48)} = 15.025$, $P < 0.001$). Mice exposed to the spontaneous cocaine withdrawal model presented a reduced number of rubbings (Figure 2C, One-way ANOVA, $F_{(4,48)} = 5.856$, $P < 0.001$) and groomings (Figure 2D, One-way ANOVA, $F_{(4,48)} = 6.248$, $P < 0.001$), which were normalized only with the lowest employed dose of CBD (10 mg·kg^{-1}).

Effects of CBD on anxiety-like behaviour induced by spontaneous cocaine withdrawal

After the cessation of cocaine treatment, cocaine + vehicle-treated mice exhibited a significant decrease in the time spent in the lighted box. The administration of CBD fully blocked this anxiety-like behaviour at 10 and 20 mg·kg^{-1} doses, and induced an additional anxiolytic effect at the dose of 40 mg·kg^{-1} (Figure 3A, One-way ANOVA, $F_{(4,48)} = 12.573$, $P < 0.001$). Furthermore, no changes were observed in the number of transitions between all the 5 groups (Figure 3B, One-way ANOVA, $F_{(4,48)} = 0.326$, $P = 0.859$).

Effects of CBD on changes in the DAT, TH, CNR1 and CNR2 gene expression induced by spontaneous cocaine withdrawal

Mice exposed to the animal model of spontaneous cocaine withdrawal showed an increase relative gene expression of DAT compared with control mice, which levels were normalized with CBD administration at the dose of 40 mg·kg^{-1} (Figure 4A, One-way ANOVA, $F_{(4,48)} = 5.019$, $P < 0.01$). In addition, spontaneous cocaine withdrawal induced an increase of TH relative gene expression, that was completely normalized with all three administered doses of CBD (10, 20 and 40 mg·kg^{-1}) (Figure 4B, One-way ANOVA, $F_{(4,48)} = 4.878$, $P < 0.01$).

One-way ANOVA revealed decreased CNR1 relative gene expression in mice exposed to the animal model of spontaneous cocaine withdrawal. CBD produced a significant up-regulation of CNR1 gene expression at the dose of 40 mg·kg^{-1} (Figure 4C, One-way ANOVA, $F_{(4,48)} = 7.292$, $P < 0.001$). In addition, CNR2 relative gene expression was increased in mice with spontaneous cocaine withdrawal, and CBD administration produced an additional increase, reaching statistical significance at the dose of 40 mg·kg^{-1} (Figure 4D, One-way ANOVA, $F_{(4,48)} = 6.677$, $P < 0.001$).

Discussion

The results of the present study reveal: 1) a new animal model of spontaneous cocaine withdrawal with specific short-term behavioural and gene expression alterations, and 2) how the administration of CBD significantly

regulated the emotional and gene expression disturbances induced by the model. These statements were supported by the following observations: i) Six hours after cessation of cocaine administration, mice presented increased motor activity and number of rearings and diggings, decreased rubbing and grooming behaviours, and increased anxiety-like behaviour, ii) The administration of CBD reduced the motor activity, normalized the spontaneous cocaine withdrawal-induced somatic signs, and induced an anxiolytic effect and iii) The administration of CBD blocked the increase of DAT and TH relative gene expression in the VTA, modulated the decrease of CNR1 gene expression in the NAcc, and produced an additional increase of CNR2 gene expression in the NAcc induced by spontaneous cocaine withdrawal.

The development of new animal models of cocaine withdrawal is critical for identifying new therapeutic targets. In the present study, a new rodent animal model of spontaneous cocaine withdrawal was induced by the administration of increasing doses of cocaine for 12 days. The augmentation of cocaine doses between days was carefully designed to increase first the morning dose to avoid coinciding with the peak of night-time activity minimizing adverse cardiac effects (Richards, Hollander et al., 2017). Furthermore, the administration of cocaine every 5 hours during mice light cycle contrasts with other reported cocaine withdrawal models where cocaine was given three times in the morning, one hour apart (Aguilar, Ledesma et al., 2017; Ledesma, Aguilar et al., 2017; Zhang, Schlussman et al., 2013). The pattern used in this study simulates more closely the intermittent consumption of cocaine described in cocaine users avoiding long periods of abstinence (Kawa, Allain et al., 2019). Finally, considering cocaine pharmacokinetic properties and the clinical onset of the abstinence, the evaluation of cocaine withdrawal-induced disturbances was carried out 6 hours after the last administration. This allowed the detection of enhanced withdrawal signs such as increased motor activity and anxiety-like behaviour (Aguilar, Ledesma et al., 2017; Ledesma, Aguilar et al., 2017; Valzachi, Teodorov et al., 2013). Interestingly, during cocaine withdrawal we found an increase in the number of rearings and diggings and a decrease in the number of rubbings and groomings. Furthermore, the evaluation of cocaine spontaneous withdrawal revealed significantly increased anxiety-like behaviour, unlike other models where anxiety-like behaviour was assessed days after the last cocaine administration (Aguilar, Ledesma et al., 2017; Ledesma, Aguilar et al., 2017; Valzachi, Teodorov et al., 2013).

In the last years, several studies focused on the potential of CBD for the treatment of substance use disorders. This was probably because of its anxiolytic, antidepressant, antipsychotic and neuroprotective actions (Campos, Moreira et al., 2012), without presenting potential as a drug of abuse (Viudez-Martinez, Garcia-Gutierrez et al., 2019). Indeed, CBD resulted useful to modulate the reinforcing and motivational properties of different drugs of abuse such as alcohol, cannabis or opiates (Katsidoni, Anagnostou et al., 2013; Navarrete, Aracil-Fernandez et al., 2018; Ren, Whittard et al., 2009; Viudez-Martinez, Garcia-Gutierrez et al., 2018a; Viudez-Martinez, Garcia-Gutierrez et al., 2018c). Also, the administration of CBD modulated context- and stress-induced cocaine seeking in rats (Gonzalez-Cuevas, Martin-Fardon et al., 2018), and reduced cocaine self-administration (Lujan, Castro-Zavala et al., 2018). In this study, the administration of CBD normalized the increase of motor activity induced by spontaneous cocaine withdrawal. Interestingly, CBD significantly reduced the number of rearings and diggings at intermediate and high doses whereas normalized the number of rubbings and groomings at the lowest dose. This dose-dependent modulation of withdrawal signs could be explained, at least in part, with the bell-shaped dose-response curve of CBD-mediated regulation of anxiety-like behaviour (Campos, Moreira et al., 2012). A recent study of our group revealed that CBD modulated hypothalamic-pituitary-adrenal (HPA) axis activation after an acute stress only at the lowest dose (Viudez-Martinez, Garcia-Gutierrez et al., 2018b). Moreover, CBD normalized the increased anxiety-like behaviour in mice exposed to the spontaneous cocaine withdrawal model reaching an additional anxiolytic action at the highest dose.

Gene expression analyses were performed to identify alterations in specific targets that may underlie the behavioural disturbances induced by the cocaine spontaneous withdrawal and its regulation by CBD. Interestingly, increased relative gene expression of DAT was observed in vehicle-treated mice exposed to the cocaine withdrawal model. The main mechanism of action of cocaine is the blockade of DAT function avoiding dopamine reuptake, leading to an up-regulation after repeated administration (Daws, Callaghan et al., 2002; Fang & Ronnekleiv, 1999; Kahlig & Galli, 2003; Mash, Pablo et al., 2002). Interestingly, CBD (40 $mg \cdot kg^{-1}$)

completely normalized DAT gene expression, suggesting a inhibitory action consistent with the suggestion of a previous study revealing that CBD may act as a low potency inhibitor of DAT in rat striatal terminals (Pandolfo, Silveirinha et al., 2011). Considering the crucial role of DAT in the actions induced by cocaine, it is possible to hypothesize that this effect may be closely related with the CBD-mediated improvement of cocaine withdrawal behaviours.

Previous results suggested that CBD modulates dopaminergic neurotransmission in the mesolimbic system: 1) Microdialysis perfusion of CBD into the lateral hypothalamus increases dopamine extracellular levels in the NAcc (Murillo-Rodriguez, Palomero-Rivero et al., 2011), 2) The administration of CBD into the NAcc blocked VTA dopaminergic neuronal sensitization induced by the amphetamine (Renard, Norris et al., 2017), and 3) CBD attenuated cocaine-induced increases in extracellular dopamine in the NAcc (Galaj, Bi et al., 2020). Accordingly, cocaine withdrawal also significantly increases TH gene expression in the VTA and this action was completely blocked by all doses of CBD. The potential of CBD to reduce enhanced TH gene expression was previously reported by our group in animal models of alcohol consumption (Viudez-Martinez, Garcia-Gutierrez et al., 2018a; Viudez-Martinez, Garcia-Gutierrez et al., 2018c). Taken together, it is tempting to suggest that the CBD-mediated regulation of cocaine-induced mesolimbic dopaminergic activation may be involved in the regulation of behavioural alterations induced during cocaine withdrawal.

CNR1 and CNR2 gene expressions in the AMY were also measured since both genes may be involved in the mechanism of action of CBD and in cocaine addiction (Arnold, 2005; Gobira, Oliveira et al., 2019). Some authors reported the involvement of CB1r in the reinforcing and motivational properties of cocaine (Liu, Canseco-Alba et al., 2017; Lopes, Bastos et al., 2020; Soria, Mendizabal et al., 2005; Vlachou, Nomikos et al., 2003). CNR1 gene expression was lower in mice exposed to the cocaine withdrawal model, and up-regulated with CBD. Interestingly, a similar effect was observed in the hippocampus of CBD-treated mice exposed to a cocaine self-administration paradigm (Lujan, Cantacorps et al., 2019). The normalization of CNR1 gene expression changes in the NAcc by CBD was previously shown by our group (Navarrete, Aracil-Fernandez et al., 2018; Viudez-Martinez, Garcia-Gutierrez et al., 2018a; Viudez-Martinez, Garcia-Gutierrez et al., 2018c). CBD is an indirect agonist of CB1r by the inhibition of $FAAH$ activity and the blockade of $anandamide$ reuptake (Bisogno, Hanus et al., 2001). Some reports also suggest that CBD might act as negative allosteric modulator of CB1r (Chung, Fierro et al., 2019; Laprairie, Bagher et al., 2015). Further studies are needed to identify the mechanism involved in the actions of CBD on CNR1 gene.

Cocaine withdrawal syndrome was associated with increased CNR2 gene expression in the AMY. Overexpression of CB2r resulted in cocaine-induced conditioned place aversion and reduced cocaine-self-administration in mice (Aracil-Fernandez, Trigo et al., 2012). Furthermore, the use of conditional knockout mice to delete CNR2 gene in midbrain DA neurons revealed the critical involvement of CB2r to attenuate psychomotor and rewarding effects of cocaine (Liu, Canseco-Alba et al., 2017). More recently, it was discovered that the CB2r agonist $JWH133$ inhibited the acquisition and expression of cocaine sensitization and conditioned place preference (Lopes, Bastos et al., 2020). A plausible hypothesis is that cocaine withdrawal-induced up-regulation of CNR2 gene expression may be related with a homeostatic compensatory effect. Interestingly, in this study the administration of CBD additionally increases CNR2 gene expression. This effect may be due, at least in part, to the inverse agonism or antagonism on CB2r produced by CBD (Thomas, Baillie et al., 2007). A recent report suggest that the attenuation of cocaine rewarding effects induced by CBD was mediated by CB2r (Galaj, Bi et al., 2020). Therefore, it is possible to argue that CBD-induced CNR2 up-regulation may be responsible, at least in part, for the modulation of cocaine withdrawal.

In conclusion, the results of this study provide unequivocal evidence of the efficacy of CBD to improve anxiety-like behaviour, motor activity and somatic signs alterations induced by a new animal model of spontaneous cocaine withdrawal. Gene expression analyses provide relevant information about the neurobiological basis of this model and of the mechanisms involved in the actions of CBD. Future studies are required to improve our knowledge about the neurobiological mechanisms involved in cocaine withdrawal as a strategy to develop new drugs to prevent relapse, and to explore the potential of CBD to treat cocaine withdrawal.

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Figure legends

Figure 1 . Timeline diagram of the experimental procedure used for the development of an animal model of spontaneous cocaine withdrawal syndrome. Cocaine administration (15 mg·kg^{-1}·day^{-1} – 60 mg·kg^{-1}·day^{-1}, i.p.) was performed during 12 consecutive days. Six hours after last cocaine dose, the effects of CBD (10, 20 and 40 mg·kg^{-1}, i.p.) on spontaneous cocaine withdrawal syndrome were evaluated by the open field and light-dark box paradigms.

Figure 2 . Evaluation of CBD (10, 20 and 40 mg·kg^{-1}) effects on motor activity and somatic signs alterations induced by the spontaneous cocaine withdrawal model, in the open field paradigm. Columns represent the means and vertical lines ±SEM of the total distance recorded (cm) (A) and the number of rearings (B), rubbings (C), groomings (D) and diggings (E). *, values from COCA-VEH treated group that are significantly different from VEH-VEH controls (One-way ANOVA, $P < 0.05$). #, values from COCA-CBD treated groups that are significantly different from COCA-VEH treated group (One-way ANOVA, $P < 0.05$). &, values from COCA-CBD (20 and 40 mg·kg^{-1}) treated groups that are significantly different from COCA-CBD 10 mg·kg^{-1} treated group (One-way ANOVA, $P < 0.05$).

Figure 3 . Evaluation of CBD (10, 20 and 40 mg·kg^{-1}) effects on increased anxiety-like behaviour induced by the spontaneous cocaine withdrawal model, in the light-dark box paradigm. Columns represent the means and vertical lines ±SEM of the time spend in the lighted box (s) (A) and the number of transitions (B). *, values from COCA-VEH treated mice that are significantly different from VEH-VEH treated mice (One-way ANOVA, $P < 0.001$). #, values from COCA-CBD treated groups that are significantly different from COCA-VEH treated group (One-way ANOVA, $P < 0.001$). &, values from COCA-CBD (40 mg·kg^{-1}) treated group that are significantly different from COCA-CBD (10 and 20 mg·kg^{-1}) groups (One-way ANOVA, $P < 0.05$).

Figure 4 . Relative gene expression analyses of dopamine transporter (DAT) (A) and tyrosine hydroxylase (TH) (B) in the ventral tegmental area (VTA), and cannabinoid receptors 1 (CNR1) (C) and 2 (CNR2) (D) in the nucleus accumbens (NAcc). Columns represent the means and vertical lines ±SEM of $2^{-\Delta\Delta^{\circ}\tau}$. *, values from COCA-VEH treated group that are significantly different from VEH-VEH treated group (One-way ANOVA, $P < 0.01$). #, values from COCA-CBD treated group that are significantly different from those treated with COCA-VEH (One-way ANOVA, $P < 0.05$). &, values from COCA-CBD (40 mg·kg^{-1}) treated group that are significantly different from groups treated with COCA-CBD (10 mg·kg^{-1}) (One-way ANOVA, $P < 0.01$).

Figure 1

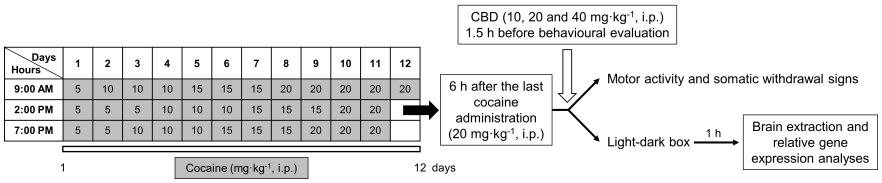

Figure 2

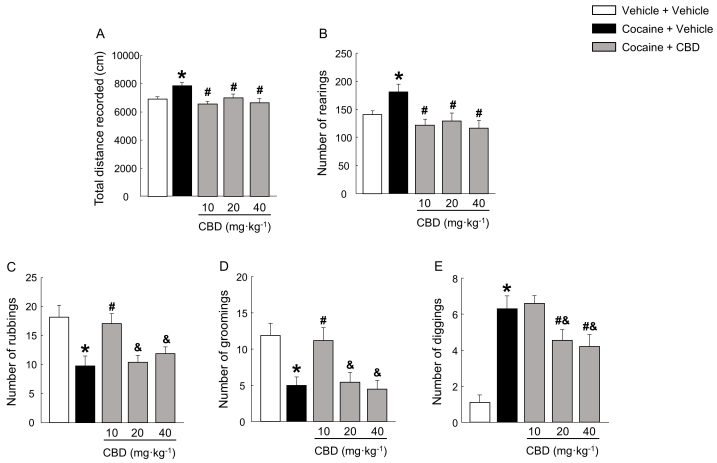

Figure 3

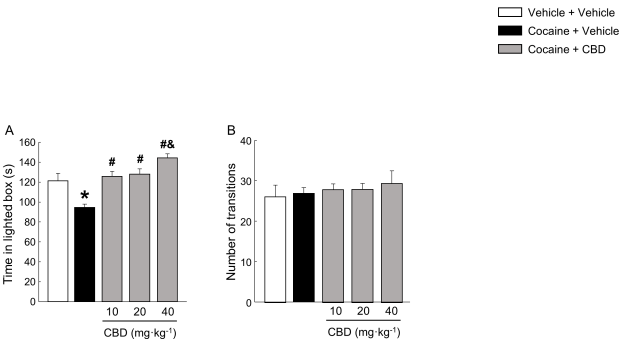

Figure 4