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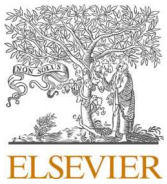

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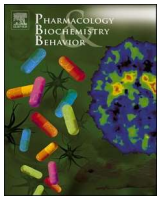

Cannabidiol treatment prevents drug reinstatement and the molecular alterations evoked by amphetamine on receptors and enzymes from dopaminergic and endocannabinoid systems in rats

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ABSTRACT

In psychostimulant drug addiction, relapse is the most concerning outcome to be managed, considering there is no approved treatment for this neuropsychiatric condition. Here, we investigated the effects of the CBD treatment on the relapse behavior triggered by stress, after being submitted to the amphetamine (AMPH)-induced conditioned place preference (CPP) in rats. To elucidate the mechanisms of action underlying the CBD treatment, we evaluated the neuroadaptations on dopaminergic and endocannabinoid targets in the ventral striatum (VS) and ventral tegmental area (VTA) of the brain. Animals received d,l-AMPH (4 mg/kg, i.p.) or vehicle in the CPP paradigm for 8 days. Following the first CPP test, animals were treated with CBD (10 mg/kg, i.p.) or its vehicle for 5 days and subsequently submitted to forced swim stress protocol to induce AMPH-CPP relapse. Behavioral findings showed that CBD treatment prevented AMPH-reinstatement, also exerting anxiolytic activity. At the molecular level, in the VTA, CBD restored the CB1R levels decreased by AMPH-exposure, increased NAPE-PLD, and decreased FAAH levels. In the VS, the increase of D1R and D2R, as well as the decrease of DAT levels induced by AMPH were restored by CBD treatment. The current outcomes evidence a substantial preventive action of the CBD on the AMPH-reinstatement evoked by stress, also involving neuroadaptations in both dopaminergic and endocannabinoid systems in brain areas closely involved in the addiction. Although further studies are needed, these findings support the therapeutic potential of CBD in AMPH-relapse prevention.

1. Introduction

Drug addiction is a chronic disorder characterized by compulsive seeking and loss of control over substance use despite serious negative consequences ensue (Koob and Volkow, 2009). Amphetamine-type psychostimulant drugs, such as amphetamine (AMPH), 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine (METH) are highly addictive, and therefore widely abused (UNODC, 2020). In recent decades, the development of new treatments for psychostimulant addiction has been the focus of several studies. However, no specific pharmacological therapy had shown effectiveness in alleviating abstinence symptoms (e.g. craving and anxiety) and preventing relapse (Cao et al., 2016).

The mesocorticolimbic system, also known as the reward system, plays an important role in drug addiction. The use of drugs such as AMPH increases dopaminergic neurotransmission in this pathway (Maldonado, 2003) being able to activate reward symptoms, and this response is related to the drug rewarding effect (Di Chiara et al., 2004). Furthermore, repeated exposure to these substances can cause transient and persistent adaptations in brain areas related to the mesocorticolimbic system (Robinson and Berridge, 2003) and the adaptive changes seem to corroborate for compulsive and uncontrolled use of drugs, as well as for the relapse occurrence (Chou and Narasimnham, 2005).

Although the dopaminergic system has been considered the most important system involved in brain reward processes for decades (Liu and Li, 2018), recent evidence suggests that the endocannabinoid system (ECS) also has a fundamental role in the signaling of rewarding events (Manzanares et al., 2018; Olière et al., 2013). Endocannabinoids (eCBs) and cannabinoid receptors are extensively expressed in brain

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areas of the mesocorticolimbic system and it is believed that they can modulate the dopaminergic signaling in this pathway (Everett et al., 2021; Lupica et al., 2004; Maldonado and Rodriguez de Fonseca, 2002; Melis et al., 2004). The eCBs also seem to participate in the mesocorticolimbic system synaptic plasticity, which is required for the development of the adaptive changes, leading to drug addiction (Gerdeman et al., 2002; Robbe et al., 2002). Also, neuroadaptations in the endocannabinoid system after chronic exposure to drugs were found (Parsons and Hurd, 2015). Thus, evaluating receptors and enzymes involved in endocannabinoid metabolism may provide interesting answers regarding the involvement of this system in the context of drug addiction. Currently, the modulation of the endocannabinoid system has been pointed as a potential target for the development of strategies for drug addiction treatment.

Cannabidiol (CBD) is a phytocannabinoid constituent of the *Cannabis sativa* plant devoid of addictive effects (Viudez-Martínez et al., 2019). CBD has multiple mechanisms of action, and its pharmacology is not completely understood. It acts on several receptors and systems including the ECS (Pertwee, 2008), serotonergic (Campos and Guimarães, 2008; Russo et al., 2005), and opioid (Kathmann et al., 2006) systems, among others (see review: Bonaccorso et al., 2019). Within the ECS, CBD has a low affinity for cannabinoid type 1 (CB1R) and 2 (CB2R) receptors (Zlebnik and Cheer, 2016), despite the fact that CBD exerts a negative modulation effect on both receptors (Ligresti et al., 2016). Furthermore, CBD decreases hydrolysis of the endocannabinoid anandamide (arachidonoylethanolamine, AEA) mediated by fatty acid amide hydrolase (FAAH) (Ligresti et al., 2016; Campos et al., 2012). Recently, CBD emerged as a promising compound and a new pharmacological strategy for a wide range of disorders (Ligresti et al., 2016), with both human and pre-clinical studies showing its potential as a therapeutic tool in the treatment of various psychiatric diseases including anxiety, depression, and psychosis (Bonaccorso et al., 2019; Campos et al., 2012). Currently, CBD has drawn the scientific community's attention due to its possible beneficial effects on substance use disorders treatment (see review: Chye et al., 2019).

Pre-clinical studies investigating the CBD use on animal models of addiction showed possibilities to reduce the behavioral and molecular manifestations of maladaptive neuroplasticity underlying drug addiction. For instance, CBD reduced the consumption and relapse to ethanol (Viudez-Martinez et al., 2017), craving and relapse to heroin (Ren et al., 2009) as well as reward and withdrawal symptoms to morphine (Bhargava, 1976; Katsidoni et al., 2013). In addition, CBD administration reduced the intake (Weiss and Gonzalez-Cuevas, 2019) and prevented reinstatement of cocaine-seeking (Gonzalez-Cuevas et al., 2018). Current evidence, including studies from our research group, has demonstrated potential anti-relapse effects of CBD on amphetamine addiction (Karimi-Haghighi and Haghparast, 2018; Karimi-Haghighi et al., 2020; Metz et al., 2021). Nonetheless, the information available on the possible neural mechanisms by which CBD acts and reduces drug use behaviors is still very scarce. The explanation regarding these processes would provide a consistent understanding of existing data and could consolidate the promise of CBD as a treatment for drug addiction.

In order to expand upon this research line, the current study aimed to evaluate the behavioral effects of CBD treatment on the stress-induced reinstatement of AMPH-CPP. Moreover, to explore a possible mechanism underlying the CBD effects, we also evaluated the molecular modifications on the dopaminergic and endocannabinoid targets in the rats' brain areas of reward.

2. Materials and methods

2.1. Animals

Twenty-four male *Wistar* rats (Universidade Federal de Santa Maria - UFSM, RS, Brazil) weighing 120-150 g were used for this study. The animals were 40-days old, which is considered by many studies as the adolescence period in rodents, and a highly vulnerable period for developing drug addiction (Teixeira-Gomes et al., 2015). The animals were housed three per cage with free access to water and food in a room with controlled temperature (22 ± 1 °C) and on a 12-h light/dark cycle. All procedures were performed according to the Animal Ethics Committee (UFSM-8850121118) guidelines, affiliated to the National Council for the Control of Animal Experimentations (CONCEA), following international standards of care and animal maintenance. The importance of current animal experiments in brain research is described in Homberg et al. (2021).

2.2. Drugs and solutions

The d, l-amphetamine (AMPH; Merck, Germany) (4 mg/mL/kg) was dissolved in saline (0.9 % NaCl; AMPH-vehicle) and was used to induce conditioned place preference (CPP). The chosen dose and time for the conditioning period were standardized in the AMPH- CPP and used in previous studies from our research group (Metz et al., 2019, 2021; Segat et al., 2016, 2017). Cannabidiol (CBD; STI Pharmaceuticals, UK) (10 mg/mL/kg) was dissolved in a solution of 2 % polysorbate 80 (Tween 80; Sigma-Aldrich, St Louis, MO, USA) and saline. CBD dose was based on a previously published study from our research group (Metz et al., 2021). CBD was administered for 5 days, considering the extinction period of the AMPH preference, which, according to our previous study, can be observed 4 days after the last AMPH exposure (Metz et al., 2021). Animals received CBD 30 min before the extinction test (Mahmud et al., 2017). All drugs were intraperitoneally (i.p.) injected at a volume of 1 ml/kg of body weight.

2.3. Experimental protocol

At the postnatal day (PND) 40, the rats (n = 24) were assigned to two experimental groups: vehicle (received saline – 0.9 % NaCl solution, i.p., n = 12) and amphetamine (AMPH) (received AMPH, 4 mg/kg, i.p, n = 12) and were exposed to the conditioned place preference (CPP) protocol (as described below). Following the first CPP test, which was performed to verify AMPH preference, half of each experimental group received the cannabidiol treatment (CBD, 10 mg/kg, i.p) or control (saline and 2 % polysorbate 80, i.p) once a day for five consecutive days. This resulted in four final groups (n = 6): i) vehicle/control; ii) vehicle/CBD; iii) AMPH/control; iv) AMPH/CBD. During the CBD treatment period, additional CPP tests were performed to assess the loss of preference for the drug. Twenty-four hours after the last treatment, animals were submitted to forced swim stress protocol to induce the reinstatement of extinguished AMPH-CPP (as described below). Then, in sequence, a CPP test was conducted. Moreover, for assessing the locomotor activity and anxiety-like symptoms, the animals were tested on the open field and elevated plus-maze tests, respectively. On the next day, the rats were anesthetized (isoflurane, the dose to the effect), euthanized by decapitation, the brains were extracted and the ventral striatum (VS) and ventral tegmental area (VTA) were dissected according to Paxinos and Watson (2007) for molecular analysis (Fig. 1).

2.4. Behavioral assessments

2.4.1. Conditioned place preference (CPP)

The CPP apparatus consists of a box with two compartments of equal size (45 × 45 × 50 cm) and an equivalent intensity of light incidence but having different environmental stimuli: one compartment has a white floor and striped walls, and the other one has a striped floor and smooth white walls. Both compartments were accessible through a central compartment (18 × 36 × 50 cm) separated by manual guillotine doors.

Initially, on the post-natal day (PND) 40, the animals were placed for 15 min in each compartment, except the neutral compartment, for habituation. The purpose of this procedure was to exclude exploratory behavior that is common in new environments to avoid

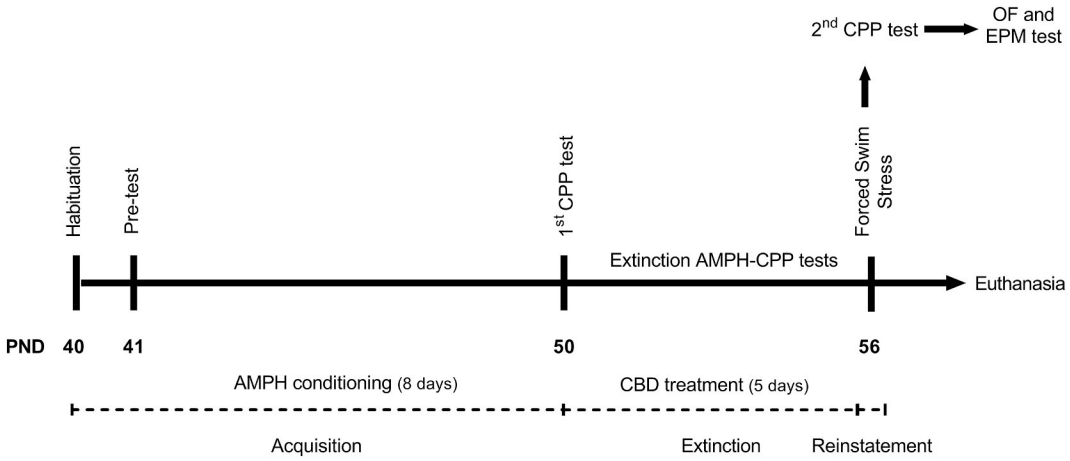

Fig. 1. Experimental design: after basal behavioral evaluation (pre-test), animals were treated with AMPH (4 mg/kg, i.p.) for 8 days, when the 1st behavioral evaluation was performed in the CPP paradigm. Subsequently, animals were treated with CBD (10 mg/kg, i.p.) for 5 days. During the CBD treatment period, additional CPP tests were performed to assess the extinction of AMPH-CPP. Twenty-four hours after the last treatment dose, animals were submitted to forced swim stress protocol to induce the reinstatement of extinguished AMPH-CPP and in sequence, a CPP test was conducted. OF and EPM tests were also performed, followed by molecular analysis in both VS and VTA. Abbreviations: AMPH: amphetamine; CBD: cannabidiol; CPP: conditioned place preference; OF: open field; EPM: elevated plus maze; VS: ventral striatum; VTA: tegmental ventral area.

misinterpretations.

One day after habituation (PND 41), the pre-test was performed. This phase consists of placing the animal into the central compartment allowing free access to the entire apparatus for 15 min and the time spent in each compartment was recorded. This procedure aimed to determine the innate environmental chamber preference. Rats that showed strong unconditioned aversion (<25 % of the session time) or preference (>75 % of the session time) for any compartment were discarded (Vasquez et al., 2006).

Following this, the conditioning phase was carried out for 8 days (PND 42–49). The animals received AMPH and were conditioned for 25 min in the compartment they spent the shortest time in the pre-test (Carlezon et al., 2002). After a 4-hour interval, they received the vehicle (0.9 % NaCl) and were confined in the opposite compartment. Vehicle-treated groups received two daily injections of NaCl solution in both compartments of the CPP in alternated turns. On PND 50, the CPP test was conducted (without drug/vehicle administration). The time spent in each compartment was assessed for 15 min and results express the percentage of time spent by the animals in the compartment associated with AMPH during the abstinence period (Metz et al., 2019, 2021; Segat et al., 2016).

CBD treatment was performed from PND 51 to PND 55. Additional CPP tests were assessed during this phase to evaluate the drug preference extinction, which was observed on the fourth day (PND54) of CBD treatment.

Reinstatement studies are considered an experimental model of drug relapse (Katz and Higgins, 2003). In the animal models, it is well established that three types of stimuli could induce drug reinstatement, as follows: i) stress exposure, ii) environmental cues associated with the drug effects, and iii) drug priming (Mantsch et al., 2016; Shaham et al., 2003). After a detailed analysis of the different animal models of stress exposure described in the literature (Karimi-Haghighi and Haghparast, 2018; Nygard et al., 2016; Sedki et al., 2015), we decided to follow the forced swim model (Haghparast et al., 2014; Mikail et al., 2012), after the AMPH-CPP extinction.

Thus, after the treatment period (PND 56), animals were submitted to forced swim stress (FSS) protocol to induce the reinstatement of preference for AMPH (Haghparast et al., 2014; Mikail et al., 2012). The forced swim stress (FSS) apparatus consists of a plastic cylindrical tank measuring 50 cm height, 30 cm width, which was filled up with 30 cm of clean tap water (23–27 °C). The rats performed individually a 6-min forced swim. After, they were dried with towels and returned to their home cages for at least 10 min before the reinstatement test (Haghparast et al., 2014; Mikail et al., 2012). Next, an additional CPP test was performed to assess relapse. Symptoms of reinstatement preference were quantified by the longer time spent in the drug-paired environment after exposure to the FSS protocol.

2.4.2. *Open field (OF) test*

Animals were individually placed in the center of an OF arena (40 × 40 × 30 cm) enclosed by black matte walls and floor divided into squares, as described by Kerr et al. (2005). The number of crossings (horizontal squares crossed) was quantified for 5 min and used to measure the locomotor activity.

2.4.3. *Elevated plus maze (EPM) test*

The anxiety-like behavior was assessed in the elevated plus-maze (EPM) paradigm. The apparatus consists of a platform elevated 50 cm above the floor. Forty-centimeter-high walls enclose two opposite arms (50 × 10 cm) while the other two arms had no walls. All arms are connected by a central intersection (10 × 10 cm). The time spent in the closed arms (s), the time spent in the open arms (s), the total arm entries (number), the frequency of head-dipping (number), and anxiety index (calculated by the following formula) were evaluated for 5 min (Montgomery, 1955):

$$\text{Anxiety index} = 1 - ((\text{Open arms time}/\text{Total time}) + (\text{Open arms entries}/\text{Total entries}))/2$$

Anxiety index values range from 0 to 1, where an increase in the index expresses increased anxiety-like behavior (Cohen et al., 2012).

2.5. *Molecular assays*

Molecular analyses were performed by western blot as described by Dias et al. (2017). Briefly, the membranes were incubated with the primary antibodies (Santa Cruz Biotechnology): anti-dopamine D1 receptor (D1R) (1:500, sc-33660), anti-dopamine D2 receptor (D2R) (1:500, sc-9113), anti-dopamine transporter (DAT) (1:500, sc-14002), anti-cannabinoid CB1 receptor (CB1R) (1:250, sc-293419), anti-diacylglycerol lipase α (DAGLα) (1:500, sc-390409), anti-monoacylglycerol lipase (MAGL) (1:500, sc-398942), anti-N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) (1:500, sc-514372), anti-fatty acid amide hydrolase (FAAH) (1:250, sc-100739), anti-

β-actin (1:50000; Sigma-Aldrich, St. Louis, USA), followed by the appropriate secondary antibody (Santa Cruz Biotechnology) IgG horseradish peroxidase conjugate. Actin was used as an internal control and data were standardized according to its values.

2.6. Statistical analysis

All the results from behavioral, biochemical, and molecular assays were expressed as the mean ± standard error of the mean (S.E.M.). CPP data before CBD treatment were analyzed by Student's *t*-test. Repeated-measures ANOVA was performed for the analysis of the extinction period and reinstatement test and post-hoc Newman–Keuls was used to obtain differences between the different treatment groups. For other analyses, two-way ANOVA followed by Newman–Keuls test was performed using Statistic® (version 7.0). Values of $P < 0.05$ were considered statistically significant for all comparisons made. GraphPad Prism® (version 6.0) was used to design the figures.

3. Results

3.1. CBD treatment prevented stress-induced reinstatement of AMPH-CPP

Behavioral assessment performed before starting CBD treatment (1st CPP test) showed that AMPH-conditioned rats remained a longer time in the drug-conditioned place in comparison to the vehicle group ($P < 0.05$), which showed no place preference (Fig. 2A).

Two-way ANOVA for repeated measures revealed a significant main effect of the drug, treatment and time on the drug preference [$F(1, 20) = 152.79$, $P < 0.001$; $F(1, 20) = 4.83$, $P < 0.05$; $F(1, 20) = 12.45$, $P < 0.001$, respectively]. Also, two-way ANOVA for repeated measures revealed a significant interaction of time with drug [$F(1, 20) = 14.72$, $P < 0.001$], treatment [$F(1, 20) = 7.28$, $P < 0.001$] and drug × treatment [$F(1, 20) = 3.93$, $P < 0.001$].

Post hoc analysis showed that there was a significant decrease of drug preference at 4th day of extinction compared to the 1th day, indicating the extinction of AMPH-CPP. As represented in Fig. 2B, AMPH-CPP showed no difference among experimental groups in the extinction test, thus confirming the drug preference extinction on the 4th day after the last AMPH administration. The drug preference in the reinstatement test was significantly higher compared to the extinction test, indicating the reinstatement of CPP. In addition, post-hoc comparison showed that CBD treatment was able to prevent stress-induced reinstatement of AMPH-CPP.

3.2. AMPH exposure and CBD treatment did not affect locomotor activity in the open field (OF) task

After stress-induced reinstatement of AMPH-CPP, the locomotor activity observed in the OF task showed no difference among the experimental groups (Fig. 3).

3.3. CBD treatment reduced anxiety parameters assessed in the elevated plus-maze (EPM) test

Two-way ANOVA of the EPM revealed a significant main effect of the drug, treatment, and treatment × drug interaction on the time spent in the closed arms [$F(1, 20) = 8.41$; 46.27 and 10.91, $P < 0.05$, respectively] and on the time spent in the open arms [$F(1, 20) = 5.00$; 427.72; and 28.11, $P < 0.05$, respectively]. Two way ANOVA of the EPM also revealed a significant main effect of treatment and treatment × drug interaction on the head dipping frequency [$F(1, 20) = 8.07$; 4.78, $P < 0.05$, respectively] and on the anxiety index [$F(1, 20) = 41.49$ and 5.15, $P < 0.05$, respectively].

Newman-Keuls test revealed that CBD treatment per se decreased the

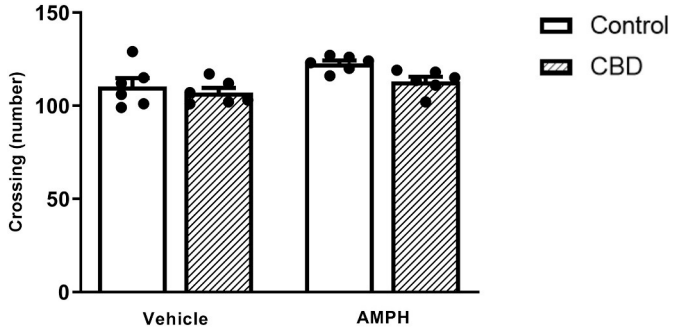

Fig. 3. CBD treatment influence (10 mg/kg, i.p., for 5 days) on the locomotor activity in the open field (OF) test after stress-induced reinstatement of AMPH-CPP. Crossing number was quantified (n = 6). Data are expressed as mean ± S.E.M. Abbreviations: AMPH: amphetamine; CBD: cannabidiol.

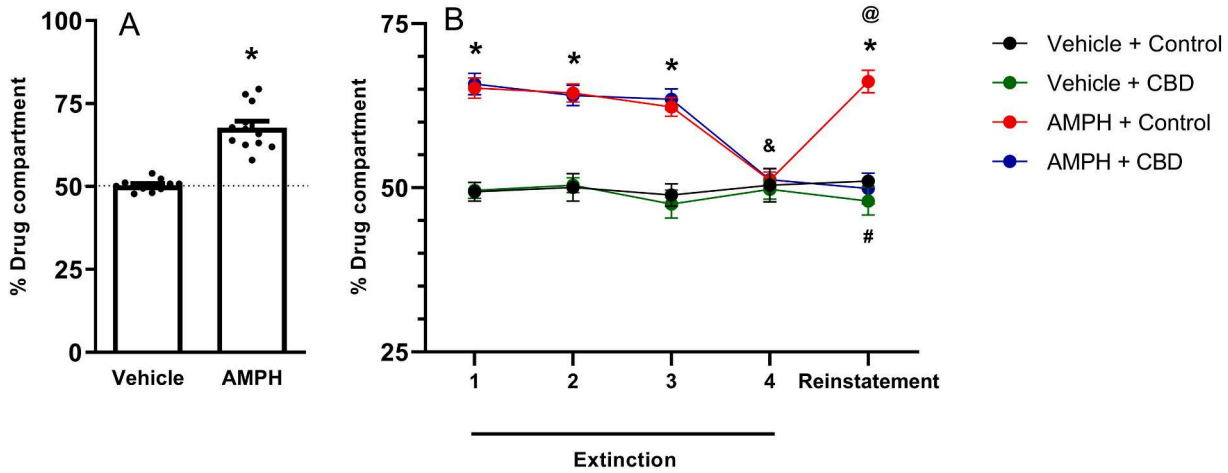

Fig. 2. Drug preference behavior induced by AMPH (4 mg/kg, i.p. for 8 days) observed in CPP paradigm before 5 days of CBD (10 mg/kg, i.p.) treatment (A), the extinction period after the last AMPH administration and drug preference behavior after stress-induced reinstatement of AMPH-CPP (B). (n = 6). Data are expressed as mean ± S.E.M. * indicates a significant difference of conditioning (vehicle/AMPH) in the same treatment (control/CBD) ($P < 0.05$). # indicates significant differences of the treatment (control/CBD) in the same conditioning (vehicle/AMPH) ($P < 0.05$). & indicates significant differences as compared to day 1 of extinction ($P < 0.05$). @ indicates significant differences as compared to day 4 of extinction ($P < 0.05$). Abbreviations: AMPH: amphetamine; CBD: cannabidiol; CPP: conditioned place preference.

time spent in the closed arms and increased the time spent in the open arms of the EPM (Fig. 4A and B). On the other hand, AMPH per se increased the time spent in the closed arms, decreased time spent and head dipping frequency in the EPM open arms when compared to the vehicle-injected group (Fig. 4A, B, and E). However, AMPH-exposed animals that were subsequently treated with CBD showed decreased time spent in the closed arms and an increase in the time spent and head dipping frequency in the open arms (Fig. 4A, B, and E). There was no statistical difference among experimental groups on the number of entries in open arms and total number of entries in both arms in the EPM test (Fig. 4C and D). Furthermore, CBD treatment reduced the anxiety index in both vehicle and AMPH-conditioned groups (Fig. 4F).

3.4. CBD treatment modified the dopamine-receptor (D1R and D2R) and -transporter (DAT) immunoreactivity in the ventral striatum (VS)

Two-way ANOVA of dopaminergic markers revealed a significant main effect of the drug, treatment, and treatment x drug interaction on D1R level [$F(1, 20) = 17.92$; 11.08 and 13.24, $P < 0.05$, respectively], as well as a significant main effect of drug and treatment on D2R level [$F(1, 20) = 6.53$ and 4.76, $P < 0.05$, respectively] and a significant main effect of the drug on DAT level [$F(1, 20) = 8.55$, $P < 0.05$].

Newman-Keuls test showed that AMPH exposure increased both D1R (Fig. 5A) and D2R (Fig. 5B) immunoreactivity in the VS when compared to the vehicle group. In contrast, AMPH-conditioned animals further treated with CBD did not show this increase induced by AMPH (Fig. 5A and B). In addition, AMPH exposure was able to reduce DAT levels when compared to vehicle, but the immunoreactivity of this transporter was preserved by CBD treatment in the AMPH-exposed rats (Fig. 5C).

3.5. CBD treatment modified cannabinoid receptor type 1 (CB1R), as well as the enzymes diacylglycerol lipase α (DAGL), monoacylglycerol lipase (MAGL), N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), and fatty acid amide hydrolase (FAAH) immunoreactivity in the ventral tegmental area (VTA)

Two-way ANOVA revealed a significant main effect of the drug, treatment and treatment × drug interaction on DAGL levels [$F(1, 20) = 26.84$; 281.47 and 10.55, $P < 0.05$, respectively] and a significant main effect of the treatment on CB1R, MAGL and FAAH immunoreactivity [$F(1, 20) = 14.95$; 20.99 and 26.84, $P < 0.05$, respectively]. Two-way ANOVA also revealed a significant main effect of both treatment and treatment × drug interaction on NAPE-PLD immunocontent [$F(1, 20) = 480.74$, $P < 0.05$; $F(1, 20) = 18.92$, $P < 0.05$, respectively].

Neuman-Keuls test revealed that AMPH per se decreased CB1R and NAPE-PLD levels (Fig. 6A and D), increasing FAAH levels (Fig. 6E) when compared to the vehicle group. CBD treatment per se increased DAGL and NAPE-PLD levels (Fig. 6B and D) and decreased MAGL and FAAH immunoreactivity (Fig. 6C and E). In animals exposed to AMPH, CBD treatment was able to restore CB1R levels (Fig. 6A), which were decreased by AMPH exposure. In addition, CBD treatment increased DAGL and NAPE-PLD immunocontent in comparison to both AMPH-conditioned and vehicle-treated groups (Fig. 6B and D) and decreased MAGL and FAAH levels in comparison to AMPH-conditioned animals (Fig. 6C and E).

4. Discussion

Here we investigated the effects of cannabidiol (CBD) treatment on stress-induced reinstatement of amphetamine (AMPH) conditioned place preference (CPP) in rats. Our findings showed that: i) CBD exerted relevant benefits on the animal behavior, being able to prevent AMPH-CPP reinstatement induced by a stressor. ii) CBD presented promising effects in relapse-promoting conditions, such as anxiety. iii) CBD can interact and stimulate significant changes in dopaminergic and endocannabinoid targets in both ventral striatum (VS) and ventral tegmental area (VTA). These brain areas are closely involved in addiction processes, where CBD promoted maintenance of the cellular homeostasis even after AMPH exposure. Taken together, these observations provide evidence that supports a therapeutic potential for CBD, especially when the addiction is associated with AMPH-type psychostimulants.

4.1. CBD treatment effects on AMPH reinstatement behavior

In the current study, AMPH-exposed animals showed drug-reinstatement behavior in the CPP paradigm after exposure to forced swimming stress while CBD treatment prevented AMPH-CPP reinstatement induced by this stressor. This potential anti-reinstatement effect

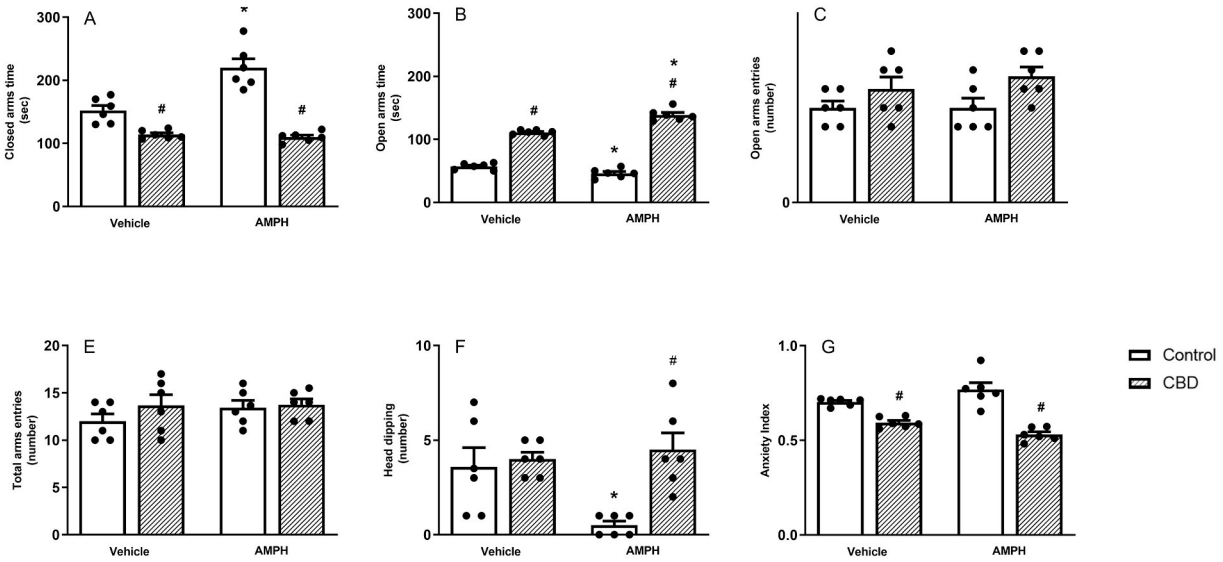

Fig. 4. CBD treatment influence (10 mg/kg, i.p.) on anxiety-like behaviors in the elevated plus-maze (EPM) test after stress-induced reinstatement of AMPH-CPP. The time spent in the closed arms (A), the time spent in the open arms (B), the number of open arms entries (C), the number of total arms entries (D), the number of head dipping (E), and the anxiety index (F) were quantified. (n = 6). Data are expressed as mean ± S.E.M. * indicates a significant difference of conditioning (vehicle/AMPH) in the same treatment (control/CBD) ($P < 0.05$). # indicates significant differences of the treatment (control/CBD) in the same conditioning (vehicle/AMPH) ($P < 0.05$). Abbreviations: AMPH: amphetamine; CBD: cannabidiol.

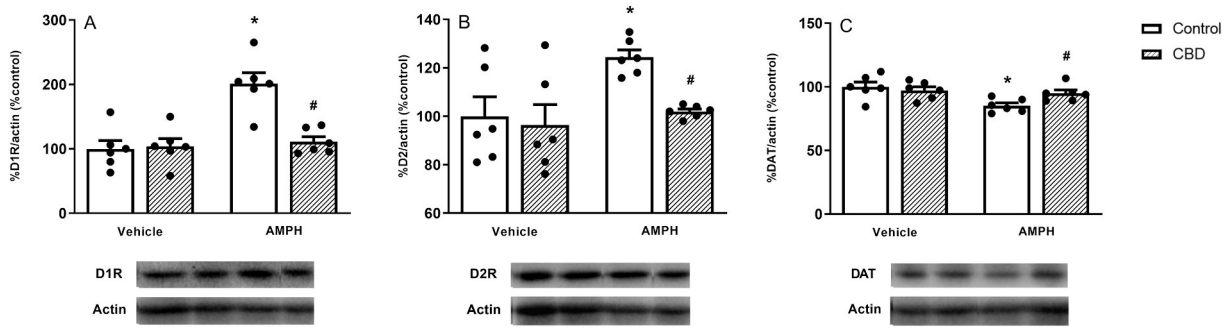

Fig. 5. CBD treatment influence (10 mg/kg, i.p.) on dopamine receptor type 1 (D1) levels (A), dopamine receptor type 2 (D2) levels (B) and on dopamine transporter (DAT) levels (C) in ventral striatum (VS) after stress-induced reinstatement of AMPH-CPP. (n = 6). Each representative band in the sequence corresponds to one bar in the figure. Data are expressed as mean ± S.E.M. * indicates a significant difference of conditioning (vehicle/AMPH) in the same treatment (control/CBD) ($P < 0.05$). # indicates significant differences of the treatment (control/CBD) in the same conditioning (vehicle/AMPH) ($P < 0.05$). Abbreviations: AMPH: amphetamine; CBD: cannabidiol.

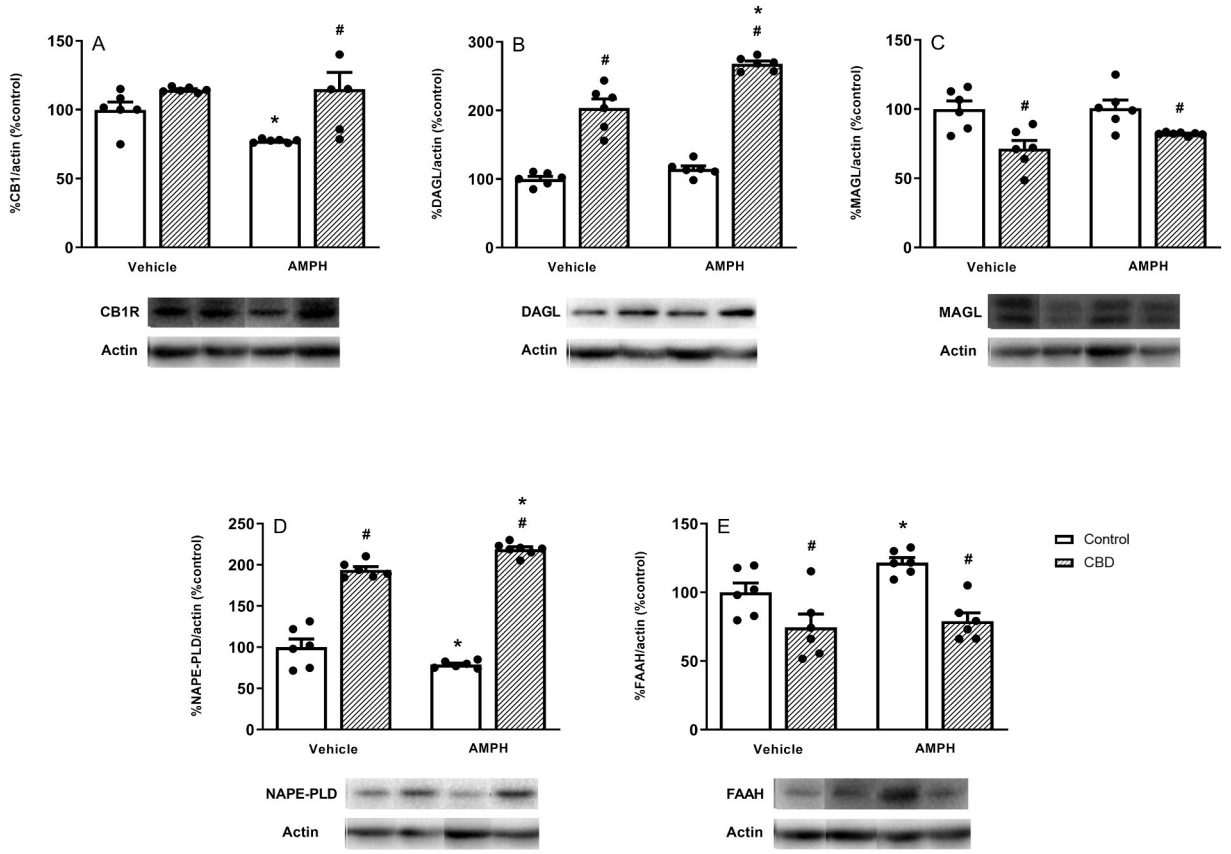

Fig. 6. CBD treatment influence (10 mg/kg, i.p.) on cannabinoid receptor type 1 (CB1) levels (A), on the enzyme diacylglycerol lipase α (DAGL) levels (B), on the enzyme monoacylglycerol lipase (MAGL) levels (C), on the enzyme N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) levels (D) and the enzyme fatty acid amide hydrolase (FAAH) levels (E) in ventral tegmental area (ATV) after stress-induced reinstatement of AMPH-CPP. (n = 6). Each representative band in the sequence corresponds to one bar in the figure. Data are expressed as mean ± S.E.M. * indicates a significant difference of conditioning (vehicle/AMPH) in the same treatment (control/CBD) ($P < 0.05$). # indicates significant differences of the treatment (control/CBD) in the same conditioning (vehicle/AMPH) ($P < 0.05$). Abbreviations: AMPH: amphetamine; CBD: cannabidiol.

demonstrated by CBD treatment is consistent and reinforces our previous data when CBD administration prevented drug-relapse induced by AMPH re-exposure (Metz et al., 2021). Also, these outcomes confirm other findings, showing that CBD is also effective in preventing relapse by other AMPH-type psychostimulants drugs in different experimental models (Hay et al., 2018; Karimi-Haghighi et al., 2020; Metz et al., 2021). Taken together, these studies report the therapeutic potential of the CBD to treat psychostimulants drug addiction, thus reiterating the importance of the continuance and advances in scientific research in this regard. Furthermore, to validate the experimental model, we evaluated the locomotor activity of the animals in the open field (OF) task, which results showed no differences in locomotor behavior among the experimental groups. This data supports the assertion that the behavioral results found in the CPP test are indeed due to the effects underlying the

treatment with CBD and are not related to locomotor artifacts (Metz et al., 2019; Segat et al., 2016; Takamatsu et al., 2011).

4.2. Anxiolytic properties of CBD and its impact on AMPH reinstatement

In humans and rodents, anxiety is an important factor involved in establishing and maintaining drug addiction, including AMPH. It was reported that 30–40 % of drug-addicted individuals also suffer from depression or anxiety disorder (Conway et al., 2006). After a prolonged exposure and subsequent detoxification, anxiety can be one of the main elements that trigger drug relapse (Biala et al., 2009). Here, we observed that AMPH administration followed by exposure to a stressor stimulus triggered anxiogenic behaviors in the animals, when they were subjected to the EPM task. Our findings are in agreement with previous experimental studies showing AMPH-induced anxiety-like behaviors (Barr et al., 2010; Metz et al., 2021; Vuong et al., 2010). Moreover, clinical and preclinical evidence has supported the anxiolytic effects of CBD, especially as a treatment for anxiety disorders (see review: Blessing et al., 2015). Our current outcomes also showed that CBD treatment was able to prevent anxiety-like behaviors in AMPH-exposed animals, but considering this anxiolytic effect was also observed in the animals not exposed to AMPH, it allowed us to attribute the anxiolytic activity to CBD per se. These findings are compatible with previous findings from our research group, when CBD showed anxiolytic properties per se, but also prevented anxiety-like behaviors in AMPH-reconditioned animals (Metz et al., 2021). Other researchers also evidenced that CBD produced anti-anxiety-like effects in rats in the context of cocaine and crack addiction (Gonzalez-Cuevas et al., 2018; Luján et al., 2018). We hypothesized that the anxiolytic properties of CBD can contribute, at least in part, to its anti-reinstatement effect, since anxiety symptoms substantially contribute to the relapse occurrence.

4.3. CBD effects on maladaptive dopaminergic molecular changes underlying AMPH exposure

In addition to the behavioral effects previously discussed, the current study also evidenced the molecular changes underlying AMPH exposure. We believe that these neural adaptations are closely related to the behavior modifications also observed in the animals. The immunoreactivity of dopaminergic targets in the VS showed that AMPH-conditioning followed by the exposure to forced swimming stress triggered the increase of dopaminergic receptors 1 (D1R) and 2 (D2R) levels in this brain region when compared to the group not exposed to the drug. D1R- and D2R are recognized for playing pivotal roles in the addiction processes as they mediate relapse and drug-seeking behavior mechanisms (Hansen et al., 2002). Besides us, other authors have recently documented a relation between the overstimulation of D1R and D2R induced by AMPH and addictive behaviors (Metz et al., 2019, 2021; Moratalla et al., 2017), supporting the outcomes shown here. Possibly the overstimulation of these receptors, which occurs due to the high levels of extracellular DA available after the AMPH administration, can cause neuroadaptations and persistent modifications in their immunoreactivity. Also, we showed the impact of AMPH exposure on DA transporter (DAT) levels. As seen in previous studies (Ares-Santos et al., 2012; Granado et al., 2011; Gou et al., 2015), exposure to AMPH triggered a decrease in DAT immunoreactivity in the VS. Likewise, DAT is a relevant dopaminergic target in AMPH addiction, once this transporter is responsible for both intracellular and extracellular DA levels maintenance (Raineri et al., 2011, 2012), whereas, AMPH acts blocking this primary DAT function (Volz et al., 2007). Considering our current outcomes, CBD treatment preserved this dopaminergic target in the animals that were previously exposed to AMPH. This finding is consistent as we found similar results in an earlier study (Metz et al., 2021). We speculate that this maintenance of dopaminergic targets and possible restoration of dopaminergic tone could be behind the beneficial behavioral effects of CBD during AMPH reinstatement. In this sense, an important study by Renard et al. (2016) demonstrated that CBD administration into the mesolimbic system decreased the AMPH-induced sensitization, thus affecting the dopaminergic neuronal activity.

4.4. CBD influence on endocannabinoid modulation of DA release

Different studies demonstrated that endocannabinoids (eCBs) are capable of modulating the mesolimbic dopaminergic pathways (Everett et al., 2021; Lupica et al., 2004; Su and Zhao, 2017) by trigger the DA release incrementing a cannabinoid type 1 receptor (CB1R)-dependent manner (Everett et al., 2021; Oleson et al., 2012; Solinas et al., 2006). Some research groups have investigated the hypothesis that a pharmacological increase in eCBs levels could be a relatively safe method of replacement therapy, especially during drug withdrawal, given that it is believed that at this stage, the presence of negative symptoms is accompanied by a decrease in DA levels. Thus, modulation of eCBs could normalize DA levels decreased and prevent negative effects that contribute to relapse (McCutcheon et al., 2012; Oleson and Cheer, 2013; Roitman et al., 2008).

Our hypothesis is in line with the idea that the increase in endocannabinoid tone can produce anti-abstinence effects and thus corroborate to prevent the occurrence of AMPH-CPP reinstatement. Our outcomes pointed out that AMPH exposure triggered persistent disturbances by decreasing CB1R immunoreactivity. This is in accordance with previously published data showing that exposure to crack and cocaine also triggers a decrease in the CB1R expression in mesolimbic brain areas (Areal et al., 2015). In addition, we observed that AMPH exposure decreased N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) and increased FAAH levels. NAPE-PLD and FAAH are responsible for the synthesis and degradation of AEA, respectively (Blankman and Cravatt, 2013). Changes in the eCB levels are known to reflect changes in the enzyme expression involved in their metabolism. A decrease in AEA levels may be a consequence of reduced synthesis by NAPE-PLD or improved degradation by FAAH (Smaga et al., 2017). Taken together, these findings seem to provide evidence that the AMPH impaired the endocannabinoid neurotransmission, at least concerning AEA. Regarding eCBs 2-AG, we found no significant changes underlying AMPH exposure in the immunoreactivity of its synthesis and degradation enzymes, diacylglycerol lipase α (DAGL), and monoacylglycerol lipase (MAGL), respectively. Conversely, CBD treatment provided an increase in NAPE-PLD, as well as a reduction in FAAH levels in both experimental groups exposed or not to AMPH. In this context, changes in these enzymes are associated with a possible increase in AEA levels (Di Marzo, 2008). Moreover, CBD was able to maintain the CB1R levels in AMPH exposed animals. Finally, we also observed that CBD treatment increased DAGL and decreased MAGL levels, regardless of AMPH exposure. Given what other studies have similarly reported (Smaga et al., 2017), we can infer that the CBD's influence on these enzymes resulted in increased levels of the 2-AG. All of these data allow us to hypothesize that the CBD treatment is capable of triggering a general increase in endocannabinoid tone. This idea is in line with our hypothesis about the potential anti-reinstatement effect of the CBD, which reflects the ability of this cannabinoid to increase endocannabinoid tone and subsequently restore a dopaminergic system compromised by the AMPH administration.

Although we investigated important dopaminergic and endocannabinoid targets in VS and VTA, we recognized that our study had some limitations. In the current study, we were unable to quantify DA and its derivatives levels as well as measure the levels of the main eCBs. Furthermore, we recognize that the use of a sequence of behavioral tests does not characterize ideal experimental conditions. Therefore, this is a limitation of our study. We hope to be able to clarify these limitations in future studies.

5. Conclusion

In summary, our results provide consistent evidence to support the CBD potential in preventing AMPH reinstatement. Furthermore, the absence of abuse potential combined with its good tolerability in general highlights CBD as a promising candidate to be used as pharmacotherapy in psychostimulant drug use disorders. Additional preclinical and future clinical studies are needed to better understand the interactions between endocannabinoid and dopaminergic systems, as well as the potential of CBD in the context of drug addiction.

Ethics approval

The experimental procedures were approved by the Research Ethics Committee of Universidade Federal de Santa Maria (UFSM-8850121118), which is affiliated to the National Council for the Control of Animal Experiments (CONCEA), following international norms of care and animal maintenance.

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CRediT authorship contribution statement

Vinícia Garzella Metz: Conceptualization, Formal analysis, Investigation; Writing - Original draft, Writing - Review & editing; **Jéssica Leandra Oliveira da Rosa:** Investigation; **Domenika Rubert Rossato:** Investigation; **Marilise Escobar Burger:** Conceptualization, Resources, Writing - Review & editing, Project administration, and Funding acquisition; **Camila Simonetti Pase:** Conceptualization, Formal analysis, Resources, Writing - Review & editing, Project administration and Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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