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Veronika Llerena, Iva Tic, Maria Llach-Folcrà, Olga Valverde & Mireia Medrano

Sex and dose-dependent effects of cannabidiol on cocaine consumption in mice

Veronika Llerena[1], Iva Tic[1], Maria Llach-Folcrà[1], Olga Valverde[1,2]* and Mireia Medrano[1]*

1. Neurobiology of Behaviour Research Group (Grenec-NeuroBio), Department of Medicine and Life Sciences (MELIS), Universitat Pompeu Fabra, Barcelona, Spain, 2. Neuroscience Research Program, Hospital del Mar Research Institute, Barcelona, Spain

*Dr. Olga Valverde and Dr. Mireia Medrano equally contributed to this work.

Correspondence:

Dr. Olga Valverde
Universitat Pompeu Fabra and Hospital del Mar Research Institute
Department of Medicine and Life Sciences and Neuroscience Research Program
Neurobiology of Behaviour Research Group (Grenec-NeuroBio)
Carrer del Doctor Aigüader 88, 08003 Barcelona, Spain
olga.valverde@upf.edu, +34933160867

Dr. Mireia Medrano
Universitat Pompeu Fabra
Department of Medicine and Life Sciences
Neurobiology of Behaviour Research Group (Grenec-NeuroBio)
Carrer del Doctor Aigüader 88, 08003 Barcelona, Spain
mireia.medrano@upf.edu, +34933160825

Abstract

Cocaine use disorder (CUD) is a neuropsychiatric disorder marked by compulsive drug-seeking and loss of control over cocaine intake, with women experiencing a faster addictive development and greater adverse outcomes despite lower incidence compared to men. Currently, there are no effective pharmacological treatments for CUD. Cannabidiol (CBD), a multitarget compound acting mainly on mediators of the expanded endocannabinoid system has emerged as a potential therapeutic agent for substance use disorders. Though its effects have been researched in preclinical studies with males, its role in females remains underexplored. Here, we investigated the effect of CBD on distinct phases of cocaine-seeking and taking behaviour using the intravenous self-administration (SA) paradigm in female mice. First, CBD's pharmacological profile was evaluated on anxiety-like and cognitive-task models. Consequently, animals received CBD at 10 or 20 mg/kg to assess its effects on the acquisition of cocaine-consummatory behaviours and compulsive drug-seeking following association to negative consequences like an electric foot-shock. Our findings reveal that CBD modulates cocaine-seeking behaviour in a dose-dependent manner: 10 mg/kg CBD attenuated the acquisition of SA by alteration of reward- and cognitive-related markers within the mesocorticolimbic pathway. Conversely, 20 mg/kg increased cocaine consumption post-punishment but reduced cocaine-seeking upon re-exposure to punishment-associated cues and induced an upregulation of *Htr1a* expression in the medial prefrontal cortex. Parallel studies in males found no effects after punishment. These results highlight the complex, dose-dependent effects of CBD on cocaine consumption patterns and underscore its potential as a modulator of specific neurobehavioral processes in CUD in female mice.

Keywords

Cocaine, cannabidiol, female mice, intravenous self-administration, expanded endocannabinoid system, punishment.

Abbreviations

5-HT1A: Serotonin 1A receptor

AEA: Anandamide

BLA: Basolateral amygdala

CB1R: Cannabinoid receptor 1

CB2R: Cannabinoid receptor 2

CBD: Cannabidiol

CP: Conditioned punishment

CPP: Conditioned place preference

CUD: Cocaine use disorder

ECs: Endocannabinoid system

EPM: Elevated plus maze

FAAH: Fatty-acid amide hydrolase

FR1: Fixed ratio 1

HIP: Hippocampus

mPFC: Medial prefrontal cortex

NOR: Novel object recognition

PN: Punishment

Post-PN SA: Post-punishment regular self-administration sessions

PR: Progressive ratio

RM: Repeated measures

ROI: Region of interest

SA: Self-administration

VEH: Vehicle

vSTR: Ventral striatum

VTA: Ventral tegmental area

1. Introduction

Cocaine is the second most consumed illicit drug in Europe after cannabis [1]. Its use can quickly lead to abuse and addiction, with serious cardiovascular and neuropsychiatric risks[1,2]. Although prevalence is higher in men[1], women progress to dependence more rapidly and are more vulnerable to comorbidities and accelerated escalation of use, a pattern known as the telescoping effect[3]. Currently, psychosocial therapy is the main treatment for cocaine use disorder (CUD), as no pharmacological options are approved despite clinical trials with compounds like modafinil and topiramate[4,5]. CUD is characterized by impaired control, intense craving, and compulsive drug-seeking during abstinence, influenced by genetic, environmental, and comorbid factors[6,7]. Notably, women often face more severe outcomes, including heightened craving and relapse risk.

As other drugs of abuse, cocaine increases dopaminergic activity in the mesocorticolimbic pathway by blocking dopamine transporters at the synaptic cleft, disrupting reuptake and producing rewarding effects[8]. Dopaminergic signalling in limbic regions is also modulated by endocannabinoids, which regulate reward processing and reinforcement learning[9]. The endocannabinoid system (ECs) includes anandamide (AEA) and 2-arachidonoylglycerol as main endocannabinoids that interact primarily with cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R), among other targets. This system comprises enzymes involved in the catabolism of endocannabinoids like fatty-acid amide hydrolase (FAAH). Several studies have demonstrated the involvement of the ECs in the modulation of reward and motivation, as well as neuroplasticity phenomena associated with the addictive process[10–12].

In the latest years there has been an increasing interest in using cannabidiol (CBD) as a potential treatment for substance use disorder as reflected in recent publications with alcohol[13–15] and nicotine[16]. Additionally, anxiolytic-like effects[17,18] and cognitive function improvement by CBD's treatment have been reported in different neuropsychiatric disorders [19,20], further driving interest in its therapeutic applications. CBD is the most abundant non-psychotropic compound from the *Cannabis sativa* plant[21]. It is a multitarget compound with several pharmacological effects as it acts as negative allosteric modulator of CB1R and CB2R, inhibits FAAH activity, and is agonist of transient receptors potential channels, peroxisome proliferator-activated receptors and serotonin 1A (5-HT1A) receptors[22,23]. Preclinical studies have explored CBD's ameliorating effects in distinct phases of cocaine consumption. Concretely, research in mice has been focused on males, where CBD has been shown to reduce cocaine-induced conditioned place preference (CPP), acquisition of cocaine self-administration (SA)[10], cue- and stress-induced reinstatement[11], motivation after a behavioural economics protocol[24], and normalization of somatic signs after spontaneous cocaine withdrawal[25]. However, the role of CBD in modulating cocaine-seeking in female mice remains unexplored. Only one study has reported no effect of CBD in the formation of contextual drug-related memories[26], leaving other phases of the cocaine consumption in females unstudied.

In the present study, we address this research gap by evaluating CBD's effects in distinct phases of cocaine consumption in female mice. First, we assessed CBD's modulatory effects in anxiety-like behaviours and cognitive performance to determine the pharmacological profile of CBD. Then, we used the SA operant paradigm to evaluate CBD's effects during the acquisition of cocaine SA and the punishment (PN) associated with cocaine consumption. Additionally, we also evaluated the effects of CBD after PN in male mice. Our results provide evidence of CBD's dose-dependent modulatory effects in attenuating cocaine-seeking and taking behaviours, depending on the addictive phase of study. We also provide insights on the underlying molecular mechanisms modulated by CBD in specific mesocorticolimbic areas implicated in the control of reward and emotional behaviours.

2. Materials and methods

2.1. Animals

A total number of 264 female and 52 male CD1 mice (postnatal day 53, 30-35 gr) were purchased from Charles River (Barcelona, Spain) and maintained in the animal facility (UBIOMEX, PRBB). Animals were maintained in a 12-h light-dark cycle in stable conditions of temperature (22 ºC± 2) and humidity (55 % ± 10 %), with food and water *ad libitum*. All behavioural experiments were conducted under inverse 12-hour light-dark cycle (lights on 19:30 h – 7:30 h). Animal Ethics Committee (CEEA-OH-PRBB) approved all animal care and experimental protocols, in line with European Community Council guidelines (2010/63/EU).

2.2. Drugs

Cocaine hydrochloride (0.6 mg/kg/inf) was purchased in Alcaliber S.A. (Madrid, Spain) and dissolved in 0.9% NaCl solution. CBD (5, 10, 20 and 30 mg/kg, intraperitoneally, i.p) was kindly provided by Phytoplant Research S. L. (Córdoba, Spain). CBD was first dissolved with ethanol absolute and then diluted with cremophor E. L. (Sigma-Aldrich) and 0.9% NaCl in a final ratio 0.5:1:18.5. VEH solution consisted of ethanol absolute, cremophor E. L. and 0.9% NaCl (ratio 0.5:1:18.5). The CBD doses were established based on previous studies from our lab [10].

2.3. Behavioural assessment

Female mice underwent first elevated plus maze (EPM) test to assess CBD's pharmacological profile in anxiety-like behaviour. After 3 days of washout, animals performed novel object recognition (NOR) test to evaluate short-term hippocampal memory. For both tests, CBD was administered at 5, 10, 20 and 30 mg/kg, i.p. For additional experiments, cocaine (7.5 mg/kg) was injected 5 minutes before test.

2.4. Cocaine self-administration

Surgery, implantation of the catheter in the jugular vein and operant SA protocols were conducted as described elsewhere [24,27]. Briefly, mice were trained for 10 days (2-hour sessions each day) to nosepoke in the active hole to obtain cocaine under a fixed ratio 1 (FR1) schedule of reinforcement. Mice were considered to have acquired a stable SA behaviour if reaching the following acquisition criteria for 3 consecutive days: 1. ≥65% of responses were received in the active hole; 2. >5 infusions per session.

2.4.1. Acquisition of operant self-administration

Two independent experiments were conducted: (i) VEH vs CBD 10 mg/kg, i.p., and (ii) VEH vs CBD 20 mg/Kg i.p. Treatment was injected immediately before entering operant boxes during acquisition and in the progressive ratio (PR) test. After PR, mice were immediately euthanised by cervical dislocation. Brains from CBD 10 mg/kg experiment were collected by snap-frozen method and from CBD 20 mg/kg experiment were fresh dissected for molecular analyses. Further explanations in Supplementary Materials.

2.4.2. Punishment of cocaine consumption

The PN protocol was followed as previously described [27], with minor modifications. Mice underwent acquisition for 10 days and animals that reached acquisition criteria performed 3 days of 1-hour/day PN sessions, consisting of an electric foot-shock delivery each time an animal nosepokes for the 3rd time the active hole. To associate the foot-shock with drug intake, an environmental light was on after each 2nd nosepoke until performing the 3rd nosepoke. After PN, half of the mice were treated with either VEH or CBD 30 minutes before starting regular SA sessions (post-PN SA) and conditioned punishment (CP) test, in which the environmental light was presented as in PN sessions, but no foot-shock was issued. After CP test, all animals from 10 mg/kg experiment were euthanised by cervical dislocation and brains were fresh dissected. For 20 mg/kg experiment, half of the animals were

transcardially perfused, and half were sacrificed by cervical dislocation and their brains dissected for molecular analyses.

2.5. qPCR and OpenArray™

Quantitative PCR (qPCR) was conducted using Power SYBR® Green (Applied Biosystems™, United States, REF: 4368708) and gene-specific primers of *Cnr1*, *Htr1a*, *Crhr1* and *Crhr2*, with primer efficiency previously verified by standard curves (95–100% efficiency). Additionally, we used OpenArray™ high-throughput qPCR platform. Custom OpenArray plates of 56 genes were used to assess expression of target genes related to the endocannabinoid and mesocorticolimbic systems. We used 4 endogenous controls (*Actb*, *B2m*, *Gapdh*, *Hprt*) to normalize the amplification of the sequences of interest. The accession numbers for the sequence corresponding to each ThermoFisher ID (**Table S1**) can be found in the ThermoFisher - TaqMan® OpenArray® Real-Time PCR Plate with Gene Expression Assays - Custom Array configurator, with a direct link to GenBank® - NIH genetic sequence database. ΔΔCt method was used to calculate relative gene expression and OpenArray data was analysed with the ThermoFisher Connect tool, using VEH group data as reference samples.

2.6. Immunofluorescence

Free-floating coronal slices at the BLA level were incubated overnight with primary antibodies for parvalbumin, a marker of GABAergic interneurons, and cFos, a marker of neuronal activity. The following day, slices were incubated with the respective secondary antibody and mounted with FluorSave Reagent (Merck Millipore, United States, REF: 345789). Images from both hemispheres were obtained using sequential laser scanning confocal microscopy (Leica TCS SP8, Leica, Germany) at 20x magnification.

2.7. Statistical analyses

Data are presented as mean ± SEM and analysed using GraphPad Prism 10.2.3. Data follow normality and homogeneity of variance criterias. One-way ANOVA with Dunnett's post-hoc test was used for EPM and NOR tests (factor: *CBD*). For supplementary EPM and NOR experiments with cocaine, we used two-way ANOVA (*Cocaine x Treatment*) with Šídák's post-hoc test. Two-tailed Fischer's exact test was used to compare acquisition ratios of SA behaviour. Acquisition of SA was analysed using two-way repeated measures (RM) ANOVA (*Day* × *Treatment*, *between subject*). Total intake and PR breaking points were analysed with two-way ANOVA and Tukey's multiple comparison test. PN protocol data were analysed with one-way RM ANOVA and Dunnett's post-hoc. Two-way RM ANOVA with Šídák's post-hoc was used to compare infusions, nosepokes, and intake across post-PN SA and CP sessions or between last day of post-PN SA and CP test (*Day x Treatment, between subject*). Gene expression was compared via unpaired two-tailed t-test on fold change values. An unpaired two-tailed t-test was used to compare cFos-positive cells between VEH and CBD groups.

3. Results

3.1. CBD reduces anxiety-like behaviour in female mice but does not alter object recognition memory

EPM and NOR were conducted to assess CBD's pharmacological profile in females (**fig 1A**). In the EPM test, locomotor activity was measured to discard that CBD's effects on locomotion could interfere other procedures, finding no differences in the distance moved between VEH- and CBD-treated mice (One-way ANOVA, $F_{(4, 44)}$ = 0.4460, P = 0.7747) (**fig 1B**). For anxiety-like behaviour evaluation, mice treated with 10 mg/kg of CBD significantly spent more time in the open arms of the EPM in comparison with the VEH group ($F_{(4, 42)}$ = 3.308, P = 0.0192, Dunnett, p = 0.0192) (**fig 1C**). However, this effect was not observed with the other CBD doses tested (5, 20, 30

mg/kg). CBD treatment had no effects in the anxiety index ($F_{(4, 43)} = 1.077$, $P = 0.3797$) but exhibits an inverted bell-shaped profile that follows a similar tendency with time spent in open arms (**fig 1D**), supporting the anxiolytic-like effects induced by CBD at 10 mg/kg. Both indicators of anxiety-like behaviour indicate an anxiolytic-like effect of CBD as anxiety index is a measure that shows an overall tendency considering individual parameters from EPM test (time spent in open arms, number of open and closed arm entries).

In the NOR test, we found no differences in the exploration time of VEH- and CBD-treated mice ($F_{(4, 45)} = 0.2027$, $P = 0.9356$) (**fig 1E**), indicating no effects of CBD on locomotion, and neither in the discrimination index for the tested doses ($F_{(4, 38)} = 1.156$, $P = 0.3453$) (**fig 1F**).

As cocaine also influences parameters like locomotion or cognition, which could affect EPM and NOR performance, supplementary experiments were conducted evaluating the effects of CBD together with cocaine injections[28] (**fig S1**). As expected, cocaine groups showed a higher locomotor activity, as observed in distance moved during EPM test (Two-way ANOVA, *Cocaine:* $F_{(1, 47)} = 66.14$, $P < 0.0001$) (**fig S1A**) and NOR training ($F_{(1, 48)} = 67.47$, $P < 0.0001$) (**fig S1D**). The EPM results showed no differences between the 10 mg/kg CBD groups (vehicle and cocaine) as no significant decrease in open arm time (**fig S1B**) or anxiety index (**fig S1C**) was observed. This suggests that this dose of CBD may be attenuating cocaine-induced anxiety-like behaviour. In NOR experiment, we report a reduction of exploration time during training in the cocaine group treated with VEH, but not with CBD (Šídák, p = 0.0120) (**fig S1E**). During the test, there were no significant differences in the discrimination index between saline versus cocaine treatment comparisons for any dose of CBD (**fig S1F**).

3.2. CBD modulates cocaine-taking and seeking during acquisition of cocaine self-administration

Mice were trained to self-administer cocaine under a FR1 schedule for 10 days while being treated with either VEH or CBD (10 or 20 mg/kg) (**fig 2A**). First, we observed significant differences in the percentage of animals that acquire cocaine SA between CBD 10 and 20 mg/kg (Fisher's exact test, p = 0.0015), but not in comparison with VEH (**fig 2B**). Two-way ANOVA revealed a significant effect of *CBD 10* in the number of infusions (*CBD factor:* $F_{(1, 84)} = 5.397$, $P = 0.0226$) (**fig 2C**) and active nosepokes (*CBD factor:* $F_{(1, 84)} = 6.360$, $P = 0.0136$) in comparison with the VEH group (**fig 2D**). No differences were found for CBD 20 mg/kg neither for infusions nor nosepokes achieved during 10 days of acquisition. Additionally, female mice treated with CBD 10 mg/kg displayed a lower consumption of cocaine than the VEH group (Tukey's multiple comparison test, p = 0.0463) but not CBD 20 mg/kg (**fig 2E**). In the PR test, significant differences are reported in the breaking point between CBD 10 and 20 mg/kg groups (Tukey, p = 0.0126) (**fig 2F**).

3.3. Differential gene expression in CBD treated animals in mesocorticolimbic areas after cocaine self-administration

Given that CBD acts on multiple molecular targets and cocaine SA engages diverse reward- and cognition-related processes, we performed a comprehensive gene expression analysis with OpenArray to capture these complex mechanisms. We examined three key regions of the mesocorticolimbic circuit like medial prefrontal cortex (mPFC), ventral striatum (vSTR) and hippocampus (HIP) to assess CBD's effects across areas critically implicated in executive control, reward processing, and drug-context associations[29].

mPFC, vSTR and HIP from mice treated with CBD 10 mg/kg during acquisition and PR were processed to explore a battery of genes related to the different systems involved in motivation and reward like glutamatergic, dopaminergic or GABAergic systems[29], apart from ECs which is direct and indirectly targeted by CBD[22]. In the

mPFC, *Slc17a6* (fold change = 1.607, p = 0.036), *Adora1* (fold change = 1.161, p = 0.045) and *Gria3* (fold change = 1.239, p = 0.036) were upregulated in samples from CBD-treated mice compared to the VEH group (**fig 3A**). Additionally, *Slc1a2* was downregulated (fold change = 0.832, p = 0.048). In the vSTR, significant differences of gene expression were only found in *Slc6a3* expression (fold change = 0.511, p = 0.031), which was decreased in animals that received CBD 10 mg/kg (**fig 3B**). In the HIP, *Grm5* and *Cnr1* were found downregulated (fold change = 0.875, p = 0.047 and fold change = 0.847, p = 0.032, respectively) (**fig 3C**).

3.4. CBD promotes cocaine consumption post-punishment and reduces cocaine-seeking in the conditioned punishment test

In PN experiments, drug-seeking was evaluated after associating the appearance of a negative stimulus to a consolidated cocaine consumption (**fig 4A**). In **fig 4B**, active nosepokes from last day of acquisition (Acq 10) and PN sessions (days 11, 12 and 13) were compared to assess PN association in each experiment. A *Day* effect was found in the number of active nosepokes during 3 days of PN (days 11, 12 and 13) (One-way RM ANOVA, *Day:* $F_{(2.417,\ 142.6)} = 51.52$, $P < 0.0001$). A significant reduction of active nosepokes was discerned in days 12 and 13 in comparison with Acq10 (Dunnett, p < 0.0001). As expected, we conclude that the PN protocol effectively reduced the number of active nosepokes in comparison to the acquisition phase.

After PN, mice were treated with VEH or CBD (10 or 20 mg/kg) for 3 days of FR1 SA sessions (days 14, 15 and 16) and in the day of CP (day 17). When comparing CBD 20 mg/kg with VEH, a *Day* effect was observed for the number of infusions (Two-way RM ANOVA, *Day:* $F_{(2.543,\ 106.8)} = 5.228$, $P = 0.0035$) (**fig 4C**), active nosepokes (*Day:* $F_{(2.453,\ 102.3)} = 3.548$, $P = 0.0244$) (**fig 4D**) and cocaine intake (*Day:* $F_{(2.543,\ 106.8)} = 5.228$, $P = 0.0035$) (**fig 4E**) achieved after PN, although Treatment and Interaction effects were not significant. Notably, CBD 20 mg/kg, but not CBD 10 mg/kg, showed an escalating pattern of cocaine consumption during the three days of post-PN SA. Focusing on the transition from Day 16 to Day 17, a *Day* factor was detected in the number of infusions (*Day:* $F_{(1,\ 54)} = 11.53$, $P = 0.0013$) (**fig 4C**), active nosepokes (*Day*: $F_{(1,\ 54)} = 9.327$, $P = 0.0035$) (**fig 4D**) and cocaine intake (*Day:* $F_{(1,\ 54)} = 11.53$, $P = 0.0013$) (**fig 4E**). Multiple comparisons analysis within CBD 20 mg/kg group showed a reduction in the number infusions (Šídák, p = 0.0030), active nosepokes (Šídák, p = 0.0134) and cocaine intake (Šídák, p = 0.0030) performed in the CP test in comparison to the last day of post-PN SA. No significant within-group differences were observed for VEH or CBD 10 mg/kg. These results indicate that CBD 20 mg/kg escalates cocaine consumption during post-PN SA sessions and reduces cocaine-seeking during the CP test within the same animals.

An additional experiment was conducted using male mice treated with CBD 20 mg/kg (**fig S2**). Similar results as in females are observed for the punishment protocol assessment (One-way RM ANOVA, *Day:* $F_{(1.836,\ 40.40)} = 12.98$, $P < 0.0001$), displaying differences only between Acq10 and day 13 (Dunnett, p = 0.0003) (**fig S2A**). While no differences were observed between the VEH and CBD groups in terms of infusions and intake (**fig S2B and D**), our results revealed a reduction in the number of nosepokes achieved by VEH-treated mice in the CP test in comparison with last day of post-PN SA (Šídák, p = 0.0236) (**fig S2C**).

3.5. CBD upregulates *Htr1a* expression in the medial prefrontal cortex after punishment without modulating neural activity at the basolateral amygdala

We evaluated expression levels of several genes involved in the control of anxiety responses at the mPFC of female mice after the PN protocol, focusing on *Cnr1*, *Htr1a, Crfr1* and *Crfr2*. *Cnr1* and *Htr1a*, encoding CB1R and the 5-HT1A receptor, are established CBD targets involved in cocaine-related behaviours[10,11] and in the regulation of anxiety and stress[30,31], respectively. *Crfr1* and *Crfr2* encode corticotropin-releasing factor receptors that modulate activation of the hypothalamic–pituitary–adrenal axis during stress and anxiety[32]. For CBD 10 mg/kg,

no differences were found in the relative mRNA expression of *Cnr1*, *Htr1a*, *Crfr1* and *Crfr2* (**fig 5A**). On the other hand, *Htr1a* was significantly upregulated in the mPFC from the CBD 20 mg/kg group in comparison with VEH (t-test, p = 0.0132). However, there were not significant changes in the expression of *Cnr1*, *Crfr1* and *Crfr2* (**fig 5B**). Moreover, no differences of mRNA expression were identified in experiments with males (**fig S2E**).

Furthermore, immunofluorescence labelling of cFos, a well stablished neuronal activation marker[33], was performed in coronal sections of the BLA, which was delimited using parvalbumin as a marker of GABAergic interneurons. We quantified the number of cFos-positive cells expressed in this region per square millimetre (**fig 5C**). Analyses showed that CBD 20 mg/kg did not induce a significant effect on cFos expression at the BLA in comparison with VEH (**fig 5D**). In contrast, results in male mice treated with CBD 20 mg/kg revealed lower expression levels of cFos in the BLA of CBD-treated vs VEH-treated mice (t-test, p = 0.0516) (**fig S2F and G**).

4. Discussion

The present study highlights the distinctive modulatory role of CBD in cocaine-seeking behaviour of female mice, depending on the dose and the distinct phases of cocaine-consumption studied. CBD at 10 mg/kg attenuates acquisition of cocaine SA, reporting gene expression changes in key mesocorticolimbic areas involved in reward and cognitive processes. Interestingly, a higher dose of CBD (20 mg/kg) enhances cocaine-seeking after the association to a negative stimulus but reduces seeking behaviour in the CP test, accompanied by an overexpression of *Htr1a* gene in the mPFC.

CBD is a multitarget compound [22,23] with dose-dependent effects[10,20,25,34]. In this study, we used the cocaine SA paradigm, which involves locomotion, cognition, and stress vulnerability [35,36], to model drug-seeking. Prior to SA, we first evaluated CBD's effects on anxiety-like behaviour and short-term memory in female mice. While previous work carried out in our lab on males showed that 20 mg/kg was effective for both tests[10], our results indicate that CBD 10 mg/kg in females reduces anxiety without altering short-term hippocampal memory. This aligns with recent reports showing anxiolytic-like effects at 10 mg/kg in females and at 20 mg/kg in males[34], though other studies highlight sex-dependent variability[18,37,38]. Additionally, although acute CBD does not improve NOR performance in healthy mice[34,38,39], it has shown cognitive benefits in disease models[40,41], underscoring the importance of sex and dose considerations.

4.1. CBD's effects on acquisition of cocaine self-administration target key mesocorticolimbic areas

To expand previous work conducted in males[10,24,42], we evaluated for the first time CBD's effects on acquisition of SA and motivation in female mice. Interestingly, while CBD 10 mg/kg reduced drug-taking and seeking behaviours during acquisition, CBD 20 mg/kg displayed an opposite effect in a motivational task, highlighting the dose-dependent effects of CBD. Our results hint that CBD at 10 mg/kg could be improving complex cognitive processes like associative learning that favour overall acquisition of the operant paradigm, as there were not significant effects for hippocampal short-term memory in the NOR test. On the other hand, it could be ameliorating cocaine rewarding effects as animals that met the acquisition criteria consumed and sought less drug, as demonstrated during both acquisition and PR test. Similar reductions in consummatory behaviours induced by CBD have been observed with alcohol [13] and nicotine [16], although responses using natural rewards (e.g., sucrose, chocolate, food) remain inconsistent [13,16,43]. This variability suggests that CBD's responses may depend on reward salience and could extend beyond drug-specific pathways, possibly affecting operant learning or motivation-related memories, as previously proposed in male studies [10].

These behavioural effects likely reflect CBD's modulation of key mesocorticolimbic circuits involved in reward and learning. Dopaminergic, glutamatergic, and GABAergic systems in the vSTR, mPFC, and HIP[44,45] regulate motivation, memory, and behavioural control, functions often disrupted in addiction [46,47]. In the mPFC, we report an upregulation of *Slc17a6* (Vesicular Glutamate Transporter 2) and *Gria3* (Glutamate Ionotropic Receptor AMPA Type Subunit 3) and a downregulation of *Slc1a2* (Excitatory Amino Acid Transporter 2), which could indicate an increase in glutamatergic signalling[48,49], consistent with previous work[50–53], whereas upregulation of *Adora1* (Adenosine receptor 1) may reflect compensatory inhibitory mechanisms in line with CBD's effects on adenosine signalling[54,55]. In the vSTR, downregulation of *Slc6a3* (Dopamine transporter) suggests normalization of cocaine-induced dopaminergic adaptations[56], likely contributing to reduced cocaine intake during acquisition. Interestingly, CBD itself exhibits a weak and transient ability to inhibit dopamine transporter function [55]. Lastly, results in the HIP showed a downregulation of *Cnr1* (CBR1) and *Grm5* (Metabotropic glutamate receptor 5), two receptors essential for synaptic plasticity mechanisms underlying memory consolidation and drug-associated learning[57,58]. This reflects potential disruption of synaptic plasticity processes involved in drug-associated memory consolidation, as reported in other behavioural studies with cocaine [12,57,59]. In line with our results, CBD 10 mg/kg could be disrupting drug-associated memories, as reflected in the overall drug-intake, but favouring the acquisition of the operant paradigm. Nonetheless, further research on neural activity of these areas should be done to fully elucidate the mechanisms by which CBD ameliorates drug-seeking and taking during acquisition of SA.

4.2. CBD's paradoxical modulation of cocaine-seeking after a punishment context

We next evaluated CBD's effects in a punishment (PN) paradigm, modelling continued drug use despite negative consequences, a hallmark of human addiction[60]. In this work, we used a behavioural paradigm based on our previous work [27]. Here, foot-shock intensity was gradually increased across sessions to strengthen the association between the aversive stimulus and cocaine, while avoiding sudden behavioural extinction as in other studies[61,62]. Importantly, CBD treatment was initiated only after consolidation of drug-taking behaviour, allowing us to assess its impact on established drug-seeking. Surprisingly, in female mice, 20 mg/kg CBD increased cocaine consumption during post-PN but reduced cocaine-seeking when re-exposed to the PN-associated cue. In males, CBD 20 mg/kg (a dose that reduced acquisition of cocaine SA [10]) did not alter cocaine-seeking after PN, highlighting a sex- and dose-dependent response consistent with previous findings on anxiolytic-like effects[34]. In contrast, a similar work with URB597, a FAAH inhibitor, showed reduced cocaine-seeking in the CP test without increasing post-PN consumption[27], suggesting that CBD's broader pharmacological profile may engage additional pathways beyond endocannabinoid tone enhancement. To explain these behavioural outcomes, we proposed two non-exclusive hypotheses: that CBD modulates either anxiety-related processes or the consolidation of aversive memories. These processes are particularly relevant in stress-associated brain regions such as the mPFC and the BLA, which together regulate behavioural inhibition, emotional memory, and fear processing[46,47,63]. To assess this, gene expression analyses were performed in the mPFC, and BLA neuronal activation was assessed with cFos as a marker of neuronal activity.

Results from *Htr1a* relative gene expression in the mPFC are consistent with our first hypothesis. *Htr1a* gene codifies for 5-HT1A receptor, a Gi protein-coupled receptor that, upon activation, plays a key role in regulating stress and anxiety by decreasing neuronal excitability [31,64]. CBD's partial agonism at this receptor may enhance inhibitory tone in the mPFC under aversive conditions. The upregulation of *Htr1a* observed in females treated with CBD 20 mg/kg could reflect increased inhibitory signalling within the mPFC, which might influence top-down regulation of subcortical circuits involved in reward and compulsive drug-seeking. This mechanism provides a possible explanation for the increased cocaine consumption observed in females despite the negative consequences associated with PN. Supporting this, previous research has shown that stress enhances drug

preference in CPP, and that intra-mPFC administration of a 5-HT1A antagonist can block these stress-induced effects [65].

In parallel, our analysis of BLA cFos expression revealed a sex-specific pattern of neuronal activation. While 20 mg/kg CBD reduced BLA cFos expression in males, no reduction was observed in females. Given that increased BLA excitability is linked to heightened anxiety and stress-related disorders [66,67], these findings suggests greater male sensitivity to CBD's anxiolytic-like effects at this dose. Correspondingly, male mice maintained cocaine-seeking despite the aversive cue, whereas females at 20 mg/kg reduced seeking, suggesting higher reactivity to the PN cues. At 10 mg/kg, females showed a behavioural pattern similar to that of males at 20 mg/kg, consistent with EPM results[10,34] and previous evidence that CBD can reduce BLA excitability[68,69]. We also hypothesize that this differences in sensitivity could also be influenced by the estrous cycle, as hormonal fluctuations are known to modulate amygdala function and anxiety-like behaviours in females[70,71]. Specifically, stress has been demonstrated to shift BLA neuronal activity across the estrous cycle[72]. Moreover, CBD has also been implicated in differential effects on panic-like behaviour depending on the estrous phase[70] and in modulation of estrogen related pathways in a sex-specific manner after chronic stress[73]. However, we did not monitor the estrous cycle, which may add some variability to our findings. Beyond immediate anxiety regulation, the consolidation of aversive memories is also relevant: CBD has been shown to disrupt fear memory consolidation when administered acutely during conditioning or before fear-retrieval test [64,74]. In our study, systemic CBD for 3 days may instead facilitate consolidation in females at 20 mg/kg, allowing association of the aversive cue with punishment and promoting avoidance in the CP test.

4.3. Study limitations

Although only the 10 mg/kg dose produced anxiolytic-like effects in the EPM, anxiety may not fully account for the distinct outcomes observed in the SA experiments. Anxiety can influence drug-taking behaviour, but it represents just one component of the broader cognitive and reward-related processes involved in cocaine SA. Supporting this interpretation, our molecular findings are more consistent with alterations in these processes rather than anxiety. Nevertheless, anxiolytic effects at 10 mg/kg may contribute to behavioural differences during acquisition. Examining how previous individual anxiety profiles correlate to subsequent cocaine-seeking in SA will help clarify how these domains interact.

The absence of estrous cycle monitoring, which constrains interpretation of the sex-specific differences observed, particularly those involving amygdala-dependent processes, could represent an additional limitation. However, estrous staging was not performed because repeated collection of vaginal smears introduce additional factors that could interfere with behavioural performance[75,76], and our study aimed to prioritise translational relevance. In clinical contexts, women with CUD would receive CBD over extended periods. For instance, in a randomized trial CBD treatment was given for 92 days, making cycle-specific fluctuations not controllable[77]. Therefore, although monitoring the estrous cycle could have provided important mechanistic insight, chronic administration of CBD without cycle adjustment may better approximate clinical conditions.

4.4 Conclusions and insights on CBD's effects in CUD

In summary, our results demonstrate that CBD exerts distinct, dose-dependent effects across different phases of cocaine-seeking behaviour in female mice. Translationally, these findings suggest that CBD's efficacy may depend on the stage of addiction and the specific behavioural context. Previous clinical trials have been conducted on other drug abuse disorders like heroin or alcohol, displaying beneficial effects on craving[14,78]. However, fewer trials have been done in CUD. For example, CBD did not alleviate cocaine craving and relapse in detoxicated subjects[77]. In contrast, our results indicate that CBD can modulate drug-taking and seeking behaviour when administered

during voluntary cocaine consumption. Interestingly, cocaine-cannabis (which contains CBD) polysubstance users were less likely to engage in heavy cocaine consumption than cocaine only users[79], which is consistent with a possible modulatory role of CBD on cocaine intake observed in our study. Still, behavioural outcomes in mice widely differ depending on the phase of addiction-like behaviour studied, once again emphasizing the complex pharmacology underlying this compound. Careful titration of dose and timing, as well as close monitoring of individual responses, would be essential for future translational studies. Lastly, our study highlights the necessity of sex-specific research on psychiatric disorders like CUD, as biological differences may significantly influence treatment outcomes.

Authors contributions

VL, MM and OV conceptualized and designed the study. VL, MM, IT and ML-F performed the behavioural experiments. VL and MM conducted the molecular and imaging studies. VL, MM and OV performed data analysis and results interpretation. VL wrote the first draft of the manuscript. IT, ML-F, MM and OV provided critical revisions. All authors edited and confirmed the contents of the final manuscript version.

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Conflicts of interest

All authors declare no competing interests.

Data availability

The data used in this study are available from the corresponding author upon reasonable request.

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6. Figure legends

Fig 1. Effects of CBD on female mice in the EPM and NOR. (**A**) Scheme of behavioural tests (**B**) Distance moved in centimetres during 5 minutes of EPM test for each dose as a locomotor activity measure (**C**) Time spent in open arms (%) (Dunnett, *p<0.05) (**D**) and anxiety index in the EPM test (**E**) Exploration time in seconds during the 10 minutes of training before performing NOR test (**F**) Discrimination index in % between novel object and familiar object in the NOR test. Dashed red line indicates the 100% of discrimination index normalised to VEH group values. Dots represent individual data points. Data are represented as the mean ± SEM. VEH, n=10; CBD, n=10.

Fig 2. CBD 10 mg/kg reduces cocaine-taking during SA and displays opposite patterns with CBD 20 mg/kg in the PR test. (**A**) Scheme of cocaine SA and PR schedule (**B**) Percentage of mice reaching acquisition criteria (Fisher, **p<0.01) (**C**) Number of infusions achieved during acquisition under a FR1 schedule of reinforcement (Two-way RM ANOVA, *CBD 10*, *p<0.05) (**D**) Number of active and inactive nosepokes performed during acquisition (Two-way ANOVA, *CBD 10*, *p<0.05) (**E**) Total cocaine intake (mg/kg) (Tukey, *p<0.05) (**F**) Breaking point achieved in the PR test. Data are represented as the mean ± SEM. Total number of mice: VEH, n=74; 10 mg/kg CBD, n=38; 20 mg/kg CBD, n=44. Animals that reached acquisition criteria: VEH, n=55; 10 mg/kg CBD, n=31; 20 mg/kg CBD, n=29.

Fig 3. Relative gene expression comparison between CBD 10 mg/kg and VEH treated mice in mPFC, vSTR and HIP. Volcano plots present the magnitude of change (fold change in log2) in the X axis, and the statistical significance (p-value in -log10) in the Y axis. Values represent the magnitude of change in CBD samples in comparison to a reference group (VEH). Gene expression differences in the mPFC (**A**), vSTR (**B**) and HIP (**C**) were considered significant establishing a fold change boundary of 1.1 to each direction (vertical lines) and p-value of 0.05 (horizontal line). Green dots represent upregulated genes and red, downregulated genes. VEH, n=16; 10 mg/kg CBD, n=16.

Fig 4. CBD 20 mg/kg increases cocaine consumption after PN and reduces seeking in CP test. (**A**) Scheme of the experimental schedule and graphical representation of PN protocol (**B**) Number of active nosepokes conducted in the last day of acquisition (Acq) and PN days in 1 hour (Dunnett, ****p<0.0001) (**C**) Number of infusions (Šídák, **p<0.01), (**D**) nosepokes (Šídák, *p<0.05) and (**E**) cocaine intake (Šídák, **p<0.01) achieved in 3 days of post-PN SA sessions (day 14, 15, 16) and in the CP test (day 17). Dots represent individual data points. Data are represented as the mean ± SEM. VEH, n=28; 10 mg/kg CBD, n=13; 20 mg/kg CBD, n=16.

Fig 5. CBD 20 mg/kg upregulates *Htr1a* expression in the mPFC, without modulating cFos activation in the BLA after PN. (**A**) Relative mRNA expression of *Cnr1*, *Htr1a*, *Crfr1* and *Crfr2* in VEH, CBD 10 mg/kg and (**B**) CBD 20 mg/kg treated mice (t-test, *p<0.05) (**C**) Representative confocal sections of BLA showing cFos (green) and parvalbumin (red) immunofluorescence signalling (**D**) Number of cFos quantified per square millimetres of the ROI (BLA), delimited by the yellow line, in VEH and CBD 20 mg/kg. Scale bar: 100 µm. Dots represent individual values. Data are represented as the mean ± SEM. qPCR: VEH (10 mg/kg CBD), n=9; 10 mg/kg CBD, n=11; VEH (20 mg/kg CBD), n=7; 20 mg/kg CBD, n=8. Immunofluorescence: VEH, n=7; 20 mg/kg CBD, n=7.

7. Figures

Figure 1

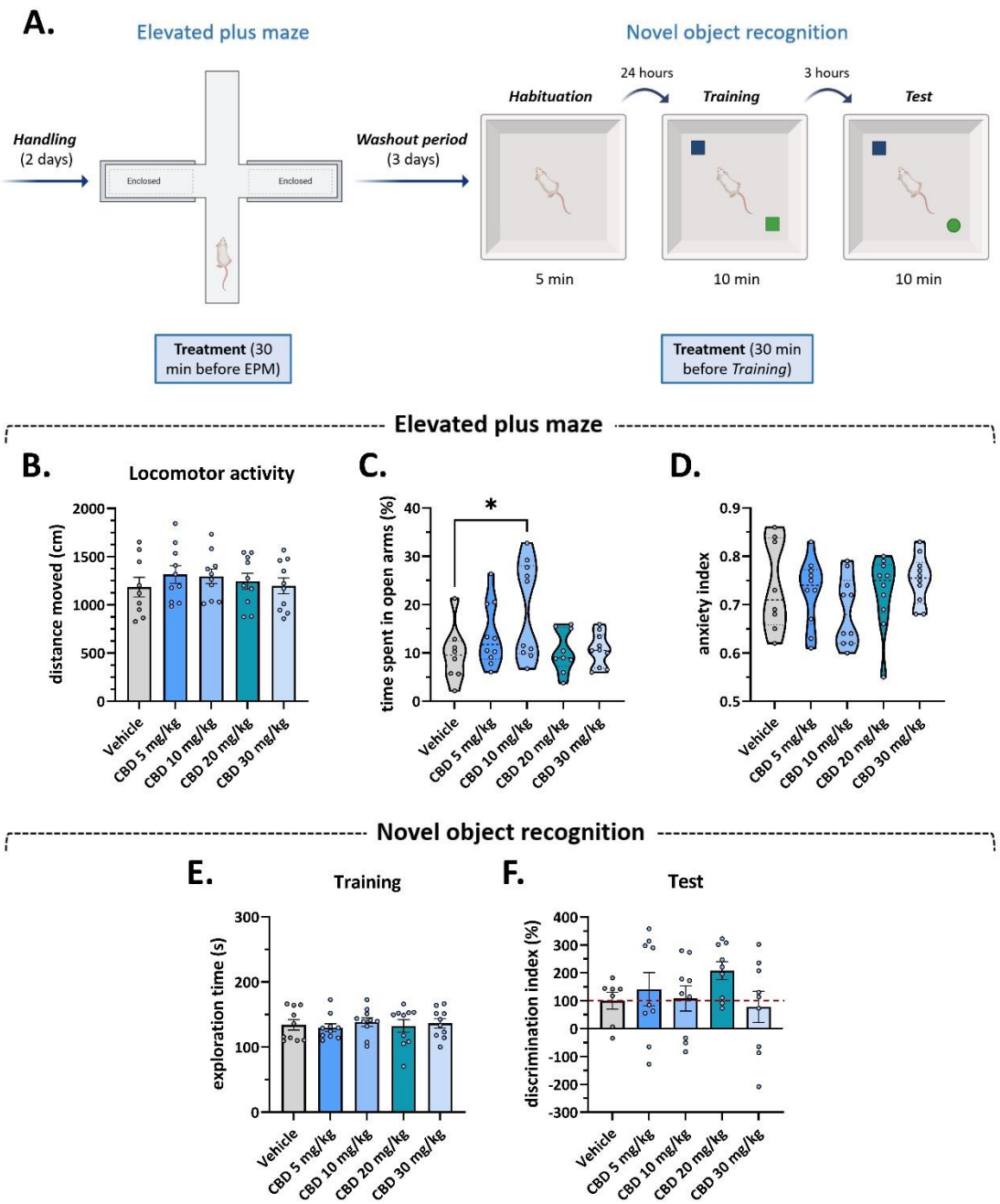


Figure 2

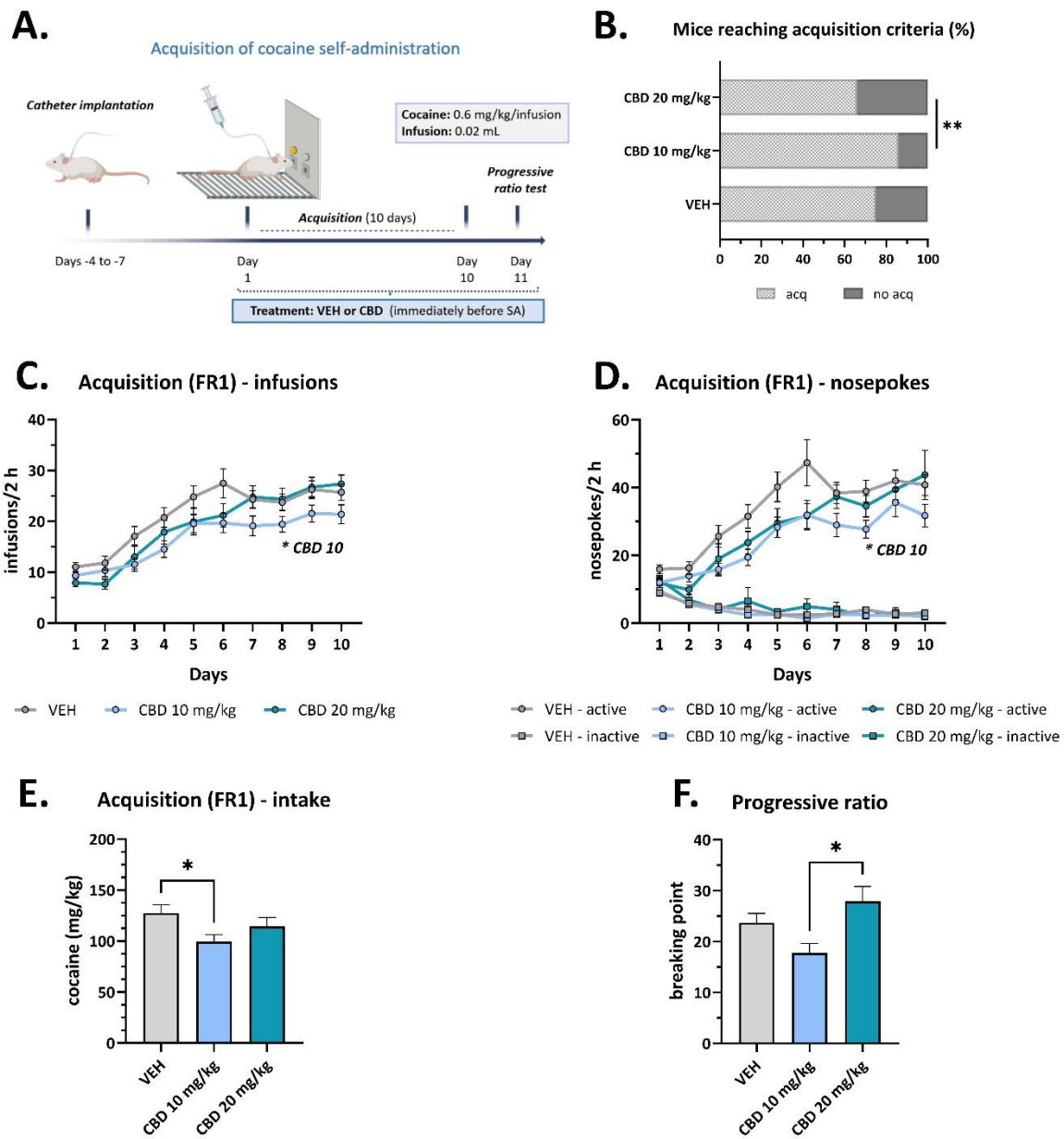
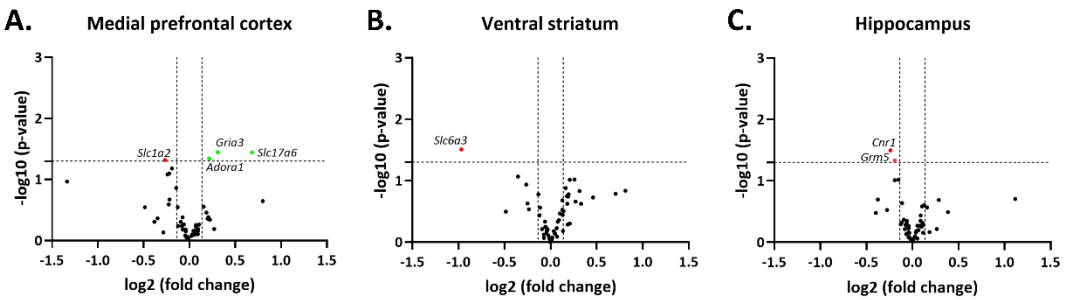

A.
Acquisition of cocaine self-administration
Catheter implantation
Cocaine: 0.6 mg/kg/infusion
Infusion: 0.02 mL
Progressive ratio test
Acquisition (10 days)
Days -4 to -7
Day 1
Day 10
Day 11
Treatment: VEH or CBD (immediately before SA)
B.
Mice reaching acquisition criteria (%)
CBD 20 mg/kg
CBD 10 mg/kg
VEH
**
acq
no acq
C.
Acquisition (FR1) - infusions
infusions/2 h
* CBD 10
Days
VEH
CBD 10 mg/kg
CBD 20 mg/kg
D.
Acquisition (FR1) - nosepokes
nosepokes/2 h
* CBD 10
Days
VEH - active
CBD 10 mg/kg - active
CBD 20 mg/kg - active
VEH - inactive
CBD 10 mg/kg - inactive
CBD 20 mg/kg - inactive
E.
Acquisition (FR1) - intake
cocaine (mg/kg)
F.
Progressive ratio
breaking point

Figure 3



Target	Fold change	P-value
Slc17a6	1,607	0,036
Adora1	1,161	0,045
Gria3	1,239	0,036
Slc1a2	0,832	0,048

Target	Fold change	P-value
Slc6a3	0,511	0,031

Target	Fold change	P-value
Grm5	0,875	0,047
Cnr1	0,847	0,032

Figure 4

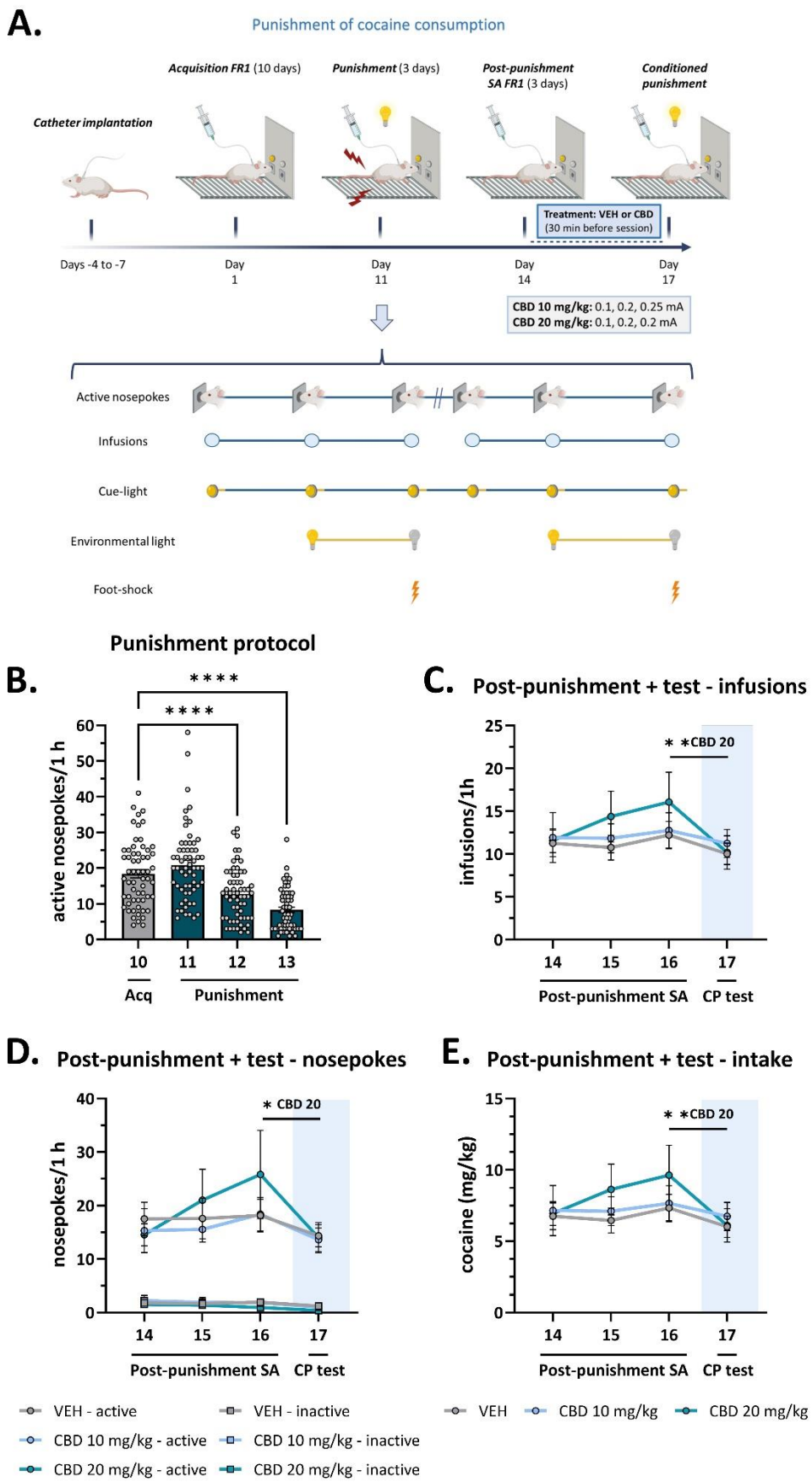

A.
Punishment of cocaine consumption
Acquisition FR1 (10 days)
Punishment (3 days)
Post-punishment SA FR1 (3 days)
Conditioned punishment
Catheter implantation
Treatment: VEH or CBD (30 min before session)
Days -4 to -7
Day 1
Day 11
Day 14
Day 17
CBD 10 mg/kg: 0.1, 0.2, 0.25 mA
CBD 20 mg/kg: 0.1, 0.2, 0.2 mA
Active nosepokes
Infusions
Cue-light
Environmental light
Foot-shock
Punishment protocol
B.
active nosepokes/1 h
Acq
Punishment
C. Post-punishment + test - infusions
infusions/1h
* *CBD 20
Post-punishment SA
CP test
D. Post-punishment + test - nosepokes
nosepokes/1 h
* CBD 20
E. Post-punishment + test - intake
cocaine (mg/kg)
VEH - active
VEH - inactive
CBD 10 mg/kg - active
CBD 10 mg/kg - inactive
CBD 20 mg/kg - active
CBD 20 mg/kg - inactive
VEH
CBD 10 mg/kg
CBD 20 mg/kg

Figure 5