


Effects of repeated long-term psychosocial stress and acute cannabinoid exposure on mouse corticostriatal circuitries: Implications for neuropsychiatric disorders

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Summary

Introduction: Vulnerability to psychiatric manifestations is achieved by the influence of genetic and environment including stress and cannabis consumption. Here, we used a psychosocial stress model based on resident-intruder confrontations to study the brain corticostriatal-function, since deregulation of corticostriatal circuitries has been reported in many psychiatric disorders. CB₁ receptors are widely expressed in the central nervous system and particularly, in both cortex and striatum brain structures.

Aims and methods: The investigation presented here is addressed to assess the impact of repeated stress following acute cannabinoid exposure on behavior and corticostriatal brain physiology by assessing mice behavior, the concentration of endocannabinoid and endocannabinoid-like molecules and changes in the transcriptome.

The first and last author share senior authorship.

The second and third author contributed equally to this work.

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Results: Stressed animals urinated frequently; showed exacerbated scratching activity, lower striatal *N*-arachidylethanolamine (AEA) levels and higher cortical expression of cholinergic receptor nicotinic alpha 6. The cannabinoid agonist WIN55212.2 diminished locomotor activity while the inverse agonist increased the distance travelled in the center of the open field. Upon CB₁ activation, *N*-oleoylethanolamide and *N*-palmitoylethanolamide, two AEA congeners that do not interact directly with cannabinoid receptors, were enhanced in the striatum. The co-administration with both cannabinoids induced an up-regulation of striatal FK506 binding protein 5. The inverse agonist in controls reversed the effects of WIN55212.2 on motor activity. When Rimonabant was injected under stress, the cortical levels of 2-arachidonoylglycerol were maximum. The agonist and the antagonist influenced the cortical expression of cholinergic receptor nicotinic alpha 6 and serotonin transporter neurotransmitter type 4 in opposite directions, while their co-administration tended to produce a null effect under stress.

Conclusions: The endocannabinoid system had a direct effect on serotonergic neurotransmission and glucocorticoid signaling. Cholinergic receptor nicotinic alpha-6 was shown to be deregulated in response to stress and following synthetic cannabinoid drugs thus could confer vulnerability to cannabis addiction and psychosis. Targeting the receptors of endocannabinoids and endocannabinoid-like mediators might be a valuable option for treating stress-related neuropsychiatric symptoms.

KEYWORDS

CB₁, Chrna6 and Slc6a4, Fkbp5, psychosocial stress

1 | INTRODUCTION

Vulnerability to psychiatric disorders is determined by the interaction of genetic and environmental factors¹ such as psychosocial stress and cannabis consumption. The dysregulation of corticostriatal circuitries is involved in the pathophysiology of psychotic disorders.² Psychosocial stress has a remarkable influence on central nervous system (CNS) and animal behavior.³ When an organism is exposed to a stressor, biological mechanisms such as the hypothalamic-pituitary-adrenal axis (HPA) and cardiovascular readjustments come into play to restore homeostasis.⁴ Under standard conditions, HPA axis function is mainly influenced by stress, which enhances its activity, and the circadian rhythm.⁵ However, when stress is prolonged over the time and the activation exceeds the capacity to keep body's homeostasis, psychopathological sequelae can appear. Among the broad spectrum of brain structures closely involved in anxiety and stress disorders (for review see Ref. [6]), we directed our efforts toward the dorsal striatum (dorsal CPu) and the prefrontal cortex (PFC) due to the fact that these areas are critically involved in social relationships.^{7,8} There is a growing body of the literature that suggests that glucocorticoid receptor(s) involve G protein-dependent mechanisms.⁹ In particular, corticostriatal activity is modulated by a variety of G protein-coupled receptors such as the cannabinoid CB₁ receptor, which is highly abundant

in the CNS.¹⁰ CB₁ receptors are the main target for endogenous endocannabinoids lipid signaling molecules and mediate both Δ^9 -tetrahydrocannabinol (*THC*) and synthetic cannabinoid drugs pharmacological actions.¹¹ The main endocannabinoids molecules for CB₁ receptors are *N*-arachidylethanolamine (AEA)¹² and 2-arachidonoylglycerol (2-AG).¹³ *N*-arachidonylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) is responsible for the production of AEA, while 2-AG is mostly synthesized by diacylglycerol lipase (DAGL- α) in the CNS.¹⁴ After activating cannabinoid receptors, the endocannabinoid AEA and related endocannabinoid-like NAEs PEA and OEA are enzymatically hydrolyzed by the fatty acid amide hydrolase (FAAH) while 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL).¹⁵

Interestingly, the endocannabinoid system has been described to have a direct effect on both neurotransmission¹⁶ and glucocorticoid signaling.¹⁷ Despite the lack of knowledge concerning the concrete corticostriatal mechanisms underlying anxiety, both pharmacologic (synthetic cannabinoid drugs) and behavioral testing have been applied. Thus, the aim of this study was to investigate the interplay between repeated long-term exposure to psychosocial stress and acute challenge with cannabinoid drugs on mice corticostriatal circuitries. For this purpose, we evaluated the animals by behavioral testing and then quantified endocannabinoid and endocannabinoid-like molecules and also changes in whole-genome gene expression profile.

2 | MATERIALS AND METHODS

All animal experimental procedures were approved by University of Göttingen Institutional Animal Care and Use Committee and also were in accordance with NIH guidelines for the use of animals in research and the European Communities Council Directive (86/609/EEC).

2.1 | Animals

A total of 120 C57Bl6/J male mice aged 7–8 weeks were purchased from Charles River Laboratories (Sulzfeld, Germany). On arrival, they were kept five per cage and maintained under standard conditions (12-hour light/dark cycle with 6:00/18:00 lights on/off, a room temperature of $21 \pm 2^\circ\text{C}$, and food and water ad libitum). After one-week habituation period, mice were subjected to the experiment. One-year-old FVB/N male mice (Charles River Laboratories, Sulzfeld, Germany) were kept individually and used as residents. FVB/N mice were selected as residents because they are more offensive than the C57Bl/6 strain.³ The FVB/N strain was kept under the same protocol conditions as the C57Bl/6J colony but housed in a separate room to prevent the C57Bl/6J mice habituation to the odor of the residents.

2.2 | Drugs

The CB_1/CB_2 receptor agonist WIN55212.2 (Sigma-Aldrich, Seelze, Germany) and the selective cannabinoid CB_1 receptor antagonist Rimonabant (Sequoia Research Products Ltd, Pangbourne, UK) were dissolved in a vehicle solution consisting of 10% DMSO (Sigma-Aldrich, Seelze, Germany) and 0.1% Tween 80 (Sigma-Aldrich, Seelze, Germany) in 0.9% saline and prepared on the 21 days of the experiment. A volume of 200 μL of drug and/or vehicle was administered intraperitoneally (WIN55212.2 and Rimonabant were administered at a concentration of 3 mg/kg) before the behavioral testing.

2.3 | Experiment design and experimental groups

The C57Bl6/J male mice were sorted into two groups: Those exposed daily to psychosocial stress for 1 hour (stress) and those who were left undisturbed (control). The stress protocol was performed daily for 3 weeks. On day 21, the animals received an appropriate drug or vehicle injection and then evaluated by behavioral testing. Indeed, control and stressed mice were split into four subgroups each (15 animals per subgroup): mice treated twice with vehicle (Veh), mice subjected first to vehicle and then WIN55212.2 (WIN), mice treated first with Rimonabant and then with WIN55212.2 (Rim+WIN), and mice injected first with Rimonabant and then vehicle (Rim). Following the last injection with either vehicle or cannabinoid drug, the animals were tested by different behavioral paradigms during the rodent active phase (ie, after 8 PM).

2.4 | Social stress procedure

An intruder (C57Bl/6J mouse) was placed in the home cage of a resident (FVB/N mouse), and then they freely interacted until the first

aggression was achieved. After the first attack, the C57Bl/6J mouse was isolated by the use of a small plastic wire-mesh cage within the FVB/N mouse's cage protecting the intruder against direct aggression; however, olfactory, visual, and some vibrissae contact with residents were maintained. After 1 hour, the C57Bl/6J mouse was placed again in its home cage. To prevent habituation, every day intruder mice had encounters with different residents. The psychosocial stress protocol was performed once per day at a similar time (after 8 PM) to enhance the stress prediction factor in intruders. In contrast, controls were left undisturbed in an empty cage every day for 1 hour. Therefore, controls were subjected to the same experimental protocol in terms of handling and exposure to a new environment (different cage), but they did not interact with the residents.

2.5 | Behavioral assessment

Mice were exposed to psychosocial stress or left undisturbed for 21 days. On day 21, they were acutely treated with different cannabinoid drugs or vehicle and finally evaluated by behavioral testing. The stress paradigm, the administration of drugs, and the behavioral assessment took place during the dark phase (active phase). We directed the behavioral analyses by measuring locomotor and anxiety-like behavior in the open-field (OF) test and testing also CNS activity and excitability by use of the functional observational battery (FOB).

2.5.1 | Functional observational battery

The FOB has been widely used and adapted for general behavioral studies in mice.¹⁸ In this study, the FOB test comprised 28 parameters by which the investigator evaluated CNS activity and excitability by recording neuromuscular and autonomic effects, and sensorimotor reactivity. There were four consecutive testing situations: (i) in the home cage, (ii) in the observer's hand, (iii) in the OF, and (iv) manipulation tests. After a brief assessment in its home cage, each mouse was removed and handled by the observer to evaluate ease-of-removal, handling reactivity, and general appearance. Then, each mouse was assessed in an OF arena (60 cm \times 90 cm) while the observer analyzed CNS activity, autonomic effects, muscle tone, equilibrium, and sensorimotor reactivity. Gait condition was scored from 1 to 8. The gait was scored as follows: normal gait, ataxia, splayed hind limbs, feet markedly splayed outward from the body, fore-limb drag, walking on tiptoes, hunched body, and body drag. The severity of gait abnormality was also evaluated on a scale from 1 (normal) to 4 (severely abnormal).¹⁸

2.5.2 | Open-field activity

In the OF test, we evaluated the spontaneous locomotor and exploratory activity of mice. The experiment was conducted in a Plexiglass arena (45 \times 45 \times 55 cm), and each mouse was left in the same starting position. Animals were allowed to examine the OF for 10 minutes without habituation. We registered the total distance travelled, percent of time moving, time spent in the center (defined as 70% of area),

hyperactivity (forward movement with speed greater than 20 cm/s), and the number of rearings by use of the ActiMot software (TSE, Bad Homburg, Germany) as described.³ The apparatus was cleaned with 70% ethanol p.a. between each test.

2.6 | Brain sample collection and tissue evaluation

Animals were sacrificed immediately after OF task finished, and brain tissue was collected accordingly. All mice were deeply anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (Sigma-Aldrich, Hamburg, Germany) and then transcardially perfused with cold 0.1% phosphate buffer saline (PBS). Finally, the PFC and the dorsal CPU were freshly isolated and frozen in liquid nitrogen for LC-MS, RNA seq, and quantitative RT-PCR.

2.6.1 | Extraction and measurement of AEA, 2-AG, OEA, and PEA levels

The endogenous lipid signaling molecules AEA and 2-AG, and endocannabinoid-related molecules *N*-palmitoylethanolamide (PEA), and *N*-oleoylethanolamide (OEA) were purified from the PFC and the dorsal CPU and then quantified as described elsewhere.¹⁹ First, samples were dounce-homogenized following with chloroform/methanol/Tris-HCl 50 mmol/L pH 7.5 (2:1:1, v/v) containing internal deuterated controls solution for AEA, 2-AG, PEA, and OEA measurement by isotope dilution ([²H]8AEA, [²H]52AG, [²H]4 PEA, [²H]4 OEA (Cayman Chemicals, MI, USA). The lipid organic phase was dried down, weighed, and prepurified on silica gel. Fractions were collected by eluting the column with 99:1, 90:10, and 50:50 (v/v) chloroform/methanol. The 90:10 fraction contained AEA, 2-AG, PEA, and OEA, and it was used for isotopic dilution liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry quantification (LC-APCI-MS), as previously described and using selected ion monitoring at *M* + 1 values for the four compounds and their deuterated homologues, as described in Ref. [20] *N* = 4 mice/group.

2.6.2 | RNA extraction

Total RNA was extracted from 80 samples (control and stress; Veh, WIN, Rim+WIN, and Rim; PFC and dorsal CPU; *N* = 5 mice/group) using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, NY, USA). The RNA was digested with RNase-free DNase (Qiagen, Düsseldorf, Germany) and checked for integrity using capillary gel electrophoresis (Bioanalyzer, Agilent Technologies, Santa Clara, USA).

2.6.3 | cDNA Library Preparation and RNA Seq

The library was prepared using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, USA) starting from 500 ng of total RNA. The cDNA libraries were quantified using the QuantiFluor dsDNA System (Promega, Madison, USA). The size range of the final cDNA libraries was determined by the Bioanalyzer 2100 (Agilent, Santa Clara, USA).

The cDNA libraries were amplified and sequenced using cBot and HiSeq2000 (Illumina, San Diego, USA).

The sequence images were converted with the Illumina BaseCaller software to bcl files, which were demultiplexed to FASTQ files with CASAVA version 1.8.2. Quality was checked via FastQC (version 0.10.0, Babraham Bioinformatics).

2.6.4 | Gene expression analyses

Sequences were aligned to the RefSeq²¹ mouse transcriptome using *bwa*,²² and raw "hits" per transcript were merged genewise. DESeq was used to analyze the counts per gene.²³ The resulting *P*-values were corrected using Benjamini-Hochberg adjustment.²⁴

2.6.5 | Quantitative RT-PCR

The most relevant top hits deregulated by stress and cannabinoid drugs were further evaluated by quantitative RT-PCR. First-strand cDNA was synthesized from 1 µg of total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Darmstadt, Germany). mRNA expression was quantified by RT-PCR using the CFX96TM Real-time PCR system (Bio-Rad, Hercules, USA). We used GAPDH mRNA as an endogenous control. TaqMan assays for mouse *Chrna6*, *Slc6a4*, and *Fkbp5* cDNAs were selected from validated and predesigned Assays-on-Demand (Applied Biosystems, Darmstadt, Germany) and used in real-time PCR amplifications to detect the expressions of *Chrna6*, *Slc6a4*, and *Fkbp5*. The reactions were performed in triplicate using 2 µL of cDNA in a 10 µL reaction volume. mRNA expression was determined using the comparative cycle threshold (Ct) method with $2^{-\Delta\Delta Ct}$, according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). cDNAs were measured relative to a "calibrator" control sample. The $2^{-\Delta\Delta Ct}$ for this "calibrator" was arbitrarily set to 1.

2.7 | Statistical analysis

A two-way ANOVA was used to examine the effects of stress and pharmacological treatment on our cohort of animals. The mean differences among the levels of one factor were determined by one-way ANOVA or Brown-Forsythe test when applicable. Bonferroni or nonparametric Tamhane post hoc test was applied for pairwise comparisons. Analysis of simple main effects was performed whether there was a significant interaction of the two main factors. Individual comparisons were made using the Student's *t* test. ANOVA for repeated measures was used to test the equality of means for mice body weight. Significance level was set to *P* < 0.05. Data are represented as mean ± SEM. Significant effects were identified by SPSS (IBM Corp, Chicago, USA).

3 | RESULTS

Results are summarized in three main categories: (i) effects of stress, (ii) effects of drugs, and (iii) effects of stress under drug influence.

Effects of psychosocial stress

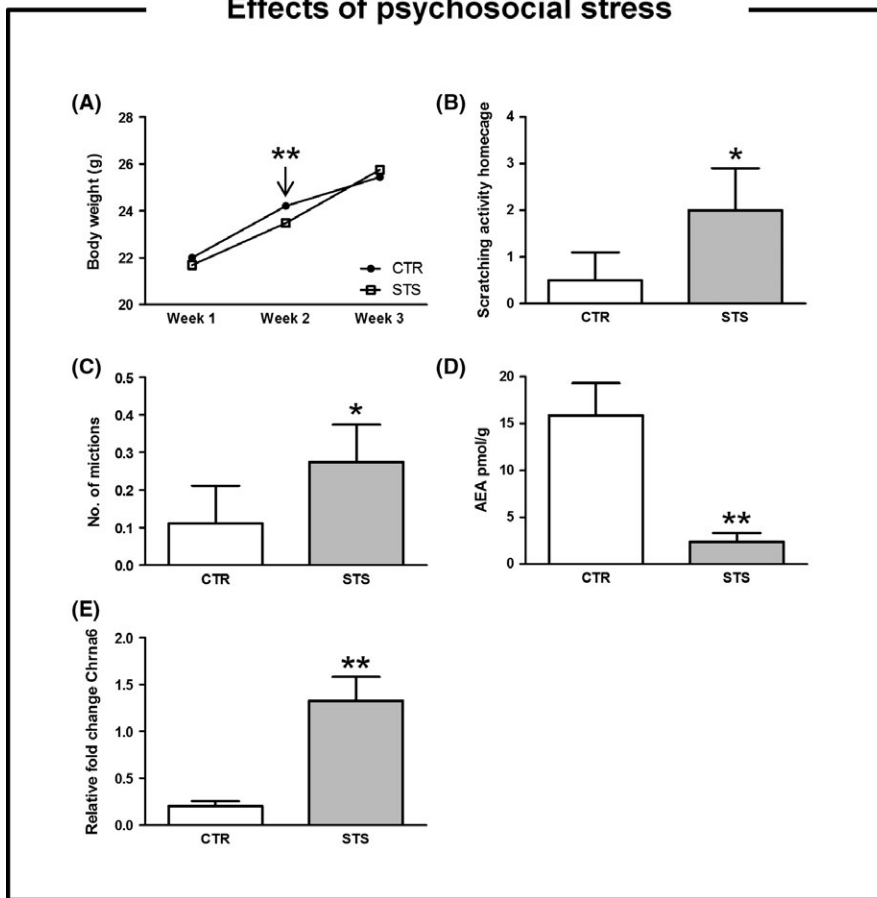


FIGURE 1 Effects of repeated exposure to psychosocial stress. Data are expressed as mean \pm SEM. Exposure to 2-wk social defeat paradigm significantly diminished body weight ($P < 0.01$), which returned to control levels by the end of the stress period (panel A). Social defeat animals displayed a pronounced scratching activity ($P < 0.05$) (panel B), urinated more frequently ($P < 0.05$) (panel C), and showed lower levels of AEA content ($P < 0.01$, in the dorsal CPu) (panel D). We determined that long-term exposure to stress increased the expression of *Chrna6* gene ($P < 0.01$, in the PFC) (panel E) when compared to controls. An * indicates significant differences between stress group and their respective control group. One or two symbols indicate $P < 0.05$; $P < 0.01$, respectively. $N = 15$ for behavioral testing, $n = 4$ for endocannabinoids quantification; $n = 5$ for whole-genome gene expression. PFC, prefrontal cortex; dorsal CPu, dorsal striatum; CTR, control; STS, stress; AEA, *N*-arachidonyl ethanolamine

3.1 | Effects of stress

We revealed a significant effect of psychosocial stress on body weight throughout the experimental period ($F(1, 123) = 4.84$, $P < 0.05$). Indeed, exposure to 2-week social defeat paradigm significantly diminished body weight ($t(122) = 2.78$, $P < 0.01$), which returned to control levels by the end of the stress period (Figure 1, panel A). Social defeat animals displayed a pronounced scratching activity ($F(1, 123) = 4.06$, $P < 0.05$) (Figure 1, panel B), urinated more frequently ($F(1, 123) = 4.97$, $P < 0.05$) (Figure 1, panel C), and showed lower levels of AEA ($F(1, 18) = 36.81$, $P < 0.01$, in the dorsal CPu) (Figure 1, panel D). Transcriptome analysis of the selected brain structures revealed 62 top hits as deregulated genes by chronic psychosocial stress before Benjamini-Hochberg adjustment (Figure S1). The cholinergic receptor nicotinic alpha 6 (*Chrna6*), as a candidate gene, was further analyzed by quantitative RT-PCR. Thus, we determined that long-term exposure to stress increased the expression of *Chrna6* gene ($F(1, 43) = 38.32$, $P < 0.01$, in the PFC) (Figure 1, panel E) when compared to the nonstressed group.

3.2 | Effects of drugs

Two-way ANOVA showed a significant main effect of the drug treatment on total distance traveled ($F(3, 121) = 8.36$, $P < 0.001$), distance traveled in center ($F(3, 121) = 3.72$, $P < 0.05$), rearing activity

($F(3, 121) = 3.07$, $P < 0.05$), PEA levels ($F(3, 17) = 7.72$, $P < 0.01$, in the dorsal CPu), and OEA levels ($F(3, 22) = 5.29$, $P < 0.01$, in the dorsal CPu). Drug administration had a remarkable effect on the relative fold change expression for *Fkbp5* ($F(3, 44) = 11.44$, $P < 0.001$, in the dorsal CPu). The main effects of drug treatment were further evaluated by one-way ANOVA or Brown-Forsythe test when applicable followed by multiple comparisons post hoc tests (Table S1). In the OF, the cannabinoid agonist WIN decreased the total distance traveled ($P < 0.01$) (Figure 2, panel A) while the administration of the inverse agonist Rim significantly antagonized such effects ($P < 0.01$) (Figure 2, panel A). Coadministration with both cannabinoid drugs clearly reduced the total distance traveled in comparison with those animals only treated with Rim ($P < 0.05$) (Figure 2, panel A). The distance traveled in center, a measure of anxiety-like behavior, was statistically elevated in animals treated with Rim alone relative to mice only treated with the agonist ($P < 0.01$) (Figure 2, panel B). Animals were also observed in their homecage by registering the vertical movements (rearings). We found that animals treated simultaneously with both drugs showed more frequent rearing behavior than WIN-treated animals ($P < 0.05$) (Figure 2, panel C). The quantification of the endocannabinoid-like molecules, PEA and OEA, revealed the highest amounts for these lipids in mice subjected to acute administration with the agonist ($P < 0.05$, in the dorsal CPu) (Figure 2, panel D & E). Genomewide transcriptional profiling revealed two top hits as deregulated genes by WIN and 32 top hits as deregulated genes

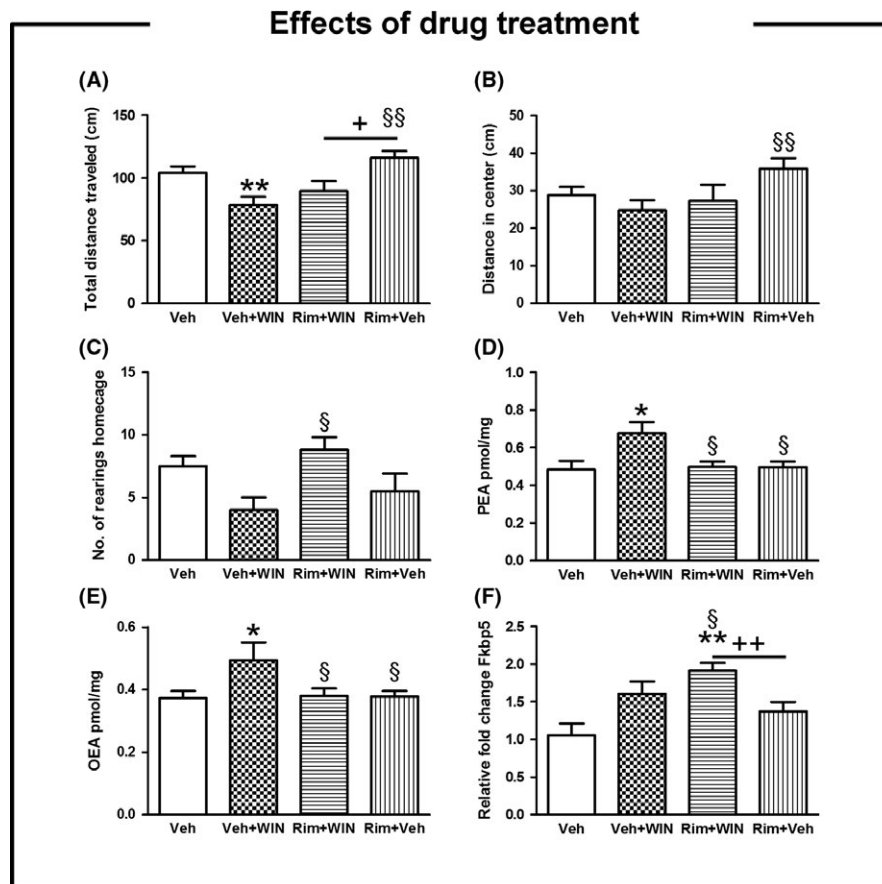


FIGURE 2 Effects of cannabinoid drugs administration. Data are expressed as mean \pm SEM. In the OF, the cannabinoid agonist WIN decreased *t* and the total distance traveled ($P < 0.01$) (panel A) while the administration of the inverse agonist Rim significantly antagonized such effects ($P < 0.01$) (panel A). Coadministration with both cannabinoid drugs clearly reduced the total distance traveled in comparison to those animals single treated with Rim ($P < 0.05$) (panel A). The distance traveled in center was statistically higher in animals treated with Rim alone than those mice single treated with the agonist ($P < 0.01$) (panel B). We reported that animals treated simultaneously with both drugs showed more frequent rearing activity in their homecage than those animals treated with WIN alone ($P < 0.05$) (panel C). We found bigger amount of PEA and OEA in mice subjected to single administration with the agonist in comparison with the remaining drug-treated groups ($P < 0.05$, in the dorsal CPu) (panel D & E). We measured higher transcription rate for *Fkbp5* gene following administration with Rim+WIN than the vehicle group ($P < 0.01$, in the dorsal CPu) or when compared to either WIN or Rim alone ($P < 0.05$, $P < 0.01$; respectively, in the dorsal CPu) (panel F). An * indicates significant differences between drug-treated mice and their respective vehicle group. Comparisons between WIN-treated mice and remaining drug treatments are indicated by an §. Otherwise underlined + indicated other significant comparison intragroup. One or two symbols indicate $P < 0.05$; $P < 0.01$, respectively. $N = 15$ for behavioral testing, $n = 4$ for endocannabinoids quantification; $n = 5$ for whole-genome gene expression. PFC, prefrontal cortex; dorsal CPu, dorsal striatum; OF, open field

by Rim before Benjamini-Hochberg adjustment (Figure S1). The candidate gene (*Fkbp5*) was validated by quantitative RT-PCR, and main results are depicted in Figure 2 (panel F). We measured higher transcription rate for *Fkbp5* after administration with Rim+WIN than vehicle ($P < 0.01$, in the dorsal CPu) or when compared to either WIN or Rim alone ($P < 0.05$, $P < 0.01$; respectively, in the dorsal CPu) (Figure 2, panel F).

3.3 | Effects of stress under drug influence

A 2-way ANOVA reported a significant interaction between the factors (stress \times drug) on total distance traveled in the OF arena ($F(3, 121) = 2.76$, $P < 0.05$), 2-AG content ($F(3, 23) = 18.91$, $P < 0.001$, in the PFC), and fold change expression for *Chma6* ($F(3, 43) = 7.38$, $P < 0.001$, in the PFC) and

Slc6a4 gene ($F(3, 43) = 3.18$, $P < 0.05$, in the PFC). Simple main effects of the interaction between the factors are depicted in Table S2.

In an OF arena, repeated long-term exposure to psychosocial stress exacerbated general motor activity following vehicle administration (Figure 3, panel A). Indeed, social defeat animals exposed to vehicle traveled longer distances than their controls ($P < 0.01$) (Figure 3, panel A). However, the pharmacological treatment with WIN reduced the total distance traveled in the OF when compared to Rim-treated animals under either control or stress conditions ($P < 0.01$) (Figure 3, panel A). Likewise, psychosocially stressed mice exposed to the CB₁ agonist WIN alone traveled smaller distances than the vehicle group ($P < 0.01$) (Figure 3, panel A). The administration of the inverse agonist in controls enhanced the total distance traveled when compared to vehicle-exposed animals ($P < 0.05$)

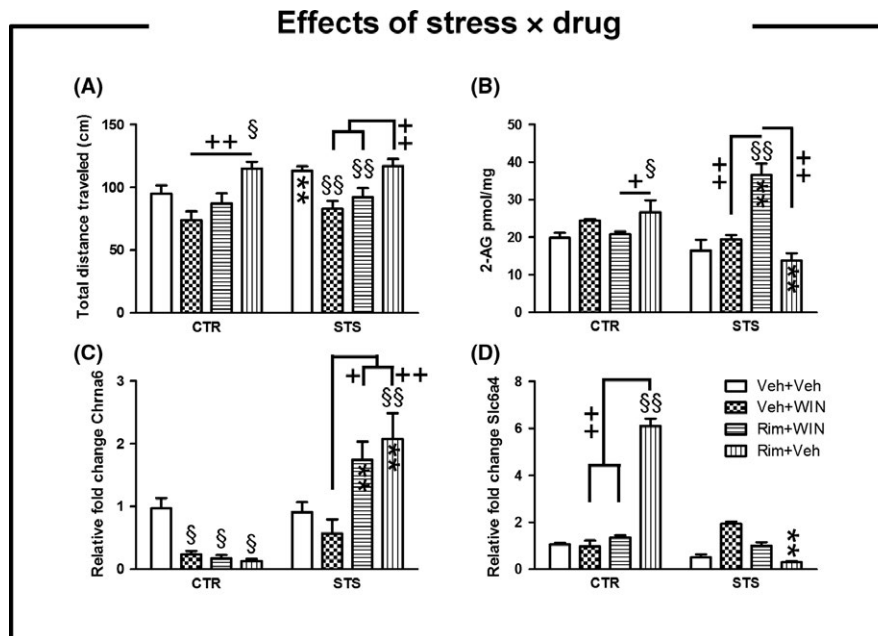


FIGURE 3 Effects of repeated exposure to psychosocial stress following acute cannabinoid drugs administration. Data are expressed as mean \pm SEM. (1) *Effects on mice behavior*: In an open-field arena, exposure to psychosocial stress exacerbated general motor activity following vehicle administration (panel A-B). However, psychosocially stressed mice exposed to the agonist WIN alone traveled smaller distances than the vehicle group ($P < 0.01$) (panel A). The administration of inverse agonist Rim in controls enhanced the total distance traveled when compared to vehicle ($P < 0.05$) (panel A). (2) *Effects on endocannabinoids levels*: Controls treated with Rim alone underwent an increase in 2-AG levels when compared to either vehicle or Rim+WIN-treated mice ($P < 0.05$, in the PFC) (panel B). The administration of the inverse agonist Rim by the end of stress period reduced the levels of the endocannabinoid 2-AG when compared to controls that received the same drug treatment ($P < 0.01$, in the PFC) (panel B). (3) *Effects on gene expression*: The use of synthetic cannabinoid drugs decreased the expression of *Chrna6* in controls ($P < 0.05$, in the PFC) (panel C) and also did not significantly when WIN was administered upon stress exposure while the inverse agonist Rim clearly antagonized such effects ($P < 0.01$, in the PFC) (panel C). Social defeat animals cotreated with Rim+WIN or treated with Rim alone underwent a remarkable upregulation of *Chrna6* expression when compared to their nonstressed counterparts ($P < 0.01$, in the PFC) (panel C). We assessed a higher transcription rate for *Slc6a4* gene in controls upon injection with the inverse agonist in comparison with the remaining control groups ($P < 0.01$, in the PFC) and also when this drug was administered in stressed animals ($P < 0.01$, in the PFC) (panel D). The remaining comparisons are depicted in the graph. An * indicates significant differences between stress groups and their respective control group. Control or stress intragroup comparisons between vehicle and treated mice are indicated by an \S . Otherwise underlined + indicated intragroup comparisons between treatment groups in control or stress conditions. One or two symbols indicate $P < 0.05$; $P < 0.01$, respectively. $N = 15$ for behavioral testing, $n = 4$ for endocannabinoids quantification; $n = 5$ for whole-genome gene expression. PFC, prefrontal cortex; CTR, control; STS, stress

(Figure 3, panel A). Upon long-lasting effects of stress, coadministration with Rim+WIN reduced the total distance traveled in comparison with vehicle ($P < 0.01$) and Rim-treated animals ($P < 0.01$) (Figure 3, panel A).

On the other hand, controls only treated with Rim exhibited higher 2-AG levels than either vehicle or Rim+WIN-treated mice ($P < 0.05$, in the PFC) (Figure 3, panel B). However, social defeat mice treated with Rim+WIN displayed higher amounts of 2-AG than their nonstressed counterparts ($P < 0.01$, in the PFC) but also when compared to either vehicle, WIN, or Rim alone under stress ($P < 0.01$, in the PFC) (Figure 3, panel B). The administration of the inverse agonist Rim by the end of the stress period reduced the levels of 2-AG when compared to controls treated with the same drug ($P < 0.01$, in the PFC) (Figure 3, panel B).

Whole-genome gene expression revealed 10 top hits genes downregulated simultaneously by stress and cannabinoid administration before Benjamini-Hochberg adjustment (Figure S1) (Table 1). Three of them (*Fkbp5*, *Chrna6*, and *Slc6a4*) were further analyzed by quantitative

RT-PCR. Two-way ANOVA showed a significant effect of the interaction on *Slc6a4* ($F(3, 43) = 3.18$, $P < 0.05$) and *Chrna6* ($F(3, 43) = 7.38$, $P < 0.001$) in the PFC but did not reveal differences in the dorsal CPu. The use of synthetic cannabinoid drugs diminished the expression of *Chrna6* in controls ($P < 0.05$, in the PFC) (Figure 3, panel C) and also did so, but not significantly, when WIN was administered in stressed animals. In contrast, the inverse agonist Rim clearly antagonized the effects of WIN on *Chrna6* expression under stress ($P < 0.01$, in the PFC) (Figure 3, panel C). Furthermore, Rim+WIN administration in stressed mice slightly decreased the expression of *Chrna6* when compared to Rim alone, although *Chrna6* expression was higher than with single WIN treatment ($P < 0.05$, in the PFC) (Figure 3, panel C). By the end of the stress protocol, acute injection with Rim increased the expression of *Chrna6* in contrast to vehicle ($P < 0.01$, in the PFC) (Figure 3, panel C). Social defeat animals exposed to either Rim+WIN or Rim alone underwent an upregulation of *Chrna6* expression when compared to their nonstressed counterparts ($P < 0.01$, in the PFC) (Figure 3, panel C). It

TABLE 1 Differentially deregulated top 10 genes in response to chronic psychosocial stress and acute cannabinoid treatment

Genes	PFC	DS	FC Stress	FC Rim	FC WIN	q value Stress	q value Rim	q value WIN
<i>Chrna6</i>	33.8	45.6	13.5	-13.2	-15.2	6.46E-11	0	0
<i>Slc6a4</i>	64.2	102.3	31.7	-34.1	-32.5	1	1	0
<i>Ldlr</i>	7.5	7.7	-0.2	-0.3	-0.1	0.7	0.01	1
<i>Sdf2l1</i>	5.6	5.3	0.5	0.5	0.2	0.002	0.02	1
<i>Lrg1</i>	4.1	2.5	0.5	0.7	0.5	0.1	0.02	1
<i>Fkbp5</i>	7.8	8.0	0.5	0.4	0.2	4.81E-06	0.03	1
<i>Dok3</i>	5.2	4.9	0.4	0.5	0.1	0.1	0.03	1
<i>Hspa5</i>	10.7	10.8	0.3	0.3	0.2	0.01	0.05	1
<i>Manf</i>	7.9	7.9	0.2	0.2	0.1	0.01	0.05	1
<i>Hspa1a</i>	6.8	6.0	0.3	0.4	0.2	0.03	0.05	1

Column titles from left to right: official gene symbol; PFC, prefrontal cortex; DS, dorsal CPU, dorsal striatum; FC stress, fold change stress; FC Rim, fold change Rimonabant; FC WIN, fold change WIN55212.2; q value Stress, *P* adjust value Stress; q value Rim, *P* adjust value Rimonabant; q value WIN, *P* adjust value WIN55212.2.

was generally observed that in the PFC, the expression of *Slc6a4* transcripts was higher in the control group treated with the inverse agonist Rim compared to all the groups ($P < 0.01$, in the PFC, Figure 3, panel D).

4 | DISCUSSION

Long-term stress confers risk to develop psychiatric disorders and symptoms.²⁵ In response to prolonged stress, glucocorticoid release has been shown to have deleterious effects on different brain regions and also is associated with the regression of synapses and dendritic spines.²⁶ Remarkably, dysfunctions in corticostriatal connectivity have been involved in the pathophysiology of psychiatric disorders.²⁷ Moreover, the G protein-coupled CB₁ is extremely abundant and a key modulator of the corticostriatal pathways.¹⁰

4.1 | Effects of stress

After 2 weeks of daily psychosocial stress, animals showed loss of body weight in line with Iniguez et al.²⁸ Our data revealed that chronic psychosocial stress exacerbates scratching activity.²⁹ The endocannabinoid system has been described to have a direct effect on serotonergic neurotransmission which in turn, modulates several behaviors and physiological functions¹⁶ such as itch sensation.³⁰ Moreover, the pronounced scratching phenotype reported here could also be attributable to the lower levels of AEA observed in the PFC.

In fact, pharmacological manipulation of the degrading enzymes leading to increased levels of AEA has shown a promising tool for reducing itch sensation.³¹ Additionally, mice with defective endocannabinoid signaling at CB₁ receptors displayed a pronounced scratching behavior (for review see Ref. [32]).

Stress is well known to affect urinary bladder function and exacerbates signs of urinary bladder dysfunction. We demonstrated that socially defeated animals urinated more frequently probably caused by reduced bladder capacity as described.³³

A decrease in motor activity is among the changes most commonly noted in subordinates and defeated animals.³ However, we found that social defeat animals traveled longer distances in the OF arena. These discrepancies could be attributable to methodological differences such as: (i) the use of mice rather than rats; (ii) housing conditions (five mice to a cage instead of just one); and (iii) the time at testing, as here the stress protocol and drug administration were performed during the active phase (after 8 PM) instead of the sleep phase. Finally, we measured an overexpression of *Chrna6* under stress. Studies of genetic linkage reported that the coding *Chrna6* region confers risk to develop neuropsychiatric disorders.³⁴ Likewise, Kimbrel et al.³⁵ reported that two SNPs linked to *CHRNA6* conferred risk for developing post-traumatic stress disorder. Thus, the results described here point out a plausible contribution of *Chrna6* to the long-lasting effects observed in social defeat animals.

4.2 | Effects of drug administration

Single administration of the agonist WIN reduced total distance traveled while acute treatment with the inverse agonist Rim slightly increased it when compared to controls an observation corroborated by Brzozka et al.³

In the OF, mice treated with the inverse agonist Rim traveled longer distances in the center than the remaining groups, which support an anxiolytic effect mediated by the drug,³⁶ even though CB₁ antagonism can also produce anxiogenic actions.³⁷ Animals treated with Rim+WIN showed more frequent rearing behavior than WIN-treated animals. Indeed, cannabis smoke exposure induced lower rearing activity, while the use of Rimonabant prevented the smoke-induced decrease in rearing.³⁸

Upon CB₁ activation by WIN, we observed an increase in the levels of both OEA and PEA, two mediators metabolically related to AEA. In line with our results, Bardou et al.³⁹ reported an increase in OEA and PEA contents after inhibiting the enzyme fatty acid amide hydrolase. Despite the fact that OEA and PEA levels often, but not always, change

in a similar manner to those of AEA under physiological or pathological conditions, we did not observe here any similar changes in AEA levels following chronic psychosocial stress. This finding is compatible with the general concept that OEA and PEA are not eCBs in as much as they do not activate directly CB1 and CB2 receptors, but instead modulate noncannabinoid receptors, such as G protein-coupled receptor 55 (GPR55), peroxisome proliferator-activated receptor- α (PPAR- α), and transient receptor potential vanilloid type-1 (TRPV1) channels. Recently, Musella et al reported that significant levels of PEA are present in the striatum, where this mediator enhances GABAergic neurotransmission via GPR55. Interestingly, PEA was previously reported to exhibit greater binding affinity for GPR55 than CB₁, CB₂, PPAR- α , and TRPV1 (for review see Ref. [40]). Thus, it is noteworthy to speculate that exposure to cannabinoid drugs, by altering PEA levels, modifies GPR55 receptor activity.

It is well known that the glucocorticoid receptor is regulated by several chaperones and cochaperones including the FKBP5 protein.⁴¹ In particular, we found that the administration of the CB₁ inverse agonist Rimonabant potentiated the expression of *Fkbp5* when compared to the remaining groups. This is in agreement with data showing that FKBP5 regulates glucocorticoid receptor sensitivity, increases its resistance, and decreases its efficiency at controlling the negative feedback in response to elevated levels of corticosterone,⁴² whereas Rimonabant can aggravate the hyperactivity of the HPA axis during stress.⁴³ Several studies attest to the idea that FKBP5 is crucial for the development of stress-related mental disorders (^{44,45}). However, it is currently unclear how the activation of the endocannabinoid system through the use of synthetic cannabinoid drugs may regulate either *Fkbp5* or glucocorticoid signaling as pointed out by Wang et al.⁴⁶ In conclusion, our understanding of FKBP5 functions is still incomplete and thus, further work must address this question.

4.3 | Effects of stress under drug influence

The inverse agonist Rimonabant in nonstress conditions antagonized the effects of WIN55212.2 on total distance travelled.³ Under experimentally induced psychosocial stress, mice treated with either WIN or Rim+WIN showed less total distance traveled than the vehicle group, while single Rimonabant administration antagonized the effects of WIN.³ We also observed that stressed mice exposed to vehicle displayed an exacerbated total distance traveled when compared to their nonstressed counterparts. Against the present findings, Brzozka et al³ found that repeated exposure to social defeat paradigm reduced the total distance traveled in the OF. The differences reported here could be explained by the influence of the circadian rhythm and the housing conditions (5 mice per cage instead of just one). Indeed, mice are nocturnal animals mainly active during the dark period.⁵ Furthermore, the absence of social support (individual housing) amplifies the anxiety induced by psychosocial stress.⁴⁷

It is widely accepted that upon HPA activation, the content of endocannabinoid lipid molecules varies according to the duration/type of stress stimuli and the brain structure. We found that social defeat animals exposed to vehicle showed similar 2-AG levels than

their controls in agreement with.⁴⁸ In contrast, Gorzalka et al⁴⁹ determined that after chronic stress, the endocannabinoid signaling was compromised because endocannabinoid levels were reduced in all limbic structures. Chronic restraint stress, for example, progressively increases 2-AG content within distinct anatomical regions such as the medial PFC, limbic forebrain, amygdala, hippocampus, and hypothalamus.⁵⁰ These differences could be explained by the influence of the circadian rhythm and also related to the stress protocol used. In fact, there is a strong evidence to suggest that in rodents circadian alterations occur in endocannabinoid brain levels,⁵¹ CB₁ expression,⁵² and the activity of enzymes controlling the metabolism of endocannabinoids.⁵¹ The administration of Rimonabant in controls as well as the pharmacological blockade of MAGL enzyme has been reported in a powerful increase of 2-AG levels in the CNS.⁵³ Here, such increase was also observed when both cannabinoids were administered but not when Rim was injected alone. Thus, the use of synthetic cannabinoids in this model warrants further investigation in order to elucidate the underlying mechanisms associated with the metabolism of 2-AG.

Not surprisingly, we found that the cannabinoid agonist WIN and the inverse agonist Rim influenced the expressions of *Chrna6* and *Slc6a4* in opposite directions, while their coadministration tended to produce a net null effect under experimentally induced psychosocial stress. Nicotinic receptor containing $\alpha 6$ subunit (CHRNA6) is expressed in distinct brain regions important for addiction behaviors and also is identified to confer susceptibility to neuropsychiatric disorders.³⁴ In line with this report, emerging data point out that the coding region for CHRNA6 confers vulnerability to drugs of abuse and their related behavioral phenotypes.⁵⁴ Interestingly, the present investigation is the first to demonstrate that simultaneous exposure to distinct environmental factors such as chronic stress and acute cannabinoid drug administration can regulate the expression of the *Chrna6* gene which in turn and could be crucial for the development of cannabis addiction and psychosis. Long-term exposure to stress constitutes a key environmental risk component for developing stress-related disorders in susceptible individuals. The underlying mechanisms disrupted in these disorders are serotonergic⁵⁵ and endocannabinoid⁵⁶ dependent. Particularly, serotonin release inhibition is attributable to the lack of CB₁ receptors which in turn, promotes higher concentration of this neurotransmitter in the synaptic cleft.⁵⁷ Then, we can speculate that CB₁ blockage by the administration of Rim could counteract the excessive serotonin neurotransmitter by increasing the expression of the serotonin transporter protein (*Slc6a4*).⁵⁸ Furthermore, SLC6A4 single nucleotide polymorphisms have been associated with stress-related psychiatric disorders,⁵⁹ which are consistent with our findings.

5 | CONCLUSIONS

In summary, the data described here highlight the effects of long-term exposure to stress on mouse corticostriatal circuitries following acute challenge with distinct cannabinoid drugs (For further details see Figure S2). Particularly, we found that psychological stress played a role in the exacerbation of micturition frequency, anxiety-related behavior,⁵⁸ and

scratching phenotype,²⁹ while the use of synthetic cannabinoids drugs interfered with locomotor activity, rearing, and anxiety-like behavior.³⁸ The glucocorticoid receptor is regulated by several chaperones and cochaperones including the FKBP5 protein⁴¹ but also is influenced by endocannabinoid system.¹⁷ Additionally, we demonstrated that psychosocially stressed animals displayed changes in the serotonergic system upon acute exposure to synthetic cannabinoid drugs.⁵⁵ The coding region for CHRNA6 confers vulnerability to drugs of abuse and their related behavioral phenotypes,⁵⁴ which in turn, could be crucial for the development of cannabis addiction and psychosis. Our findings in both PFC and dorsal CPU need to be corroborated by further studies if we want to understand the role of CB₁ and endocannabinoids and related mediators in corticostriatal signaling under psychosocial stress.

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CONFLICTS OF INTEREST

The author reports no potential conflict of interests.

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