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Deletion of *Cryab* increases the vulnerability of mice to the addiction-like effects of the cannabinoid JWH-018 via upregulation of striatal NF- κ B expression

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Synthetic cannabinoids have exhibited unpredictable abuse liabilities, especially self-administration (SA) responses in normal rodent models, despite seemingly inducing addiction-like effects in humans. Thus, an efficient pre-clinical model must be developed to determine cannabinoid abuse potential in animals and describe the mechanism that may mediate cannabinoid sensitivity. The *Cryab* knockout (KO) mice were recently discovered to be potentially sensitive to the addictive effects of psychoactive drugs. Herein, we examined the responses of *Cryab* KO mice to JWH-018 using SA, conditioned place preference, and electroencephalography. Additionally, the effects of repeated JWH-018 exposure on endocannabinoid- and dopamine-related genes in various addiction-associated brain regions were examined, along with protein expressions involving neuroinflammation and synaptic plasticity. *Cryab* KO mice exhibited greater cannabinoid-induced SA responses and place preference, along with divergent gamma wave alterations, compared to wild-type (WT) mice, implying their higher sensitivity to cannabinoids. Endocannabinoid- or dopamine-related mRNA expressions and accumbal dopamine concentrations after repeated JWH-018 exposure were not significantly different between the WT and *Cryab* KO mice. Further analyses revealed that repeated JWH-018 administration led to possibly greater neuroinflammation in *Cryab* KO mice, which may arise from upregulated NF- κ B, accompanied by higher expressions of synaptic plasticity markers, which might have contributed to the development of cannabinoid addiction-related behavior in *Cryab* KO mice. These findings signify that increased neuroinflammation via NF- κ B may mediate the enhanced addiction-like responses of *Cryab* KO mice to

cannabinoids. Altogether, *Cryab* KO mice may be a potential model for cannabinoid abuse susceptibility.

KEYWORDS

cannabinoid, *Cryab*, JWH-018, neuroinflammation, NF- κ B, synaptic plasticity, addiction susceptibility, drug abuse

1 Introduction

The development of synthetic cannabinoids was initially aimed at understanding the endocannabinoid system and developing potential pharmacotherapies for cannabinoid-induced disorders (de Luca and Fattore, 2018; Diao and Huestis, 2019). However, the growing number of novel synthetic cannabinoids has become a major public health concern as the identification of specific synthetic cannabinoids consumed by abusers became difficult. Additionally, novel cannabinoids are classified under the highest drug schedule due to their adverse effects and lack of medical use (Drug Enforcement Administration, 2015). Prevalence of their abuse might be due to their stronger effects compared to trans- Δ^9 -tetrahydrocannabinol (THC), the main psychoactive component of marijuana (Weinstein et al., 2017), and being “legally” available online. Despite inducing adverse effects (MacDonald and Pappas, 2016; Schmitz and Richert, 2020; National Institute on Drug Abuse, 2021), synthetic cannabinoids have persistently circumvented legal regulations and initial detection owing to their varying chemical composition, resulting from the constant modification of functional groups. Thus, evaluating the potential dangers of these substances has piqued the interest of the scientific community.

The pharmacological properties of synthetic cannabinoids are similar to those of exogenous phytocannabinoids, with synthetic cannabinoids being more potent due to their higher affinity for cannabinoid receptors (Tai and Fantegrossi, 2014). Recent studies have suggested that CB₁ receptors are ubiquitously expressed throughout the central nervous system, forming part of the endocannabinoid system (Fagundo et al., 2013), which is interconnected with the mesolimbic dopaminergic pathway, a signaling network implicated in mediating drug addiction (Adinoff, 2004; Vlachou and Panagis, 2014). The endocannabinoid system may mediate addiction-like behaviors by regulating γ -Aminobutyric acid (GABA)/glutamate neurotransmission *via* retrograde signaling of CB₁ receptors expressed on GABAergic/glutamatergic neurons, thus influencing dopaminergic neuronal activity (Parsons and Hurd, 2015). This may provide the basis for cannabinoid-induced addiction in humans, leading to cannabinoid use disorders. However, some studies have demonstrated that not all synthetic cannabinoids can induce significant addiction-like behaviors in animal models (Polissidis et al., 2009; Hyatt and Fantegrossi, 2014; Tampus et al., 2015; Rodríguez-Arias et al., 2016; Bilel et al., 2019), especially in self-administration (SA) paradigms, contradictory to clinical reports and personal accounts (Le Boisselier et al., 2017; de Luca and Fattore, 2018; Leung et al., 2020). This presents several challenges in screening the abuse potential of novel cannabinoids and the involvement of complex neural mechanisms in the pathophysiology of cannabinoid abuse. Thus, there is a need to develop an

efficient tool for evaluating the abuse potential of synthetic cannabinoids.

Accumulating evidence suggest the involvement of neuroinflammation in cannabinoid addiction, potentially entailing both pro- and anti-inflammatory activities (Kozela et al., 2010; Kinsey et al., 2011; Javed et al., 2016; Bayazit et al., 2017). Some cannabinoids also seem to mitigate the addictive effects of abused drugs (Xi et al., 2011; Delis et al., 2017; Lin et al., 2018). Increased neuroinflammation induced by drugs of abuse may modulate glutamate synapses *via* increased astroglia glutamate reuptake (Boycott et al., 2008; Ramos et al., 2010), thus affecting glutamate-dependent synaptic plasticity (Park et al., 2015), which may reinforce drug-seeking behavior (Kauer and Malenka, 2007). Since some cannabinoids may reduce cytokine production (Xu et al., 2007) and deactivate glial cells (Rodrigues et al., 2014), their potential effects on neuroinflammation may provide another possible mechanism that may also mediate cannabinoid addiction development. This further suggests their inconsistent addiction-inducing effects in rodent models to probably involve inflammatory processes.

Transgenic mice models have been widely used to elucidate the pathophysiology of substance abuse disorder (Spanagel and Sanchis-Segura, 2003; Sora et al., 2010). These models are commonly developed based on genes that are differentially expressed in response to drugs of abuse. These genes are then modified in mice to generate transgenic rodent models (Yazdani et al., 2015; Szumlinski et al., 2017). Preliminary findings have suggested that treatment with methamphetamine or cocaine modulates the expression of *Cryab* in mice (Ministry of Food and Drug Safety, 2021). *Cryab* codes a small heat shock protein (CRYAB) with anti-inflammatory functions, such as reducing apoptosis through enhancing PI3K and activating AKT signaling (Ren et al., 2018), and inhibiting inflammatory responses in glial cells by downregulating pro-inflammatory mediators (Kuipers et al., 2017; Guo et al., 2019). Although initially, CRYAB functions as a molecular chaperone and aggregates misfolded proteins (Dai et al., 2022), thus preventing detrimental protein accumulation during stress (Calderwood et al., 2019). Furthermore, it may also participate in various types of cancer (Zhang et al., 2019), providing it a potential anti-inflammatory and anti-apoptotic role. Although the administration of addictive drugs may alter the expression of *Cryab*, the role of *Cryab* in cannabinoid-induced addiction has not yet been investigated. Therefore, a *Cryab* transgenic mouse model may be possibly used to determine the abuse potential of novel substances and identify susceptibility to potentially addictive drugs, such as synthetic cannabinoids.

Thus, this study aimed to provide an animal model that would be susceptible to cannabinoid abuse by determining the responses of *Cryab* knockout (KO) mice to 1-naphthalenyl(1-pentyl-1H-indol-3-yl)-methanone (JWH-018; a representative cannabinoid) in

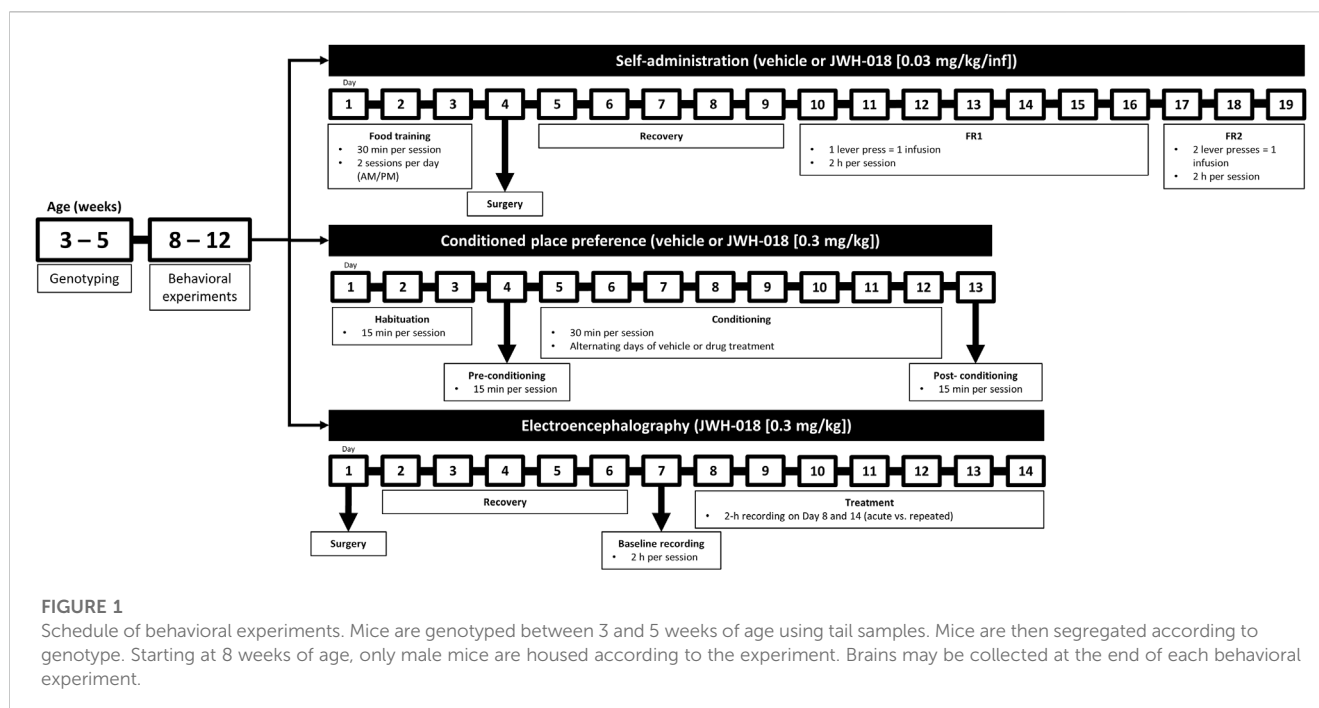


FIGURE 1

Schedule of behavioral experiments. Mice are genotyped between 3 and 5 weeks of age using tail samples. Mice are then segregated according to genotype. Starting at 8 weeks of age, only male mice are housed according to the experiment. Brains may be collected at the end of each behavioral experiment.

addiction-associated behavioral paradigms. Given the effect of *Cryab* on inflammation and the involvement of inflammation in addiction development, *Cryab* KO mice might exhibit increased sensitivity to JWH-018 abuse. As neural electrical activity indicates physiological responses to drugs of abuse, the electroencephalogram of *Cryab* KO mice may perhaps also differ from those of wild-type (WT) mice after repeated JWH-018 exposure. Since cannabinoid addiction may also involve endocannabinoid, dopaminergic, and neuroinflammatory pathways, repeated exposure to JWH-018 might differentially modulate endocannabinoid-related, dopamine-related, and neuroinflammation-related genes and/or proteins in *Cryab* KO mice, contributing to their divergent cannabinoid-induced behaviors when compared with WT mice.

2 Materials and methods

2.1 Animals

The Department of Pharmacy, Chungbuk National University (Cheongju City, Korea) provided two male and two female *Cryab* KO mice (aged 8 weeks). Transgenic mice were bred with C57BL/6N mice (aged 8 weeks, 22–25 g) obtained from Hanlim Animal Laboratory Co. (Hwasung, Korea), to obtain heterozygous *Cryab* (*Cryab* Het) mice. All procured mice were acclimatized in the animal room for 1 week prior to being used. Male and female *Cryab* Het mice (aged 8 weeks, F1) were subsequently bred to obtain male *Cryab* KO mice (F2), which were used for all experiments in this study (aged 8–12 weeks, 25–30 g). Newborn pups were genotyped at 3–4 weeks old using DNA from the tail. Only male mice were used for behavioral and molecular experiments. Mice for experiments were housed together in cages (4–6 mice per cage). To obtain next generation mice (F3), male and female *Cryab* Het mice (F2) were

bred. All mice were housed in a room under controlled conditions (circadian cycle, 12-h light/dark cycle (7 AM–7 PM); temperature, 22°C ± 2°C) and had access to food *ad libitum*. The schedule of experiments is presented in Figure 1. Animal treatment and maintenance procedures were performed according to the Principles of Laboratory Animal Care (NIH Publication No. 85–23, revised 1985) and the Animal Care and Use Guidelines of Sahmyook University, South Korea (SYUIACUC 2021-020 and SYUIACUC 2022-001).

2.2 Drugs

JWH-018 was synthesized according to reported procedures (Huffman et al., 2005). In brief, indole was reacted with pentyl bromide and potassium hydroxide in DMF. The obtained *N*-pentylindole was acylated with 1-naphthoyl chloride in the presence of Me₂AlCl in dichloromethane to produce JWH-018. The structure was confirmed by the following spectroscopic analyses: ¹H-NMR (500 MHz, CDCl₃) δ 8.52–8.48 (m, 1H), 8.20 (d, *J* = 8.6 Hz, 1H), 7.95 (t, *J* = 8.3 Hz, 1H), 7.90 (d, *J* = 8.0 Hz, 1H), 7.65 (dd, *J* = 6.9, 1.1 Hz, 1H), 7.54–7.45 (m, 3H), 7.40–7.35 (m, 4H), 4.05 (t, *J* = 7.4 Hz, 2H), 1.82–1.76 (m, 2H), 1.32–1.22 (m, 4H), 0.85 (t, *J* = 7.2 Hz, 3H); ¹³C-NMR (125 MHz, CDCl₃) δ 192.1, 139.2, 138.1, 137.1, 133.8, 130.9, 130.1, 128.3, 127.1, 126.8, 126.4, 126.1, 125.9, 124.7, 123.7, 123.0, 122.9, 117.6, 110.1, 47.3, 29.6, 29.0, 22.3, 14.0; HR-MS calculated for C₂₄H₂₃NO [M + H]⁺ 342.1852, found 342.1852; HPLC purity 99.46%. In all experiments, JWH-018 was dissolved in a solution comprising Tween 80, absolute ethanol, and 0.9% (w/v) saline (SAL) in the ratio 1:1:48 to obtain the desired dosage (0.3 mg/kg body weight). JWH-018 was selected as it has been used in several early cannabinoid-based studies, and the dose was selected based on reports that demonstrated significant

addiction-related effects of JWH-018 and other cannabinoids in rodents (de Luca et al., 2015; Ossato et al., 2017).

2.3 Behavioral tests

2.3.1 Open-field test (OFT)

Baseline locomotor activity was evaluated using a square Plexiglas open-field arena (42 cm × 42 cm × 42 cm), as per the methodology described in previous studies (Custodio et al., 2018; dela Peña et al., 2019; Kim et al., 2019). Mice were placed at the center of the arena and allowed to explore for 12 min for three consecutive days. The recorded data of the first 2 min were excluded from the analysis, as this period was considered a habituation period. The distance moved (cm) and the movement duration (s) of each mouse was measured using an automated system (EthoVision, Noldus, Netherlands).

2.3.2 Y-maze test

Basic cognition in mice was assessed using a “Y”-shaped maze comprising three identical arms (each arm spaced at an angle of 120°) with each arm measuring 5 cm × 35 cm × 10 cm. Each mouse was placed at the end of an arm and allowed to explore the maze freely for 8 min. Spontaneous alternating behavior and total entry in each arm were obtained following a previous study (Custodio et al., 2018). The alternation behavior (actual alternation) was described as the consecutive entry into three arms (continuous exploration of three different arms (e.g., ABC, BCA, or CAB) with stepwise sequence combinations. The maximum number of alternations was defined as the total number of arms entered minus two. The percentage spontaneous alternation behavior was calculated as follows: spontaneous alternation behavior (%) = (actual alternations/maximum alternations) × 100.

2.3.3 Rota-rod test

General motor balance and coordination of mice were measured using a rotating rod (Ugo Basile, Varese, Italy) at a fixed speed of 36 rpm, as per the methodology described in previous studies (Custodio et al., 2018; dela Peña et al., 2019). Two consecutive days before the experiment, the mice were allowed to habituate and were trained to run on the rotating rod for 3 min (T1 and T2). On the day of the experiment, “challenge day” (Ch), the mice were placed on the rotating rod for 10 min. Mice that fell during the experiment were gently placed back onto the rota-rod. The falling latency and frequency were recorded.

2.3.4 Cliff avoidance test

The impulsive behavior of the mice was assessed using a 50 cm long cylindrical Plexiglass rod support with a round Plexiglass platform (diameter = 20 cm; thickness = 1 cm) on both ends. Each mouse was placed at the center of the platform, and its behavior was observed for 10 min. The first fall latency and falling frequency were recorded.

2.3.5 Elevated plus-maze test (EPM)

Inherent anxiety-like behavior in mice was determined using a plus-maze comprising four arms, two open and two closed arms measuring 30 cm × 6 cm (closed arms were enclosed by 20 cm high

walls). The center of the maze contained a 6 cm × 6 cm delimited area. At the start of each test, individual mice were placed at the center, facing one of the open arms. The percentage of entries (100 × open/total entries) and time spent in the open arms were calculated after each 5 min test, similar to a previous study (dela Peña et al., 2019). The EthoVision system was used for automated recording and analysis.

2.3.6 Electroencephalography (EEG)

The mice were anesthetized with 0.02 mL Zoletil® (50 mg/mL) and Rompun® (xylazine 23.32 mg/mL) prepared in SAL. A three-channel tethered head mount (8200K3-iS/iSE) was placed and fixed with stainless screws positioned in the frontal region (A/P: −1.0 mm, M/L −1.5 mm) and posterior brain (A/P: −1.0 mm, M/L ±1.5 mm), conductive epoxy, and dental cement. Standard operating procedures were performed to minimize animal discomfort. The mice were allowed to recover for 5 days before recording. Data acquisition and analyses were performed according to the methodology described in previous studies (Botanas et al., 2021; Custodio et al., 2021) but with some modifications. On the last recovery day, mice were allowed to acclimatize to the EEG apparatus for 2 h (unrecorded). On the next day, a baseline recording for drug-naïve mice was performed for 2 h with an initial 10 min of habituation using a computer-based software (Pinnacle Technology, Inc., Lawrence, KS, United States). One group of mice was treated with JWH-018 (0.3 mg/kg body weight) or vehicle (VEH) for 7 days. After the initial baseline recording (Day 0), EEG was recorded on the first and last day of treatment. EEG wave changes relative to the baseline recording were calculated.

2.3.7 Intravenous self-administration (SA) test

The SA test was performed as reported previously (Custodio et al., 2022). Lever pressing training, which was based on food pellet reward under continuous reinforcement, was performed for three consecutive days [30 min per session (AM and PM sessions)]. In this study, lever pressing behavior was determined when mice earned 15 reinforcers at the end of the session on the third day and 75% of responses were on the active lever (Thomsen and Caine, 2011). After training, surgery was performed to insert a 0.28 mm inner diameter catheter into the jugular vein and mid-scapular region of mice anesthetized with Zoletil® (50 mg/mL) and Rompun® (xylazine 23.32 mg/mL) prepared in SAL. The catheterized mice were allowed to recover for 5 days. During recovery, the catheters were infused daily with 0.02 mL of 0.9% SAL containing heparin (20 IU/mL) and gentamicin (0.08 mg/mL). Catheter patency was determined by flushing catheters with a 0.02 mL of the Zoletil®/Rompun® mixture, and the loss of muscle tone was observed in mice within 10 s of infusion. During the SA experiment, catheters were flushed with SAL containing gentamicin or heparin immediately before and after each SA session. Mice were assessed daily in 2-h SA sessions under a fixed-ratio (FR) 1 schedule for 7 days, followed by assessment with an FR2 schedule for 3 days (Graphic State Notation 4; Coulbourn Instruments, Whitehall, PA, United States). Mice were fed a pellet diet (2 g) daily. In each session, both left and right levers were available. Left (active) lever pressing was followed by infusion of 0.05 mL JWH-018 (0.03 mg/kg body weight/infusion) or SAL over 10 s. During the infusion, the stimulus light over the lever was illuminated for 20 s. Lever presses during “time-out” periods were

recorded but had no effect. Right (inactive) lever presses were also recorded, but not reinforced. Active and inactive lever presses and the number of infusions for each daily session were recorded for evaluating SA behavior.

2.3.8 Conditioned place preference (CPP) test

The apparatus comprised two compartments (dimensions = 17.4 cm × 12.7 cm × 12.7 cm) with a removable guillotine door that separated the compartments. One compartment had smooth black walls and white flooring, whereas the other had white-dotted black walls and textured white flooring. An illumination of 12 lux was maintained throughout the experiment. A computer system (EthoVision) was used for recording animal movements and stay durations in the compartments. The protocol was performed according to previous studies with some modifications (Kim et al., 2019; Custodio et al., 2020; Sayson et al., 2020). The test comprised the following three phases: (A) habituation (days 1–3) and pre-conditioning (day 4; 15 min), (B) conditioning (days 5–12; 30 min), and (C) post-conditioning (day 13; 15 min). During habituation, mice were allowed to freely explore the entire apparatus. An initial trial (pre-conditioning) was performed to determine the stay duration of each mouse in each of the compartments. Mice were assigned to groups based on the pre-conditioning phase such that their non-preferred side was designated as the drug-paired compartment. In the conditioning phase, mice were administered JWH-018 (0.3 mg/kg body weight) or VEH and placed in the drug-paired compartment. On alternate days, the mice received VEH and were confined to the VEH-paired compartment. During the post-conditioning phase, the mice were not treated and were allowed to explore both compartments (similar to the pre-conditioning phase). The CPP score was calculated as the difference in the time spent by the mice in their respective drug-paired compartments between the post-conditioning and pre-conditioning phases.

2.4 Molecular experiments

2.4.1 RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Drug-naïve or JWH-018-treated mice (7 days) were euthanized and decapitated to excise the brain 30 min after the last treatment. The ventral tegmental area (VTA) and nucleus accumbens (NAC) were isolated using a mouse brain matrix on ice. The samples were rapidly frozen at -80°C before further processing. The subsequent steps followed previous methods (Custodio et al., 2022) and were according to MIQE guidelines. Total RNA was isolated with TRIzol® (Invitrogen, Carlsbad, CA, United States), following the manufacturer's protocol, and purified using the Hybrid-RTM kit (Geneall Biotechnology, Seoul, Korea). RNA concentrations were determined using Colibri Microvolume Spectrometer (Titertek-Berthold, Pforzheim, Germany). Total RNA (1 μg) was reverse-transcribed into complementary DNA (cDNA) using AccuPower® CycleScript RT PreMix (Bioneer, Seoul, Korea), following the manufacturer's instructions. Aliquots of

cDNA were stored at -20°C . Target genes were amplified using custom sequence-specific primers (Cosmogenetech, Seoul, Korea) and detected using SYBR® Green (Solgent, Daejeon, Korea). The primer sequences are shown in Supplementary Table S1. The input cDNA concentration was 2.5 $\mu\text{g}/\mu\text{L}$. The PCR conditions were as follows: 94°C for 1 min (denaturing step), followed by annealing at primer-specific temperature for 1 min, and 72°C for 45 s. qRT-PCR was performed using samples in triplicate. The expression of target genes was normalized to that of *Gapdh*. The results are expressed as relative expression calculated using the $2^{-\Delta\Delta\text{CT}}$ method (VanGuilder et al., 2008).

2.4.2 Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Brains of a separate cohort of VEH or JWH-018-exposed (7-day treatment) mice were collected and isolated similarly as described in the previous section. Protein extraction was done according to previous methods with slight modifications (Sayson et al., 2020). Brain tissues were lysed in 400 μL homogenization buffer [radioimmunoprecipitation assay buffer (Biosesang Inc., Seongnam, Korea) supplemented with cComplete™ ULTRA protease inhibitor cocktail tablets (05892791001, Sigma-Aldrich) and PhosSTOP™ phosphatase inhibitor cocktail tablets (04906845001, Sigma-Aldrich)]. The tissue extracts were centrifuged at 16,000 g at 4°C for 20 min. Dopamine concentration was determined using the dopamine ELISA kit (KA1887, Abnova, Taiwan), following the manufacturer's instructions. Briefly, standards, controls, and samples were acylated and incubated with dopamine antiserum for 2 h at room temperature (20°C – 25°C) on a shaker (approximately 600 rpm). Next, the samples in the wells were washed and incubated with the enzyme conjugate for 30 min. After washing the samples, the substrate was pipetted into individual wells, and the samples were incubated for 25 min. The stop solution was then added to each well. The absorbance of the reaction mixture was measured at 450 nm using an E_{Max} Plus Microplate Reader (Molecular Devices; San Jose, CA, United States). All measurements were performed in duplicates.

2.4.3 Western blotting

The striatum (STR) of VEH or JWH-018-exposed (7-day treatment) mice was isolated. Procedure was done according to previous studies (Sayson et al., 2020; Custodio et al., 2021). The samples were then heated at 95°C for 5 min. Protein lysates (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% gel. The resolved proteins were transferred onto nitrocellulose membranes. The membrane was blocked with 5% bovine serum albumin (BSA) prepared in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h and then incubated overnight with specific primary antibodies at 4°C . Then, the membrane was washed with TBST and incubated with horseradish peroxidase-conjugated anti-rabbit (1:3,000) or anti-mouse secondary antibodies (1:5,000) for 1 h. Protein bands were visualized based on enhanced chemiluminescence (Clarity Western ECL; Bio-Rad Laboratories, Hercules, CA, USA) using the ChemiDoc Imaging System (Image Lab

software, version 6.0; Bio-Rad). The levels of phosphorylation-independent proteins were normalized to those of β -Actin. The levels of the phosphorylated form of proteins were normalized to those of the total form of proteins. Fold change was determined by normalizing the values of the test groups to those of the WT VEH group. The antibodies used for the Western blot analysis are listed in [Supplementary Table S2](#).

2.4.4 Immunofluorescence

Standard protocols were used, following previously described methods ([Custodio et al., 2022](#)). After the last SA session, the mice were intracardially perfused with perfusion solution [0.05 M phosphate-buffered saline (PBS)] and perfusate [4% paraformaldehyde (PFA) in 0.1 M phosphate buffer]. The brain tissues were stored in PFA solution at 4°C overnight, followed by incubation with 30% sucrose solution at 4°C. The brain samples were sectioned to a thickness of 35 μ m using a Leica CM1850 cryostat (Wetzlar, Germany), following mouse brain stereotaxic coordinates ([Paxinos and Franklin, 2001](#)). For this experiment, the STR was selected for analysis as this region is commonly implicated in inflammation-associated behavioral alterations, such as addiction ([Krasnova et al., 2016](#); [Vonder Haar et al., 2019](#)). The brain sections were carefully washed with 1 \times PBS and subsequently incubated in a protein-blocking solution (5% BSA and 0.3% Triton X-100 in 1 \times PBS) for 1 h at room temperature. Next, the sections were incubated with primary antibodies diluted in a protein-blocking solution for 3 days at 4°C. The primary antibody used in this analysis is listed in [Supplementary Table S2](#). After washing, the samples were incubated overnight with Alexa fluor-555-conjugated goat anti-mouse (Thermo Fisher Scientific A32727, RRID: AB_2633276) antibody at 4°C. The samples were washed, incubated with Hoechst for 10 min, and mounted on 76 mm \times 26 mm \times 1 mm clean positively charged microscope slides (Walter Products Inc., Canada). Mounted sections were cured with PermOUNT[®] Mounting Medium UN1294 (Fisher Chemical, NJ, United States), covered with 24 mm \times 50 mm microscope cover glasses (Marienfeld Laboratory Glassware, Germany), and allowed to dry for 24 h at room temperature. The corrected total cell fluorescence (CTCF) level was measured in each sample using ImageJ 1.53k (NIH, Maryland, United States) as reported previously ([Hammond, 2014](#)).

2.5 Statistical analyses

All mice were randomized for treatment. The researchers were blinded to the treatment of the animals while conducting the tests and analyzing the data. Statistical analyses were performed using GraphPad Prism v7 (GraphPad Software Inc., San Diego, CA, United States). Data are presented as the mean \pm standard error of the mean (S.E.M.), unless specified otherwise. The means were analyzed using one-way or two-way analysis of variance (ANOVA) with or without repeated measures (RM), followed by Tukey's or Bonferroni's multiple comparison test, when appropriate. Differences were considered statistically significant at $p < 0.05$. The p -value and detailed statistical analysis (e.g., genotype, treatment, time, days, etc.) are indicated.

3 Results

3.1 *Cryab* KO mice exhibited hypoactivity

Although controversial, inherent aberrant behaviors were previously correlated with substance abuse co-morbidity ([Herrero et al., 2008](#); [Volkow, 2009](#); [Kabir et al., 2016](#)), therefore we screened the general behavior of *Cryab* KO mice in various behavioral assays. OFT revealed that *Cryab* KO mice innately possessed lower spontaneous locomotor activity than WT mice, as evidenced by their lower total distance moved ([Figure 2A](#); genotype: $F_{1,18} = 29.6$; $p < 0.001$; test day: $F_{2,36} = 27.0$; $p < 0.001$) and movement duration ([Figure 2B](#); genotype: $F_{1,18} = 37.3$; $p < 0.001$; test day: $F_{2,36} = 35.1$; $p < 0.001$), even across multiple testing sessions. Spontaneous alternation ([Figure 2C](#)) in the Y-maze were similar between mice genotypes, while total entry ([Figure 2D](#); $t = 5.14$, $df = 17$) also suggested lower locomotor activity in *Cryab* KO mice. Other behaviors in rota-rod ([Figures 2E, F](#)), cliff avoidance ([Figures 2G, H](#)), and EPM ([Figures 2I, J](#)) exhibited no differences between WT and *Cryab* KO mice. Drugs of abuse also modulate neural electrical activity ([Newton et al., 2003](#); [Malyshevskaya et al., 2017](#); [Zanettini et al., 2019](#); [Nukitram et al., 2021](#)), suggesting that EEG alterations may co-manifest alongside the addiction-inducing effects of some psychoactive drugs. Aberrant EEG patterns may also indicate potentially modified neuropsychological responses to addictive drugs. Accordingly, 2-h baseline EEG recording data of mice ([Figure 2K](#)) showed that the delta-, theta-, alpha-, beta-, and gamma-wave levels of *Cryab* KO mice were lower than WT mice [absolute power (μV^2) of WT mice was set to 100%]. Sample EEG traces of mice ([Figure 2L](#)) showed comparable patterns between genotype.

3.2 *Cryab* KO mice exhibited increased responses in addiction-related behavioral assays and divergent gamma-wave power

To evaluate the responses of mice to the reinforcing effect of JWH-018, we exposed WT and *Cryab* KO mice to JWH-018 SA. *Cryab* KO obtained higher active lever responses ([Figure 3A](#)) in FR1 ($F_{3,38} = 3.94$; $p < 0.05$) and FR2 [treatment ($F_{3,38} = 2.99$; $p < 0.05$); SA days ($F_{2,76} = 4.38$; $p < 0.05$)], along with greater number of infusions ([Figure 3C](#)) and FR1 ($F_{3,38} = 4.9$; $p < 0.01$) and FR2 ($F_{3,38} = 3.49$; $p < 0.05$), compared to WT mice. Average obtained infusions ([Figure 3D](#)) all throughout the SA sessions were also significantly higher ($F_{3,24} = 51.3$; $p < 0.001$) in *Cryab* KO mice compared to their WT and VEH counterparts. The varying behavior of mice during SA indicate the manifestation of JWH-018-induced reinforcing effects in *Cryab* KO mice. Aside from that, the rewarding property of JWH-018 was also evaluated in WT and *Cryab* KO mice through the CPP paradigm. JWH-018 appeared to induce rewarding effects in *Cryab* KO mice ([Figure 3E](#)), as evidenced by their higher CPP score ($F_{3,24} = 5.59$; $p < 0.01$) compared to their VEH counterpart, but not in WT mice.

Gamma-wave generation is also altered in chronic cannabis users owing to their continuous intake of cannabinoids ([Skosnik et al., 2012](#); [Liu et al., 2022](#)), as evidenced by *in vivo* and *in vitro*

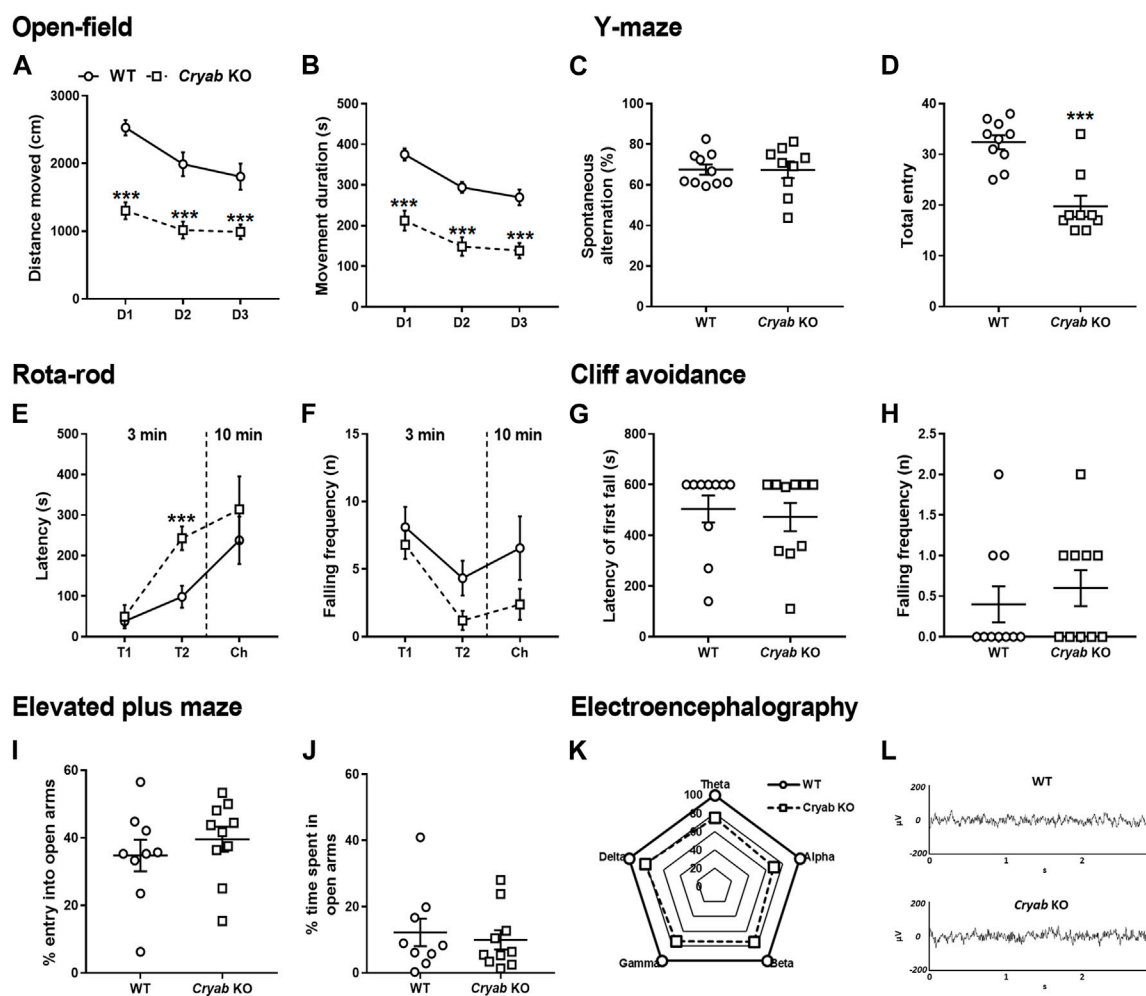


FIGURE 2

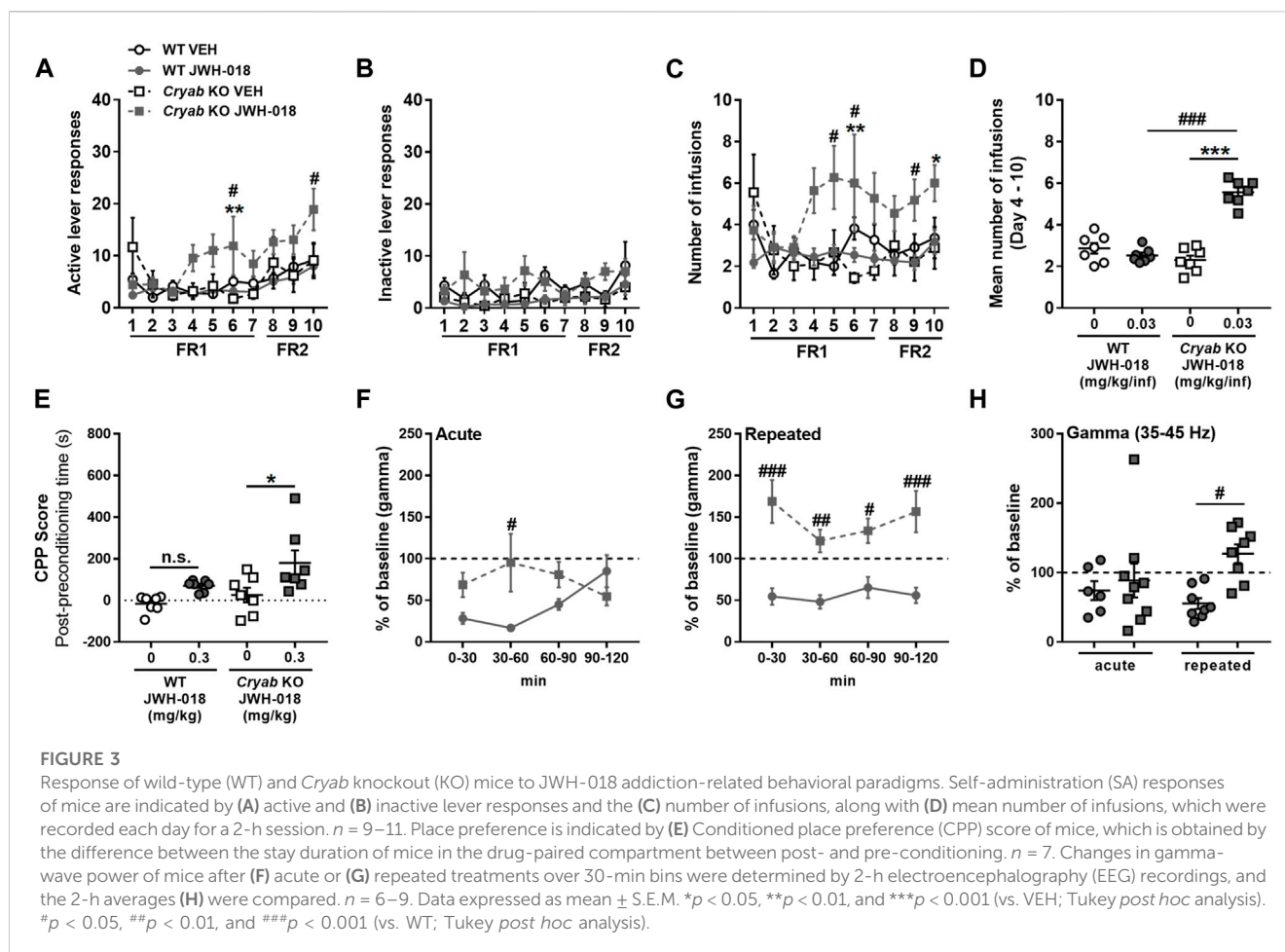
General behavior of wild-type (WT) and *Cryab* knockout (KO) mice. At 5–7 weeks old, pups were tested for their baseline behaviors. The open-field test shows the locomotor activity indicated by (A) distance moved (cm) and (B) movement duration (s) recorded for 10 min over three consecutive days. Y-maze indicates short-term memory in terms of (C) spontaneous alternating behavior (%) and (D) total entries. Latency of first fall (s) and falling frequency (n) in the (E,F) rota-rod and (G,H) cliff avoidance tests determine motor balance/coordination and impulsive tendencies, respectively. Anxiety is measured by the (I) percentage entry (%) and (J) percentage time spent (%) in the open arms in the elevated maze plus test. (K) Baseline electroencephalogram (EEG) results reveals the power of delta, theta, alpha, beta, and gamma in mice, recorded for 2 h (drug-free). (L) Sample EEG traces shows a 2-s activity during a 2-h recording. Data expressed as mean \pm S.E.M., except (K) and (L). $n = 9-10$. *** $p < 0.001$ (vs. WT; Bonferroni *post hoc* analysis or *t*-test).

studies (Sales-Carbonell et al., 2013). Thus, gamma wave alterations in mice following repeated JWH-018 administration were also determined through EEG. Gamma-wave power after acute JWH-018 treatment (Figure 3F) was significantly different among mice with a treatment-time interaction ($F_{3,42} = 5.71$; $p < 0.01$), specifically the change in gamma-wave power in *Cryab* KO mice was lesser than WT mice only after 60 min post-treatment. Repeated JWH-018 administration also induced significant differences in change in gamma-wave power (Figure 3G) among mice ($F_{3,42} = 5.71$; $p < 0.01$), showing that gamma-wave power in *Cryab* KO mice increased significantly from baseline, unlike WT mice, all throughout the recording period. The change in gamma-wave power in mice during the 2-h recording (Figure 3H) was significantly different among groups ($F_{3,27} = 3.21$; $p < 0.05$).

The change in gamma-wave power of *Cryab* KO mice was significantly higher than WT mice after repeated JWH-018 treatment, but not after acute exposure.

3.3 Expressions of endocannabinoid system-related and dopamine-related genes, including accumbal dopamine concentration, were not markedly different between genotypes

The abuse liabilities of THC and synthetic cannabinoids are usually attributed to their influence on CB₁ receptors expressed in the VTA and NAC, which are major brain regions associated with the development of addiction-related behaviors (Nestler,



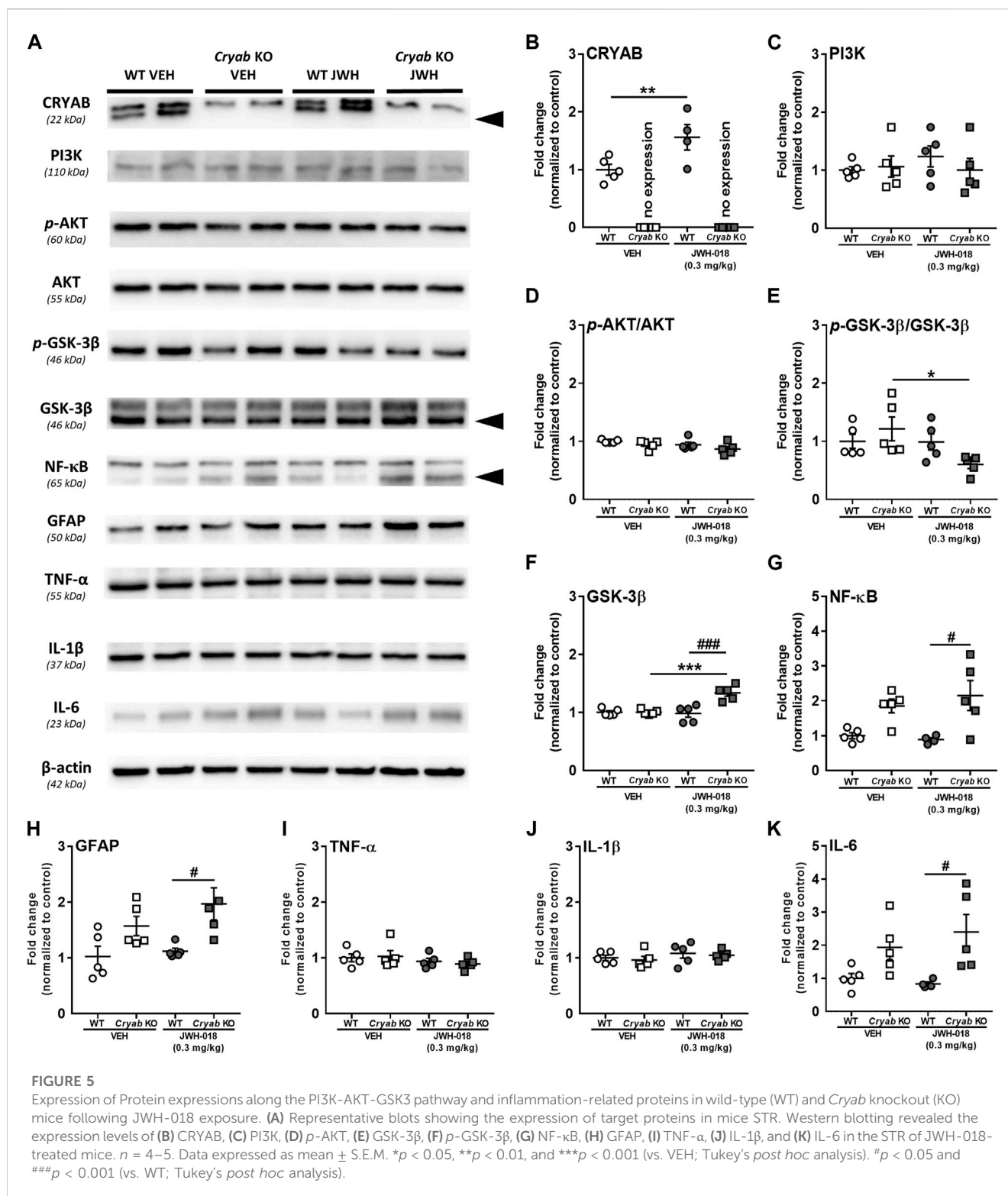
2001). Cannabinoids may also affect the expression of CB₂ receptors (Sun et al., 2017), along with enzymes that degrade endocannabinoids (Li et al., 2019). Thus, we measured the mRNA levels of endocannabinoid-related genes in the VTA and NAC of mice repeatedly treated with JWH-018. *Cnr1* (Figure 4A; $F_{1,20} = 38.8$; $p < 0.001$), *Cnr2* (Figure 4B; $F_{1,16} = 26.2$; $p < 0.001$), and *Mgll* (Figure 4D; $F_{1,16} = 41$; $p < 0.001$) mRNA levels were downregulated in the VTA of WT and *Cryab* KO mice following repeated JWH-018 treatment. However, *Cnr1*, *Cnr2*, *Faah*, and *Mgll* mRNA levels in the VTA were not significantly different between WT and *Cryab* KO mice. In the NAC, *Cnr2* (Figure 4F; $F_{1,17} = 10.9$; $p < 0.01$) and *Mgll* (Figure 4H; $F_{1,18} = 16.4$; $p < 0.001$) mRNA levels were also downregulated only in WT mice following repeated JWH-018 exposure. Similar to the VTA, *Cnr1*, *Cnr2*, *Faah*, and *Mgll* mRNA levels in the NAC were also not significantly different between genotypes. *Faah* expressions in both VTA and NAC (Figures 4C, G) were unaltered, although *Cnr1* expression in NAC (Figure 4E) exhibited treatment differences between groups ($F_{1,20} = 6.65$; $p < 0.05$).

Since endocannabinoid signaling within the VTA may influence dopaminergic neurotransmission, divergent addiction responses in mice genotypes might be reflected by differential cannabinoid-mediated regulation of the VTA and distinct changes in dopamine-related markers in the NAC.

Accordingly, we determined the mRNA levels of dopamine-related genes in the NAC of mice following repeated JWH-018 treatment. No significant alterations were detected in *Drd1* (Figure 4J) and *Drd2* (Figure 4K) mRNA levels, whereas the expression levels of *Dat* (Figure 4I; $F_{1,16} = 8.23$; $p < 0.05$) and *Vmat2* (Figure 4L; $F_{1,20} = 8.63$; $p < 0.01$) in WT mice were downregulated. We also determined the accumbal dopamine concentration in mice following acute and repeated JWH-018 administration to verify the potential effect of cannabinoid treatment on dopamine-mediated responses. Accumbal dopamine concentrations in mice showed no significant differences between the genotypes following repeated JWH-018 treatment (Figure 4N), although significant differences (treatment: $F_{1,16} = 10.4$; $p < 0.01$; genotype: $F_{1,16} = 6.04$; $p < 0.05$) were observed among groups following acute JWH-018 treatment (Figure 4M).

3.4 *Cryab* KO mice demonstrated potentially higher neuroinflammatory state after JWH-018 exposure

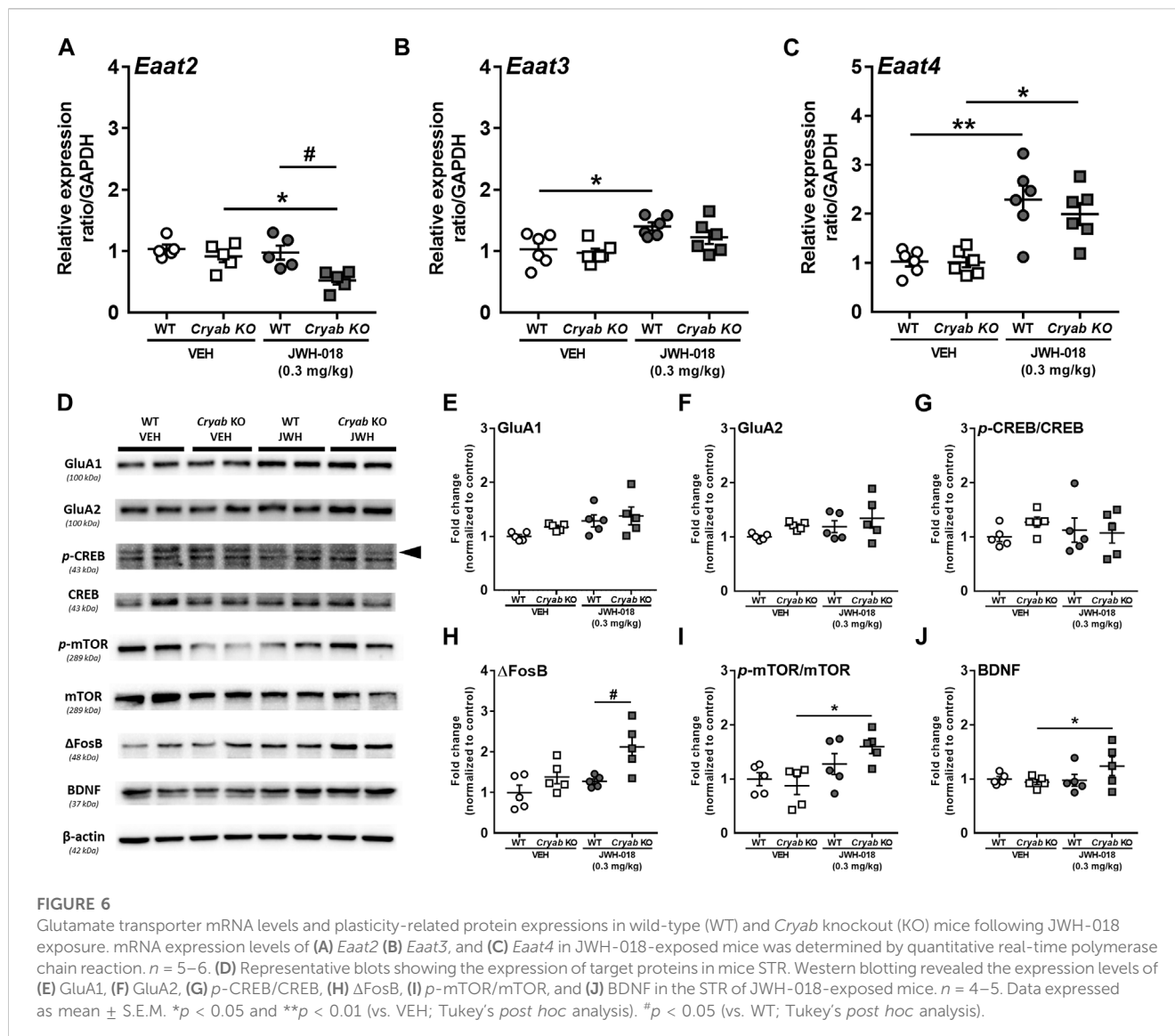
Striatal neuroinflammatory processes may possibly potentiate addiction-related behavior (Rodrigues et al., 2014; Kohno et al., 2019). Studies have mentioned that CRYAB may



(Figure 5D) were not significantly different between groups. *p*-GSK-3 β levels (Figure 5E; treatment: $F_{1,16} = 4.85$; $p < 0.05$) were decreased in *Cryab* KO mice following repeated JWH-018 administration, while GSK-3 β expression (Figure 5F; genotype: $F_{1,16} = 15$; $p < 0.01$, treatment: $F_{1,16} = 11.5$; $p < 0.01$) was significantly higher in *Cryab* KO mice than their WT and

VEH counterpart. Interestingly, repeated JWH-018 administration in the absence of functional CRYAB resulted in a higher NF- κ B expression (Figure 5G) in *Cryab* KO mice than WT mice ($F_{1,15} = 17.2$; $p < 0.001$).

NF- κ B may also influence neuroinflammation by mediating inflammatory cytokine gene transcription (Liu et al., 2017).



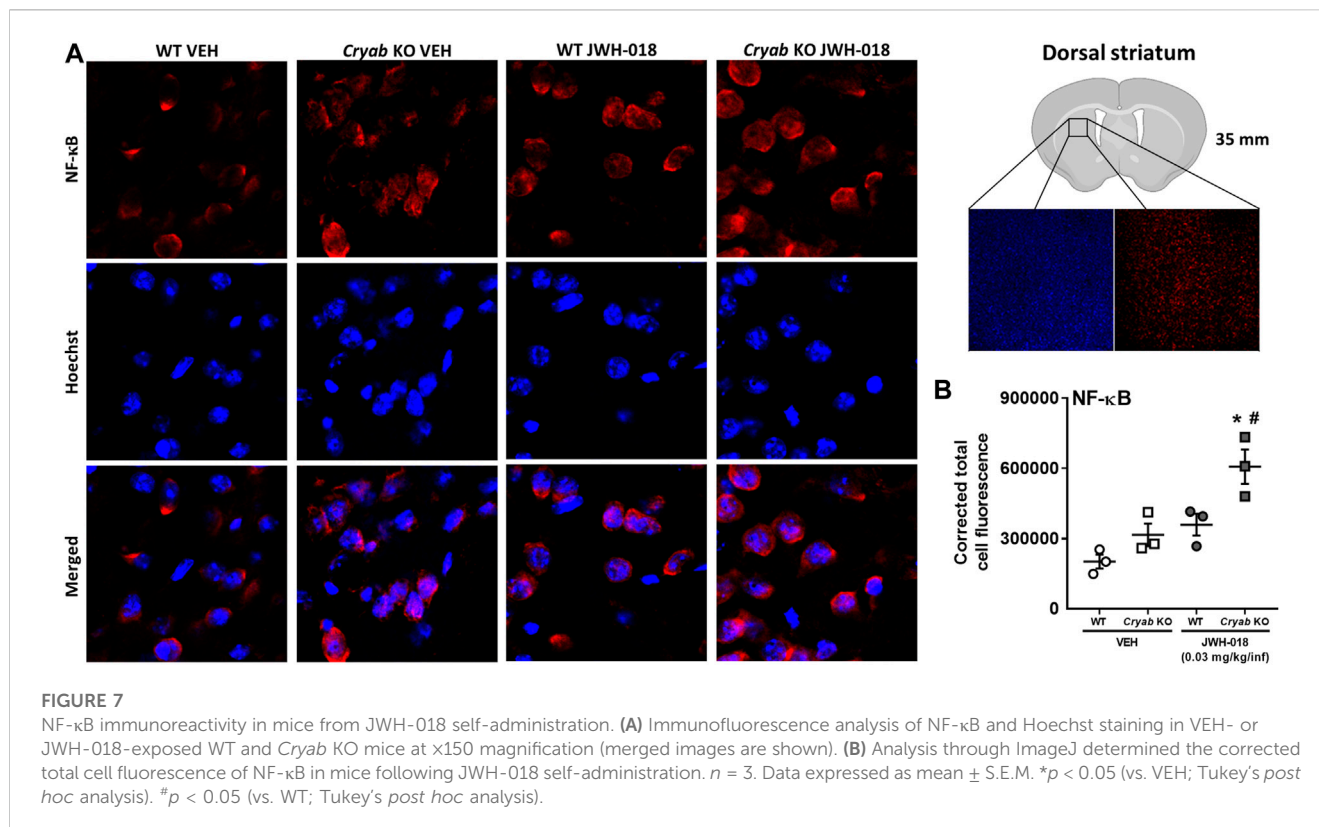
Accordingly, we verified the expression of glial cell activity and neuroinflammatory markers in mice STR. While TNF- α (Figure 5I) and IL-1 β (Figure 5J) were unaltered in mice after JWH-018 exposure, GFAP (Figure 5H; $F_{1,16} = 13.1$; $p < 0.001$) and IL-6 (Figure 5K; $F_{1,15} = 12.7$; $p < 0.01$) expressions were higher in *Cryab* KO mice than WT mice.

3.5 *Cryab* KO mice exhibited potentially higher glutamate-dependent synaptic plasticity after JWH-018 exposure

Glutamate reuptake in synapses is said to decrease, via GLT-1 downregulation, following an increase in cytokine expression and astrocyte activation (Boycott et al., 2008; Ramos et al., 2010), possibly altering glutamate concentration. Thus, we determined the mRNA expressions of glutamate transporter subtypes in JWH-018 exposed mice. Expression level of *Eaat2* mRNA (Figure 6A) was significantly decreased in *Cryab* KO mice and

was lower than WT mice (genotype: $F_{1,16} = 10.4$; $p < 0.01$; treatment: $F_{1,16} = 6.51$; $p < 0.05$) after repeated treatment of JWH-018. *Eaat3* (Figure 6B) and *Eaat4* (Figure 6C) mRNA levels were both increased in WT mice (*Eaat3*: $F_{1,20} = 12.5$; $p < 0.01$, *Eaat4*: $F_{1,20} = 32.5$; $p < 0.001$), while only *Eaat4* mRNA was upregulated in *Cryab* KO mice after repeated JWH-018 administration.

Alterations in glutamate neurotransmission have been previously associated with the development of drug addiction, including those induced by psychostimulants (Kalivas, 2007) and cannabinoids (Cohen et al., 2019). These are generally characterized by changes in glutamate receptors and markers of synaptic plasticity in brain areas mediating addiction-related behaviors (Kauer and Malenka, 2007; Park et al., 2015). In here, we determined the expressions of glutamate receptors and synaptic plasticity markers in the STR (Figure 6D). GluA1 (Figure 6E; $F_{1,16} = 5.92$; $p < 0.05$), but not GluA2 (Figure 6F), expression showed significant differences among treatment groups, but only an increased trend in JWH-018-exposed *Cryab* KO mice can be observed. While *p*-CREB expressions



of mice (Figure 6G) exhibited no significant variations among groups, ΔFosB expression (Figure 6H; genotype: $F_{1,16} = 7.69$; $p < 0.01$; treatment: $F_{1,16} = 11.4$; $p < 0.05$) was significantly higher in JWH-018-exposed *Cryab* KO mice than WT. *p*-mTOR (Figure 6I; treatment: $F_{1,16} = 10.6$; $p < 0.01$) and BDNF (Figure 6J) genotype/treatment interaction: $F_{1,15} = 5.21$; $p < 0.05$) levels were increased in *Cryab* KO mice after repeated JWH-018 administration.

3.6 *Cryab* KO mice displayed higher NF-κB immunoreactivity in the STR following JWH-018 SA

To verify the potential involvement of increased NF-κB expression in the manifestation of cannabinoid-induced addiction-related behaviors, we determined the striatal NF-κB expression of mice that underwent JWH-018 SA. NF-κB CTCF (Figure 7B) was significantly greater in *Cryab* KO mice (genotype: $F_{1,8} = 12.2$; $p < 0.01$; treatment: $F_{1,8} = 18.8$; $p < 0.01$) compared to their WT and VEH counterparts after JWH-018 SA.

4 Discussion

Cryab KO mice displayed generally similar baseline behavior with WT mice (Figures 2C, E–J), other than the marked hypoactivity in OFT (Figures 2A, B). This kind of phenotype was previously observed to occur alongside other aberrant behaviors, such as anxiety (Wood et al., 1987), depression (Ota et al., 2018), or

autism spectrum disorder (Wang et al., 2018; Angelakos et al., 2019). Animal models for depression, which were also observed to possess inherently low spontaneous locomotor activity (Skalisz et al., 2002; Rygula et al., 2005; Ota et al., 2018), demonstrated increased sensitivity to cocaine (Lepsch et al., 2005; Ribeiro Do Couto et al., 2009; Xu et al., 2020) and nicotine (Cruz et al., 2008). The hypoactive behavior of *Cryab* KO mice might then indicate a possibility for them to likewise exhibit aberrant responses to psychoactive drugs. Furthermore, *Cryab* KO and WT mice might differentially respond to addictive drugs also due to their divergent baseline EEG recordings (Figure 2K). Human EEG data demonstrated persistent changes in the delta, alpha, and gamma waves of subjects repeatedly exposed to addictive drugs, such as cocaine (Reid et al., 2006) and marijuana (Herning et al., 2008), indicating the involvement of EEG waves in the neuropsychological effects of addictive drugs. Together, the hypoactivity and baseline EEG of *Cryab* KO mice might render them potentially sensitive to psychoactive substances, probably including synthetic cannabinoids. We then subsequently investigated the behavioral responses of *Cryab* KO mice to the effects of the synthetic cannabinoid JWH-018 using paradigms that generally indicate the rewarding and reinforcing effects of drugs.

Cryab KO mice exhibited potentially higher sensitivity to the addiction-related effects of JWH-018, as evidenced by their increased responses in intravenous SA (Figures 3A–C) and CPP (Figure 3E) tests compared to WT mice. While several studies have reported the addiction-like effects of synthetic cannabinoids, including JWH-018 (Hur et al., 2021), in healthy rodent subjects (Cha et al., 2014; de Luca et al., 2015), some cannabinoids still do not exhibit significant abuse potential in animal behavioral paradigms

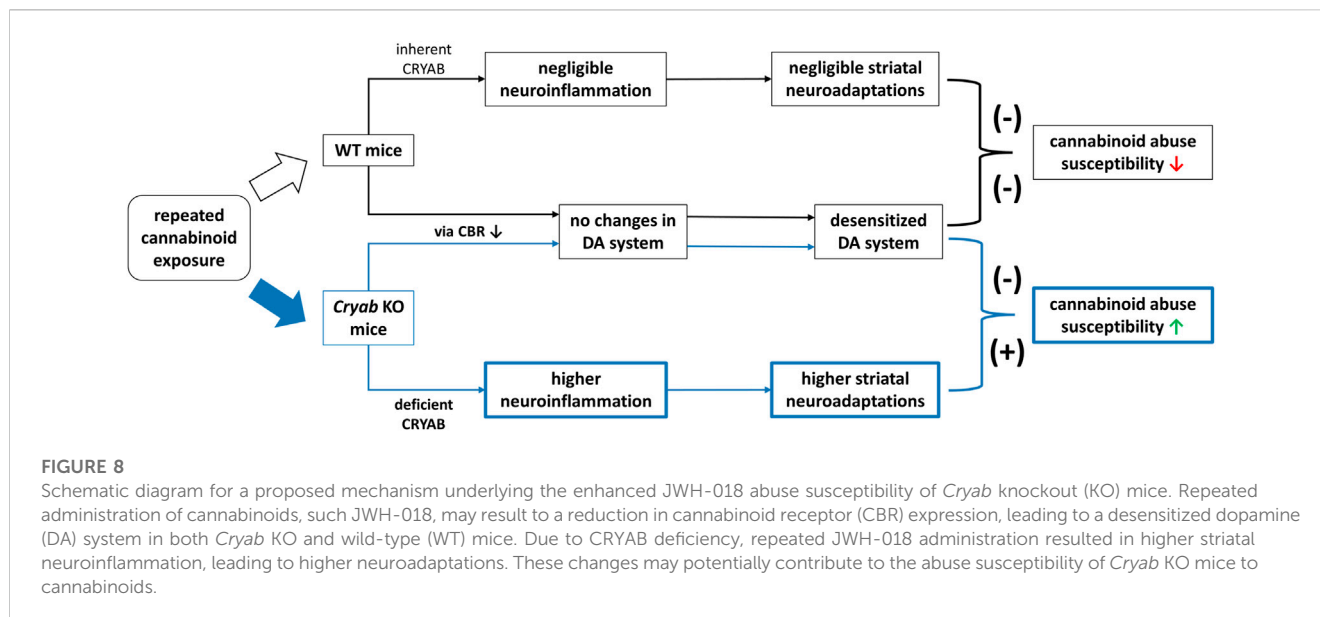
(Tampus et al., 2015; Bilel et al., 2019). This suggests the involvement of complex neurological mechanisms (that mediate the addictive effects of cannabinoids) other than the typical mesolimbic dopaminergic pathway. The results of the SA and CPP tests were consistent with this hypothesis, as WT mice did not exhibit significant preference or tendency for self-administering JWH-018 at 0.03 mg/kg/infusion or 0.3 mg/kg, unlike with previous studies. This discrepancy among studies investigating intravenous JWH-018 SA in WT mice might be attributed primarily to methodological differences, given that some studies required long-term exposure of mice in JWH-018 SA (de Luca et al., 2015), with some using adolescent mice with potentially different drug sensitivity (Margiani et al., 2022). However, the fact that *Cryab* KO mice exhibited modest SA responses and place preference for JWH-018 indicated cannabinoid-induced addiction-like behaviors, suggesting cannabinoid abuse susceptibility.

EEG recordings revealed significantly different gamma-wave power changes in WT and *Cryab* KO mice following repeated exposures to JWH-018 (Figures 3F–H). Gamma oscillations are involved in both cognition and perceptive functions and may be mediated by CB₁ receptors via GABA interneurons (Skosnik et al., 2012), indicating the potential involvement of gamma-wave alterations in cannabinoid-mediated effects, which may include addiction. Previous studies have also reported that repeated exposure to various addictive drugs increases gamma-wave production (Abiero et al., 2020; Custodio et al., 2020), which was also observed in *Cryab* KO mice after repeated JWH-018 exposure. However, gamma-wave production is generally associated with attention and perception (Müller et al., 2000; Tallon-Baudry et al., 2005), which may implicate the JWH-018-induced increase in *Cryab* KO mice gamma-wave power as an improvement in receptivity and cognition. While this may not entirely indicate an addiction response or vulnerability, the divergent changes in gamma-wave power between WT and *Cryab* KO mice might still indicate different neurophysiological modifications in brain substrates that may contribute to the observed disparity in the cannabinoid-induced, addiction-like responses between genotypes. Thus far, behavioral and EEG data suggest the *Cryab* KO mice to be a potential animal model of cannabinoid abuse susceptibility.

As previously mentioned, psychoactive cannabinoids generally activate CB₁ receptors on GABA/glutamatergic neurons located in the VTA and NAC, thus modulating the release of GABA/glutamate (Cristino et al., 2020; Spanagel, 2020) and influencing dopamine-mediated activities, such as addiction responses. Thus, a dysregulation in these pathways may influence cannabinoid-induced responses. Endocannabinoid-related mRNA expression levels (Figures 4A–H) suggest a possible downregulation of endocannabinoid signaling in WT and *Cryab* KO mice VTA, and perhaps also in WT mice NAC, after repeated JWH-018 treatment. While frequent exposure to cannabinoids potentially downregulates cannabinoid receptors in some brain regions (Scherma et al., 2016; van de Giessen et al., 2017; Kesner and Lovinger, 2021), similar to our qRT-PCR data, comparable JWH-018-induced mRNA alterations were detected between *Cryab* KO and WT mice, indicating possibly similar effects on dopaminergic signaling. Since alterations in cannabinoid receptor-encoding gene expressions may indicate GABA/glutamate signaling adaptations

and dopaminergic activity dysregulation (Maldonado et al., 2011; Colizzi et al., 2016; Zehra et al., 2018; Pintori et al., 2021), comparable modifications in both *Cryab* KO and WT mice might suggest similarities in the expression of cannabinoid-induced behaviors. As this was not the case, given the SA and CPP results, the disparity between the JWH-018-induced behavioral responses of WT and *Cryab* KO mice might involve divergent modifications in dopaminergic signaling.

Drug addiction is frequently attributed to alterations in dopamine neurotransmission, along with upregulated accumbal dopamine levels (Fleckenstein et al., 2007; MacNicol, 2017; Jiménez-González et al., 2022). Expressions of dopamine-related mRNA in the NAC of JWH-018-exposed mice (Figures 4I–L) suggest a possible dampening of dopaminergic activity. The same phenomenon was also observed in previous studies showing the blunting of dopaminergic activity following chronic cannabinoid exposure (van de Giessen et al., 2017; Kesner and Lovinger, 2021). It was previously suggested that genes influencing dopamine availability tend to also downregulate following chronic cannabinoid treatments (Perdikaris et al., 2018). The *Dat* and *Vmat2* downregulation in WT mice may suggest a compensatory response to promote dopamine availability, which is a phenomenon also found during drug tolerance and in chronic methamphetamine users (Wilson et al., 1996; Graves et al., 2021). Although there are only limited studies describing the effect of cannabinoids on *Vmat2* mRNA, its downregulation may denote possible dopamine release dysregulation (German et al., 2015) in WT mice in response to cannabinoids. One study suggested that reduced *Vmat2* mRNA expression may lead to inhibition of vesicular dopamine and potentially increase cytosolic dopamine levels (Sun et al., 2014). The lack of such changes in *Cryab* KO mice may imply their resilience to cannabinoid-induced dopaminergic alterations, further suggesting dopamine-independent mechanisms mediating their higher cannabinoid sensitivity. Nevertheless, downregulation of endocannabinoid signaling may have contributed to possible dopaminergic desensitization following repeated JWH-018 administration, which is a potential theory behind cannabinoid addiction pathophysiology (Zehra et al., 2018). On the other hand, the involvement of dopamine mediation might still not be ruled out completely. Studies demonstrating dopamine level upregulation after acute cannabinoid administration (de Luca et al., 2015; Ossato et al., 2017; Pintori et al., 2021), along with our ELISA results following acute JWH-018 treatment (Figure 4M), may still implicate transient dopamine increments in addiction-related brain regions to the addictive properties of cannabinoids. However, probably due to the absence of sustained dopaminergic activation via endocannabinoid signaling downregulation, some cannabinoids may have appeared to lack robust abuse potential following prolonged exposure (Cha et al., 2014; Tampus et al., 2015), as opposed to recognized abused drugs that show persistent increases in dopamine concentration and activity even after repeated exposures (Nestby et al., 1997; Cadoni et al., 2000), resulting in evident abuse liability. Therefore, the differential responses between *Cryab* KO and WT mice to the addiction-inducing effects of JWH-018 may implicate the involvement of other neurological mechanisms beyond the endocannabinoid or dopaminergic systems.



Studies have suggested the involvement of CRYAB and cannabinoids in the anti-inflammatory action of the PI3K-AKT-GSK3 pathway (Ozaita et al., 2007; Xu et al., 2013; Zhu et al., 2015), which may also modulate the expression of NF- κ B (Bathina and Das, 2018), a transcription factor broadly associated with immune system-related and inflammation-related gene regulation (Beurel et al., 2015; Liu et al., 2017). Several factors (Beurel et al., 2015; Liu et al., 2017; Feng and Lu, 2021), including some heat shock proteins (Schell et al., 2005), were reported to potentially influence neuroinflammation by regulating NF- κ B expression/activity, and when modulated, altered complex behaviors such as addiction (Rodrigues et al., 2014; Nennig and Schank, 2017; Morcuende et al., 2021). More recently, CRYAB was described to potentially regulate NF- κ B nuclear translocation (Shao et al., 2013; Qiu et al., 2016), which is essential for inflammatory cytokine and chemokine transcription (Somade et al., 2019). Based on the possible correlation between cannabinoid addiction and neuroinflammation, CRYAB, via NF- κ B, may potentially influence addiction-related behaviors. Increased CRYAB (Figure 5B) and negligible changes along the PI3K-AKT-GSK3 pathway (Figures 5C–F) in JWH-018-exposed WT mice may imply the occurrence of only minor neuroinflammation. In contrast, JWH-018-exposed *Cryab* KO mice seemed to suggest potential downstream neuroinflammatory alterations, given by increased GSK-3 β (Figure 5F) and NF- κ B (Figure 5G) expressions. From this observation, functional CRYAB expression might have a role in the anti-inflammatory effect of cannabinoids, as the presence or absence of CRYAB resulted in significant genotype differences in the expression of NF- κ B following JWH-018 exposure. Furthermore, NF- κ B expression and translocation may also influence neuroinflammation by mediating inflammatory cytokine gene transcription and astroglia activation (Young et al., 2011; Liu et al., 2017). While JWH-018 exposure induced no changes in WT mice, the higher expression of glial fibrillary acidic protein (GFAP; Figure 5H) and IL-6 (Figure 5K)

in *Cryab* KO mice may indicate a higher state of neuroinflammation compared to WT mice, which may have been a consequence of increased NF- κ B expression following repeated JWH-018 administration. Intriguingly, this had no effect on TNF- α and IL-1 β expressions, unlike in previous reports (Nennig and Schank, 2017), which could suggest that the alteration of CRYAB expression may have specificity to IL-6 transcription. Our results contradict some previous studies, given that cannabinoids are understood to be anti-inflammatory and JWH-018 seemed to potentially induce inflammation in *Cryab* KO mice. However, according to previous studies (Pintori et al., 2021; Margiani et al., 2022), JWH-018 may potentially promote neuroinflammation in mice following previous repeated exposures. Thus, the absence of CRYAB might have exacerbated the neuroinflammatory effect of JWH-018 in *Cryab* KO mice, resulting in the observed alterations in NF- κ B, GFAP, and IL-6 expressions, given also that CRYAB, possessing an anti-inflammatory function, might have rendered the *Cryab* KO mice susceptible to inflammatory insults.

Increased neuroinflammation, specifically due to increased cytokine and astrocyte activation (Boycott et al., 2008), may result to the downregulation of glutamate transporter (GLT-1) (Ramos et al., 2010), potentially increasing synaptic glutamate concentration. Glutamate reuptake was also reported to be reduced in the STR of cocaine-exposed rodents (Parikh et al., 2014; Smaga et al., 2020), potentially increasing the availability of glutamate in the synapse. Taking together the alterations in glutamate transporter mRNA (Figures 6A–C), there might be an overall decrease in glutamate reuptake in *Cryab* KO mice, suggesting potentially higher glutamate levels in the STR of *Cryab* KO mice, compared to that in WT after JWH-018 exposure, which may have implications in glutamate-mediated long-term potentiation. While glutamate-mediated cannabinoid addiction may entail their regulation of glutamate activation of medium spiny neurons in the NAC (Cohen et al., 2019), other drugs of abuse elicit

glutamate-mediated addiction through long-term potentiation of the glutamatergic synapse (LaLumiere and Kalivas, 2008; Gipson et al., 2014; Griffin et al., 2015) via potential neuroinflammatory mechanisms (Hutchinson et al., 2012; Zhu et al., 2018). Similarly, the expressions of synaptic plasticity markers in *Cryab* KO mice (Figures 6G–J) may suggest the occurrence of neuronal adaptations following JWH-018 exposure, which may have contributed to the development and maintenance of drug-induced addiction (Tsai, 2007; Post and Kalivas, 2013; Zhang et al., 2014; Sutton and Caron, 2015; Ucha et al., 2020; García-García et al., 2021) in *Cryab* KO mice. While it is still uncertain whether these changes were indeed a result of increased glutamate receptor activation, given the lack of genotype variations in GluA1 and GluA2 expressions, although with marked differences among groups (Figures 6E, F), the lack of CRYAB during JWH-018 administration might still ultimately induce positive effects on drug cue reinforcement. Since glutamate receptor activation may reinforce addiction-related behaviors through postsynaptic alterations (Gass and Olive, 2008; Joffe et al., 2014; Nennig and Schank, 2017), the greater striatal synaptic adaptations in drug-exposed *Cryab* KO mice may have induced stronger improved habit-associated learning from neuroinflammation-mediated long-term potentiation. This can be supported by NF- κ B fluorescent signaling in *Cryab* KO mice from JWH-018 SA (Figures 7A, B), implying that upregulated NF- κ B expression might indeed be involved in the manifestation of cannabinoid-induced addiction-like behaviors in *Cryab* KO mice, even through various means of JWH-018 exposure (daily repeated treatment or self-administered). Therefore, functional CRYAB deficiency may have enhanced the occurrence of increased striatal synaptic plasticity via NF- κ B-modulated neuroinflammation following JWH-018 exposure, potentially corroborating *Cryab* in neuroinflammation-mediated cannabinoid addiction.

In conclusion, *Cryab* KO mice exhibit higher cannabinoid-induced addiction than WT mice. Their divergent responses to cannabinoids compared to WT may involve neuroinflammation-mediated synaptic plasticity, due to functional CRYAB deletion (Figure 8). Such behavior might not also involve genotype differences in cannabinoid-induced alterations in the endocannabinoid or dopaminergic systems. Given these findings, future studies are imperative to investigate the behavioral responses of *Cryab* KO mice to other types of cannabinoids to further verify the involvement of *Cryab* in general cannabinoid addiction. Taken together, the *Cryab* KO mice might represent one of the potential mechanisms for enhanced cannabinoid abuse susceptibility and be an efficient tool for screening the abuse potential of novel synthetic cannabinoids, contributing to more reliable and accurate evaluation regarding the potential dangers of these substances in humans.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Guidelines of Sahmyook University.

Author contributions

LS, JC, and HK were responsible for the study design. LS, DO, HL, MK, RC, JY, CL, YL, and HC contributed to data acquisition, analysis, and interpretation of data. LS drafted the manuscript. All authors contributed to and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2023.1135929/full#supplementary-material>

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