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Optogenetic brain-stimulation reward: A new procedure to re-evaluate the rewarding *versus* aversive effects of cannabinoids in dopamine transporter-Cre mice

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Abstract

Despite extensive research, the rewarding effects of cannabinoids are still debated. Here, we used a newly established animal procedure called optogenetic intracranial self-stimulation (ICSS) (oICSS) to re-examine the abuse potential of cannabinoids in mice. A specific adeno-associated viral vector carrying a channelrhodopsin gene was microinjected into the ventral tegmental area (VTA) to express light-sensitive channelrhodopsin in dopamine (DA) neurons of transgenic dopamine transporter (DAT)-Cre mice. Optogenetic stimulation of VTA DA neurons was highly reinforcing and produced a classical "sigmoidal"-shaped stimulation–response curve dependent upon the laser pulse frequency. Systemic administration of cocaine dose-dependently enhanced oICSS and shifted stimulation–response curves upward, in a way similar to previously observed effects of cocaine on electrical ICSS. In contrast, Δ^9-tetrahydrocannabinol (Δ^9-THC), but not cannabidiol, dose-dependently decreased oICSS responding and shifted oICSS curves downward. WIN55,212-2 and ACEA, two synthetic cannabinoids often used in laboratory settings, also produced dose-dependent reductions in oICSS. We then examined several new synthetic cannabinoids, which are used recreationally. XLR-11 produced a cocaine-like increase, AM-2201 produced a Δ^9-THC-like reduction, while 5F-AMB had no effect on oICSS responding.

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AUTHORS CONTRIBUTION

ZXX designed the study. BAH, CJJ, XH, BH, and GHB carried out oICSS and sucrose self-administration experiments. HYZ, HS, and EG carried out the IHC and RNAscope ISH assays. MHB provided the new synthetic cannabinoids (XLR-11, AM-2201, and 5F-AMB). BAH, CJJ, and ZXX wrote the manuscript. MHB revised the manuscript. All co-authors read the manuscript and provided feedback.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest.

Immunohistochemistry and RNAscope *in situ* hybridization assays indicated that CB_1Rs are expressed mainly in VTA GABA and glutamate neurons, while CB_2Rs are expressed mainly in VTA DA neurons. Together, these findings suggest that most cannabinoids are not reward enhancing, but rather reward attenuating or aversive in mice. Activation of CB_1R and/or CB_2R in different populations of neurons in the brain may underlie the observed actions.

Keywords

brain-stimulation reward; cannabinoids; cocaine; dopamine; intracranial self-stimulation; optogenetics; ventral tegmental area; WIN55; 212-2; Δ^9-THC

1 | INTRODUCTION

Cannabis is the second most commonly used psychotropic substance, following alcohol.[1,2] Cannabis is also the most commonly used illicit substance in the United States.[2] There are around 37.6 million people who use cannabis in the United States, and of these, 11.8 million are young adults.[2,3] About 30% of those who use cannabis develop cannabis use disorder, indicating that for many individuals, cannabis can be addictive.[3,4] However, people report both positive and negative experiences following cannabis use. Some individuals report a sense of euphoria, relaxation, or perceptual distortions,[5–7] but not all of those who try cannabis enjoy it. Others experience dysphoria, delusions, anxiety, fear, panic, or depression after cannabis use.[8,9]

Similar paradoxical effects of cannabinoids have been observed in experimental laboratory animals. Intravenous drug self-administration, conditioned place preference (CPP), or aversion (CPA), and electrical intracranial self-stimulation (ICSS) (eICSS) are the most commonly used behavioral procedures to evaluate the rewarding or aversive effects of drugs of abuse. Δ^9-Tetrahydrocannabinol (Δ^9-THC) is reportedly self-administered by squirrel monkeys,[10,11] but not by rhesus monkeys.[12,13] As with rhesus monkeys, it is challenging to train rodents to self-administer Δ^9-THC.[14,15] In the CPP procedure, most reports show aversion to Δ^9-THC when it is on board, although a few reports show CPP.[15–19] In eICSS, acute Δ^9-THC administration is reported to facilitate brain-stimulation reward (BSR),[20,21] inhibit BSR,[22–25] or produce biphasic effects such that low doses facilitate whereas high doses inhibit BSR.[26,27] Thus, it appears that cannabis or cannabinoids can be either rewarding or aversive in animals, depending upon the species, doses employed, or experimental conditions.

Given the essential role of dopamine (DA) in drug reward[28,29] and recent progress in optogenetic techniques,[30,31] we successfully established a new procedure called optogenetic ICSS (oICSS) in transgenic mice to re-evaluate the rewarding or reward-enhancing *versus* aversive or reward-attenuating effects of cannabinoids.[32–34] In this procedure, an adeno-associated viral (AAV) vector carrying a Cre-dependent channelrhodopsin gene is microinjected into the ventral tegmental area (VTA) to express light-sensitive channelrhodopsin 2 (ChR2) protein selectively in DA neurons of transgenic DA transporter (DAT)-Cre mice. In contrast to classical eICSS, which nonspecifically stimulates multiple types of neurons or nerve fibers in the vicinity of an implanted electrode,[27,35,36] oICSS

is cell type-specific and mediated selectively by stimulation of VTA DA neurons[33,34] or glutamate neurons.[32] However, what remains unknown is whether and how cannabinoids alter such DA-dependent oICSS behavior.

In the present study, we examined the effects of multiple cannabinoids on DA-dependent oICSS, including the phytocannabinoids Δ^9-THC and cannabidiol (CBD), the synthetic cannabinoids WIN-55,212-2 and arachidonyl-2′-chloroethylamide (ACEA) used in research studies, and several new synthetic cannabinoids (XLR-11, AM-2201, and 5F-AMB) that are sold in recreational drug markets and used by humans.[37–39] Cocaine was included in the study as a positive reinforcer control, to replicate the validity of this procedure. Given that the majority of the cannabinoid compounds exhibit high binding affinities to CB_1R and CB_2R (Table 1), we used RNAscope *in situ* hybridization (ISH) and immunohistochemistry (IHC) assays to examine cellular distributions of CB_1R and CB_2R in different types of neurons in the VTA, including DA, glutamate, and GABA cells, to elucidate the possible cellular targets underlying cannabinoid action. We found that most of the tested cannabinoids produce reward attenuation or aversive effects on oICSS, likely due to the activation of CB_1R and CB_2R mainly on VTA glutamate and DA neurons.

2 | MATERIALS AND METHODS

2.1 | Animals

Adult male (n = 18) and female (n = 6) heterozygous DAT-Cre mice, aged 4–24 weeks (~30 g in weight), were used in the oICSS experiments, whereas wildtype (WT), GABA-CB1-KO (Vgat-$Cre^{+/-}$ × $CB1^{flox/flox}$) and their WT littermates (Vgat-$Cre^{+/-}$), glutamate-CB1-KO (VgluT2-$Cre^{+/-}$ × $CB1^{flox/flox}$) and their WT littermates (VgluT2-$Cre^{+/-}$) with C57BL/6 genetic background were used in the IHC and RNAscope ISH assays.[32] Heterozygous DAT-Cre (B6.SJL-*Slc6a3*$^{tm1.1(Cre)Bkmn}$/J; stock # 006660), VgluT2-Cre (Slc17a6$^{tm2(cre)\ Lowl}$/J, stock # 016963) and Vgat-Cre (B6J.129S6(FVB)-Slc32a1$^{tm2(cre)\ Lowl}$/MwarJ, stock# 028862-) knock-in mice were purchased from the Jackson Laboratory. $CB1^{flox/flox}$ mice were provided by Dr Qing-Rong Liu at the National Institute on Aging (NIA), as we reported previously.[32] All of the transgenic mice used in this study were bred at the National Institute on Drug Abuse (NIDA) and maintained on a reverse 12 h light–dark cycle (lights off 7:00 a.m./lights on 7:00 p.m.) with food and water available *ad libitum.* The mutant lines were bred for >10 generations on the background of C57BL/6 mice from Charles River Laboratories (Frederick, MD, USA). Mouse genotyping was performed by Transnetyx, Inc. (Cordova, TN) using reverse transcription quantitative polymerase chain reaction (RT-qPCR) on tail snips. All experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the US National Research Council and were approved by the NIDA Animal Care and Use Committee.

2.2 | Surgeries

Mice were anesthetized with ketamine (90 mg/kg, intraperitoneal [ip]) and xylazine (10 mg/kg, ip) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). For intra-VTA microinjection of virus, a custom-made 30-gauge stainless injector was used to infuse Cre-inducible recombinant AAV that encodes ChR2 and enhanced

yellow fluorescent protein (EYFP; AAV- EF1α-DIO-ChR2-EYFP; 150 nl, ~2×10^{12} genomes/ml, University of North Carolina Gene Therapy Center) unilaterally into the VTA (AP −3.28; ML 0.43; DV −4.41 mm relative to Bregma) using a micropump (WPI 2000 Ultra-MicroPump, Sarasota, FL, USA) with a speed of 50 nl/min. For oICSS, a custom-built optrode (200-μm multimode optical fiber, Thorlabs, Newton, NJ, USA) tethered to an intracranial ceramic ferrule (MM-FER2007C-2300, Precision Fiber Products, Inc., Milpitas, CA, USA) was implanted into the VTA approximately 1 mm above the AAV injection site. Figure 2A shows a graphic of the approach. To fix the optrode assembly to the skull, two screws were placed toward the front of the skull followed by a small layer of superglue. While the glue was still wet, dental cement was used to fix and seal the assembly together. Once the AAV vector injection and optrode implantation were finished, mice were given at least 4 weeks to recover from the surgery and to enable full AAV expression and ChR2 trafficking (as shown in Figure 1C,D) before oICSS experiments began.[34]

2.3 | oICSS apparatus

Standard mouse operant conditioning chambers (Med Associates, Fairfax, VT, USA) were used for the oICSS experiments (Figure 1B). Each operant chamber had two wall-mounted levers, two cue lights mounted above the levers, and a house light. Prior to oICSS sessions, each mouse was gently wrapped with a small piece of fabric, and the head mount was connected to a cable linked to an optical swivel (Doric Lenses Inc, Quebec, Canada). The optical swivel was connected to a 473-nm laser (OEM Laser Systems, Inc., Draper, UT, USA) which delivered light pulses in a controlled manner. To control the parameters of the laser stimulation, a customer-programed computer software was used to generate different pulse frequencies.

2.4 | oICSS procedure

The general procedures for oICSS were modified based on those reported previously[32,34] and on eICSS experiments.[27] After recovery from surgery, mice were placed into operant chambers for oICSS training. Animals were initially trained on a fixed-ratio 1 (FR1) reinforcement schedule; each active lever response led to delivery of a 1-s pulse train of light stimulation (473 nm, 20 mW, 25 Hz) accompanied by a 1-s illumination of the cue light above the lever. While inactive lever presses were counted, they had no programmed consequence. Each daily training session lasted 60 min.

2.5 | Rate-frequency oICSS procedure

Following establishment of lever pressing for oICSS, animals were presented with a series of six different stimulation frequencies (100, 50, 25, 10, 5, and 1 Hz) in descending order to obtain rate–frequency response curves (Figures 1E to 2F). Animals were allowed to respond for 10 min per stimulation frequency. We used a within-subjects design, which included three cohorts of mice, each randomly assigned to treatment with the drugs described in Table 2. The ip doses administered and pretreatment times were as follows: cocaine (0, 2, and 10 mg/kg, 5 min prior to testing), Δ^9-THC (0, 1, and 3 mg/kg, 30 min prior to testing), CBD (10 and 20 mg/kg, 30 min prior to testing), WIN55,212-2 (0.3 and 1 mg/kg, 30 min prior to testing), ACEA (1 and 3 mg/kg, 30 min prior to testing), XLR-11 (0.3 and 1.0 mg/kg, 30 min prior to testing), AM-2201 (0.01, 0.03, and 0.1 mg/kg, 30 min prior to testing),

or 5F-AMB (0.01 and 0.03 mg/kg, 30 min prior to testing). We chose to give cocaine 5 min, not 30 min, prior to the ICSS test because cocaine is a fast-acting DAT inhibitor, which produces an increase in extracellular DA in the nucleus accumbens within a minute after systemic cocaine administration.[47] The doses of each compound were chosen based on literature reports or pilot experiments, to ensure that drug treatments did not produce significant sedation or locomotor inhibition. After each test, animals received 2 to 5 days of oICSS re-stabilization until baseline lever responding was re-established. The order of testing for the various doses of the drugs was counterbalanced. Each group of animals received 3–10 drug injections throughout the whole experiment (Table 2).

2.6 | Sucrose self-administration

To determine whether the reduction in oICSS could be due to nonspecific sedation or locomotor impairment caused by cannabinoids, we examined the effects of Δ^9-THC or WIN55,212-2 on operant sucrose self-administration in mice. The procedures for oral sucrose self-administration were the same as we reported previously,[34] except that active lever presses under a FR1 reinforcement schedule led to a delivery of 0.08 ml of 5% sucrose solution into a liquid food tray located on the operant chamber wall. An additional group (n = 12) of mice was used for sucrose self-administration training and testing. Sucrose deliveries were capped at 100 per session to prevent food satiation and a reduction in motivation for sucrose-taking. After training, the mice with stable self-administration behavior were selected for testing with vehicle treatment or one of two doses of Δ^9-THC (1 or 3 mg/kg, ip, 30 min prior to test) or WIN55,212-2 (1 mg/kg, ip, 30 min prior to test). The treatment was counterbalanced in each mouse, and each test was separated by two additional training sessions on two consecutive days. The total number of sucrose deliveries during the 3-h self-administration session was used to evaluate the effects of Δ^1-THC or WIN55,212-2 on sucrose self-administration.

2.7 | IHC assays

We have previously reported that CB_2Rs are expressed in VTA DA neurons,[47] but it is unknown whether CB_2Rs are also expressed in VTA GABA neurons and glutamate neurons. To further address this issue, we used IHC to examine CB_2-immunostaining in these three phenotypes of neurons in mice. The IHC procedures were performed as reported previously.[48] Briefly, mice were deeply anesthetized with 100 mg/kg pentobarbital and transcardially perfused with cold saline followed by 4% paraformaldehyde in 0.1-M phosphate buffer. Brain tissue was transferred to 20% sucrose in phosphate buffer at 4°C overnight. Coronal sections were cut at 25 μm on a cryostat (CM3050S, Leica Microsystems Nussloch GmbH, Nussloch, Germany). Tissue sections containing the VTA were blocked and floated in 5% bovine serum albumin and 0.5% Triton X-100 phosphate buffer for 2 h at room temperature. Dual-labeling IHC was performed using a CB_2R antibody (Alomone, #ACR-002, 1:250) and an anti-tyrosine hydroxylase (anti-TH) monoclonal antibody (1:500; Millipore, Billerica, MA, USA). Sections were washed and incubated with a mixture of secondary antibodies, goat anti-rabbit Alexa 488 for CB_2R, and goat anti-mouse Alexa 568 for TH (Millipore, #MAB318; 1:500), vGluT2 (Abcam, #ab79157, 1:500), or GAD67 (Abcam, #ab26116,1:500) in 5% bovine serum albumin and 0.5% Triton X-100 phosphate buffer for 2 h at room temperature. Sections were washed, mounted, and cover slipped.

Fluorescent images were captured using a fluorescence microscope (Nikon Eclipse 80i) equipped with a digital camera (Nikon Instruments Inc., Melville, NY, USA). All images were captured under identical optical conditions.

2.8 | RNAscope ISH

Using IHC methods, we have previously reported that CB_1R-immunostaining was undetectable in the cell bodies of VTA DA neurons.[32] To further determine the cellular distributions of CB1 receptor within the VTA, in this study, we used a highly sensitive RNAscope ISH assay to examine CB_1R mRNA expression in VTA DA neurons, glutamate neurons, and GABA neurons. Mice were deeply anesthetized, and the whole brain was removed and rapidly frozen on dry ice. Fresh-frozen tissue sections (14 μm thick) were mounted on positively charged microscopic glass slides (Fisher Scientific) and stored at −80°C until RNAscope ISH assays could be performed. Multiple target gene-specific RNAscope probes were designed and provided by Advanced Cell Diagnostics (Newark, CA, USA). The riboprobes were used to observe the cellular distributions of cannabinoid receptor mRNA in VgluT2-expressing glutamate neurons, TH-expressing DA neurons, and GAD1-expressing GABAergic neurons, including the CB_1R RNAscope probe (Cat #: 420721, targeting 530–1,458 bp of the mouse Cnr1 mRNA sequence, NM_007726.3), the VgluT2 RNAscope probe (Cat #: 319171-C3, targeting 1,986–2,998 bp of the *Mus musculus* VgluT2 mRNA sequence, NM_080853.3), the TH-specific RNAscope probe (Cat #: 317621-C2, targeting 483–1,603 bp of the *Mus musculus* TH mRNA sequence, NM_009377.1), and the GAD1-specific RNAscope probe (Cat#: 400951-C3, targeting 62–3,113 bp of NM_008077.4). The RNAscope mRNA-staining steps were performed following the manufacturer's protocols. Stained slides were cover-slipped with fluorescent mounting medium (ProLong Gold Anti-fade Reagent P36930; Life Technologies) and scanned into digital images with an Olympus FluoView FV1000 confocal microscope at 40× or 60× magnification using manufacturer-provided software.

2.9 | Drugs

Cocaine HCl was provided by the NIDA IRP Pharmacy. Δ^9-Tetrahy-drocannabinol (Δ^9-THC) and CBD were provided by NIDA Drug Supply Program. WIN55,212-2 and ACEA (arachidonyl-2′-chloroethylamide) were purchased from Sigma-Aldrich. AM-2201 [1-(5-fluoropentyl)-1H-indol-3-yl](1-naphthyl)methanone, 5F-AMB (methyl 2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3-methylbutanoate), and XLR-11 (5″-fluoro-UR-144 or 5F-UR-144) were provided by Dr Michael H. Baumann at the NIDA IRP. The stock solution of Δ^9-THC was 50 mg/ml (w/v) diluted in 100% ethanol, which was evaporated prior to dilution for injections. The vehicle used to prepare these cannabinoids was 5% Cremophore (C5135, Sigma-Aldrich) in sterile saline.

2.10 | Data analysis

Data are available on request due to privacy/ethical restrictions. All data are presented as mean ± SEM. Data analysis was performed with SigmaPlot 12.0 (Systat Software, Inc. San Jose, CA, USA). Two-way ANOVA with repeated measures for drug dose and stimulation frequency were used to analyze the significance of the effects after each drug treatment. Post-hoc individual group comparisons were made using the Student–Newman–

Keuls method. A value of $p < 0.05$ was chosen as the minimum criterion for statistical significance.

3 | RESULTS

3.1 | Cocaine enhances oICSS

Figure 1 shows the general experimental procedures (Figure 1A,B), representative AAV-ChR2-eYFP expression in the midbrain (Figure 1C) and in DA neurons in the VTA (Figure 1D). Examples of lever response patterns to different frequencies of laser stimulation are shown in Figure 1E, indicating that mice readily responded for optogenetic stimulation of VTA DA neurons in a frequency-dependent manner (Figure 1E,F). We first compared oICSS responses between males and females. We found that male mice showed more robust responses than females (Figure 1F). Two-way ANOVA revealed a significant sex main effect ($F_{1,10} = 14.54$, $p < 0.01$), stimulation frequency main effect ($F_{5,50} = 140.09$, $p < 0.001$), and a sex × frequency interaction ($F_{5,50} = 14.60$, $p < 0.001$). Based on this finding, we mainly used male mice ($n = 18$) in the following pharmacological experiments, only three females showing the same robust oICSS responses as males were included in one group of mice (Table 2).

We have previously reported that cocaine dose-dependently enhances BSR using eICSS.[27,49] Therefore, in the present study, we determined whether cocaine produces a similar effect in oICSS. Consistent with our previous finding with eICSS,[27,49] systemic administration of cocaine produced a significant dose-dependent increase in the number of lever responses for oICSS and upward shifted the stimulation–response curve compared with vehicle (Figure 1G). Two-way ANOVA with repeated measures for cocaine dose and stimulation frequency revealed significant main effects for cocaine treatment ($F_{2,10} = 64.13$, $p < 0.001$) and frequency ($F_{5,25} = 21.2$, $p < 0.001$), and a dose × frequency interaction ($F_{10,50} = 6.7$, $p < 0.001$). Post-hoc testing revealed that 2 and 10 mg/kg cocaine significantly increased active lever responding at the 10-, 25-, and 100-Hz frequencies ($p < 0.05$). The 10 mg/kg cocaine dose also significantly increased oICSS responding at 5 and 50 Hz ($p < 0.001$).

3.2 | Δ^9-THC decreases oICSS

To determine whether Δ^9-THC, the major psychoactive component in cannabis, is rewarding or aversive, we pretreated mice with one of two doses of Δ^9-THC prior to oICSS sessions. Contrary to the effects of cocaine, Δ^9-THC significantly inhibited DA-dependent oICSS responding and dose-dependently shifted the oICSS curve downward (Figure 1H). Two-way ANOVA with repeated measures for Δ^9-THC dose and stimulation frequency revealed a significant main effect of dose ($F_{2,10} = 36.23$, $p < 0.001$), frequency ($F_{5,25} = 112.09$, $p < 0.001$), and a dose × frequency interaction ($F_{10,50} = 7.12$, $p < 0.001$). Post-hoc testing indicated that compared with vehicle treatment, 3 mg/kg Δ^9-THC significantly reduced oICSS responding at the 10-, 25-, 50-, and 100-Hz frequencies ($p < 0.001$).

3.3 | CBD has no effects on oICSS

Next, we examined the impact of CBD, a well-characterized non-psychomimetic component of cannabis with no reported abuse potential,[50] on oICSS responding. We found that

neither 10 nor 20 mg/kg CBD significantly shifted the oICSS curve compared with vehicle treatment (Figure 2A). Two-way ANOVA did not reveal a significant main effect of CBD treatment ($F_{2,8} = 3.75$, $p > 0.05$) or a dose × frequency interaction ($F_{10,40} = 1.075$, $p > 0.05$), suggesting that CBD is neither rewarding/reinforcing nor aversive/reward attenuating.

3.4 | WIN55,212-2 inhibits oICSS

We then tested the effects of two synthetic cannabinoids (WIN55,212-2 and ACEA) that are used as tools in preclinical laboratory research. Consistent with Δ^9-THC's effects, systemic administration of WIN55,212-2 significantly shifted the oICSS curve downward in a dose-dependent manner (Figure 2B). Two-way ANOVA with repeated measures for dose and stimulation frequency revealed a significant WIN55,212-2 treatment main effect ($F_{2,14} = 4.32$, $p < 0.05$), a significant frequency main effect of ($F_{5,40} = 198.05.1$, $p < 0.001$) and a significant treatment × frequency interaction ($F_{10,80} = 6.62$, $p < 0.001$). Post-hoc testing indicated that the 1.0 mg/kg dose significantly reduced responding for the 25-, 50-, and 100-Hz frequencies compared with vehicle ($p < 0.01$).

3.5 | ACEA inhibits oICSS

ACEA is a highly selective CB_1R agonist with >1,400-fold selectivity for CB_1R (K_i = 1.4 nM) over CB_2R ($K_i \geq 2{,}000$ nM).[43] Unexpectedly, systemic administration of ACEA also inhibited oICSS in DAT-Cre mice in a dose-dependent manner (Figure 2C). Two-way ANOVA with repeated measures for dose and frequency revealed significant main effects of ACEA dose ($F_{2,8} = 135.7$, $p < 0.001$), frequency ($F_{5,20} = 70.2$, $p < 0.001$), and a dose × frequency interaction ($F_{10,40} = 40.3$, $p < 0.001$). Post-hoc analysis indicated a significant reduction in oICSS after 3 mg/kg ACEA when the stimulation frequency was 25, 50, or 100 Hz ($p < 0.001$).

3.6 | AM-2201 inhibits oICSS

AM-2201 is a naphthoylindole synthetic cannabinoid that binds with high affinity to both CB_1R (K_i = 1.0 nM) and CB_2R (K_i = 2.6 nM)[51] and produces cannabimimetic effects similar to Δ^9-THC.[52] In the present oICSS assays, systemic administration of AM-2201 also produced a reduction in oICSS and shifted the stimulation–response curve downward (Figure 2D). Two-way ANOVA with repeated measures for dose and frequency revealed significant main effects of AM-2201 treatment ($F_{3,18} = 4.13$, $p < 0.05$), frequency ($F_{5,30} = 78.33$, $p < 0.001$), and a dose × frequency interaction ($F_{15,90} = 5.86$, $p < 0.001$). Post-hoc testing indicated that, compared with vehicle, the 0.1 mg/kg dose of AM-2201 suppressed oICSS responding at the 50- and 100-Hz frequencies ($p < 0.05$).

3.7 | 5F-AMB fails to alter oICSS

5F-AMB is a newer indazole-based synthetic cannabinoid that is abused in the United States and elsewhere.[53] The agonistic activity of 5F-AMB at CB_1R is reportedly 90 times more potent than Δ^9-THC.[37] Compared with other cannabinoids, 5F-AMB appeared very toxic. At the doses of 0.1 mg/kg or above, it produced significant sedation or locomotor impairment. Therefore, a very lower dose range of 5F-AMB was used in this experiment. Systemic administration of 5F-AMB, at the doses of 0.01–0.03 mg/kg, did not significantly alter

oICSS responding compared with vehicle (Figure 2E). Two-way repeated measures ANOVA did not reveal significant main effects of treatment ($F_{2,10} = 0.13$, $p > 0.05$) or a dose × frequency interaction ($F_{10,50} = 0.36$, $p > 0.05$).

3.8 | XLR-11 potentiates oICSS

XLR-11 is another new synthetic cannabinoid with higher affinity for CB_2R ($K_i = 2.1$ nM) over CB_1R ($K_i = 24$ nM).[44] Unexpectedly, pretreatment with XLR-11 produced a modest cocaine-like enhancement of oICSS responding and shifted the oICSS curves upward (Figure 2F). Two-way repeated measures ANOVA revealed a significant dose × frequency interaction ($F_{10,50} = 3.1$, $p < 0.01$). Post-hoc testing revealed that, compared with vehicle, the 1 mg/kg dose of XLR-11 increased oICSS responding for the 25-, 50-, and 100-Hz frequencies ($p < 0.05$).

3.9 | Δ^9-THC or WIN55,212-2 does not alter oral sucrose self-administration

To determine whether the reduction in oICSS was due to non-specific sedation or locomotor inhibition after cannabinoid administration, we evaluated the effects of the same doses of Δ^9-THC and WIN55,212-2 on operant lever responding for sucrose self-administration. We found that Δ^9-THC, at 1 or 3 mg/kg, failed to alter sucrose self-administration as assessed by the total number of sucrose deliveries per session (Figure 3A, $F_{2,10} = 2.34$, $p = 0.124$) or by percentage changes in lever response over baseline (Figure 3B, $F_{2,10} = 2.23$, $p = 0.156$). Similarly, pretreatment with WIN55,212-2, at the dose (1 mg/kg) that inhibits oICSS, also failed to alter sucrose self-administration (Figure 3C, $F_{2,12} = 1.44$, $p = 0.274$; Figure 3D, $F_{2,12} = 1.95$, $p = 0.185$).

3.10 | CB_1R mRNA is found in VTA GABA and glutamate neurons

We have previously reported that CB**1**R-immunostaining is detected mainly in cell membranes or never fibers but not in the cell bodies of VTA DA neurons.[32] To further determine which cell types in the VTA express CB_1R, here we used RNAscope ISH assays to examine the cellular distributions of CB_1R mRNA in the VTA. In contrast to the findings in the IHC assays,[32] CB_1R mRNA (green) was detected the cell bodies of neurons in the VTA (Figure 4). Triple-staining RNAscope assays for labeling CB_1R, TH, and GAD1 mRNA indicate that CB_1R mRNA is not co-localized with TH mRNA (Figure 4A), but co-localized with GAD1 mRNA in VTA GABA neurons (Figure 4A). Selective deletion of CB_1R from GABA neurons in GABA-CB_1-KO mice almost completely abolished CB_1 mRNA-staining (Figure 4B), suggesting that the detected mRNA signal is CB_1-specific. Quantitative assays show that ~60% (57.78 ± 6.26%, from 251 GABA neurons in three mice) GABA neurons in the VTA express CB_1 mRNA.

We also examined CB1 mRNA in Vglut2-positive glutamate neurons in the midbrain, which were found mainly in the medial VTA (close to the midline). We found that CB_1 mRNA is also co-localized with VgluT2 mRNA in VgluT2-Cre mice, but not in glutamate-CB_1-KO mice (Figure 5), a finding similar as we reported previously.[32]

3.11 | CB_2R is found mainly in VTA DA neurons, not in VTA GABA or glutamate neurons

Lastly, we used the IHC assays as we reported previously to examine the cellular distributions of CB_2R in VTA DA neurons, GABA neurons, and glutamate neurons.[47] We found that CB_2R-immunostaining was expressed in ~90% (88.69 ± 2.65%, from 1,401 DA neurons in four mice; Figure 6A) of DA neurons, but not in VgluT2-positive glutamatergic neurons (Figure 6B) or GAD67-labeled GABAergic neurons (Figure 6C).

4 | DISCUSSION

The major findings from this study include (1) multiple cannabinoids (Δ;^{9}-THC, WIN55-212,2, ACEA, and AM-2201) induce suppression of oICSS responding maintained by VTA DA neuron activation, suggesting reward-attenuating or aversive effects; (2) XLR-11 is the only cannabinoid that produced a cocaine-like enhancement of oICSS responding, suggesting possible rewarding or reward-enhancing effects; (3) CBD and 5F-AMB did not significantly impact oICSS, suggesting that these compounds lack rewarding or reinforcing effects at the drug doses tested; and (4) CB_1R and CB_2R exhibit different cellular distributions. CB_1R mRNA is highly expressed in VTA GABA and glutamate neurons, not in DA neurons, while selective deletion of CB1 receptor from GABA neurons or glutamate neurons abolished CB1 mRNA in VTA GABA or glutamate neurons, respectively. In contrast, CB_2Rs are expressed mainly in VTA DA neurons, not in VTA GABA or glutamate neurons. Together, these findings suggest that most cannabinoids are not rewarding or reward enhancing, but aversive or reward attenuating, and activation of CB_1 and CB_2 receptors in multiple types of neurons may underlie cannabinoid action on BSR.

Electrical ICSS (eICSS) is a commonly used behavioral procedure to study brain reward function both in rats[27,35,36,49,54] and mice.[55] In this procedure, animals respond for brief electrical pulses to the medial forebrain bundle via an implanted electrode. Drugs of abuse, such as cocaine and amphetamine, cause a decrease in the stimulation threshold for electrical brain-stimulation reward (BSR) and shift the stimulation–response curve leftward or upward immediately after acute administration, indicating enhanced BSR and a summation between BSR and drug reward.[27,35,36,55–57] Similarly, systemic administration of GBR12935 (a selective DAT inhibitor) or SKF82958 (a DA D_1R-like agonist) also produced a dose-dependent decrease in BSR threshold and a leftward or upward shift of the eICSS curve.[55,58] This effect was potent immediately after acute drug administration. Furthermore, a subthreshold effective dose of SKF-82958 potentiated the rewarding effects of low doses of cocaine. Repeated administration of cocaine or SKF82958 did not cause progressive changes in their ability to decrease BSR thresholds.[55] These findings suggest that cocaine, DAT inhibitors, or D_1R agonists each potentiate the rewarding effects of eICSS and imply that these drugs have rewarding effects of their own. In contrast, withdrawal from chronic cocaine or amphetamine administration is associated with depression-like effects and deficits in brain reward function, as assessed by BSR threshold elevation or a rightward/downward shift of eICSS.[56–59] Based on these findings, if a test drug produces a cocaine-like leftward or upward shift in ICSS curve, we interpret the drug to be rewarding

or reward enhancing. In contrast, if a drug, such as Δ^9-THC, produces an opposite rightward or downward shift in ICSS curve, it is often interpreted as having reward attenuation or aversive effects.[27,32]

We note that we didn't measure oICSS threshold (θ_0) in a way similar as to that used previously in eICSS. However, the lack of θ_0 does not affect the conclusions based on the ICSS curve shift assays in this study because both θ_0 and ICSS curve shift describe the same drug effects. In addition to θ_0, many other measures such as M25, M50, and M75 are also used in eICSS assays. We did not apply θ_0 analysis in this assay because of a technical reason. In eICSS, we used 16 different electrical pulse frequencies ranging from 141 to 25 Hz to generate a stimulation–response curve, which allowed us to accurately calculate θ_0 using best-fit mathematical algorithms as reported previously. [27] In the present oICSS study, we can only generate six different laser pulse frequencies ranging from 1 to 100 Hz using the currently available laser stimulators to establish a stimulation–response curve. Thus, more efforts are needed to optimize the oICSS procedure, particularly, by increasing the range of laser stimulation frequencies.

As stated above, the effects of cannabinoids on eICSS behavior are mixed. In some studies, Δ^9-THC produced enhanced BSR,[20,21] while in others, Δ^9-THC and other cannabinoid receptor agonists reduced electrical BSR,[26,60,61] or had no effect.[24] In a number of investigations which examined dose–response relationships, cannabinoids produced biphasic effects such that lower doses were rewarding, while higher doses were aversive.[27] The reasons underlying these disparate findings are unclear. One possible explanation is that electrical pulses into the brain may nonspecifically stimulate multiple types of neurons or nerve fibers and therefore complicate data interpretations. To overcome this limitation, we recently established a new animal procedure called oICSS to re-evaluate cannabinoid-induced rewarding *versus* aversive effects. In this procedure, animals press a lever to earn laser pulses that selectively stimulate VTA DA neurons[33,34] or glutamate neurons[32] via an optrode implanted. Consistent with findings using eICSS,[27,36,49] we found that cocaine produced a significant increase in oICSS responding and shifted the stimulation–response curve leftward or upward. It is also in accordance with our previous finding that cocaine enhances oICSS maintained by photostimulation of VTA glutamate neurons.[32] In contrast to cocaine, opioids such as oxycodone produce dose-dependent biphasic effects — low doses enhance and high doses reduce oICSS in DAT-Cre mice.[34] These findings suggest that this new oICSS procedure is reliable in its ability to predict the rewarding *versus* aversive effects and abuse potential of psychotropic drugs as we discussed above in detail.

Compared with eICSS, the oICSS procedure has several advantages. First, the neurobiological basis of the behavior is clear, that is, it is DA neuron-specific or glutamate neuron-specific, depending upon the transfected cell type.[32–34] Second, laser stimulation is safer than electrical stimulation for *in vivo* experiments. Little evidence indicates that laser stimulation (10–20 mW) of ChR2-expressing neurons leads to cell death.[30] Third, oICSS responding that is driven by optogenetic stimulation of VTA DA neurons is more robust and stable over time than eICSS. Mice quickly learn to lever press for oICSS, and once they have acquired the behavior, responding may last up to 5 months, whereas eICSS behavior in rats usually lasts 1–2 months, based on our many years of experience.[27,32–34,49,68] Lastly, oICSS

appears to be more sensitive than eICSS in detecting subtle changes in BSR and enables the testing of multiple drugs in the same subjects (with appropriate washout periods), with the added benefit of reducing animal numbers. Therefore, oICSS could be especially suitable for screening a large number of compounds for abuse potential.

Using this oICSS procedure, we found, unexpectedly, that males responded more robustly than females, suggesting that males may be more sensitive to laser stimulation of VTA DA neurons. The mechanisms underlying such a sex difference are not fully understood. It is likely that midbrain DA neurons in females are less sensitive to laser stimulation. It is well documented that female rats acquired cocaine (heroin or nicotine) self-administration more rapidly than males and consumed significantly greater amounts of cocaine, heroin, or nicotine than did males under the same experimental conditions.[62–64] The neural mechanisms underlying sex differences in drug intake are not fully understood. A prevailing hypothesis is that estrogen effects on the mesolimbic DA systems may underlie such sex differences in drug-taking behavior.[64–66] Accordingly, such an estrogen-related mechanism may also explain why females respond less for VTA DA neuron stimulation. Whatever the mechanisms, the present finding suggests that increased drug intake observed in females might be a compensatory response to blunted DA neuron responses to drugs of abuse.

We used this new oICSS to re-evaluate the rewarding *versus* aversive effects of various cannabinoids in DAT-Cre mice. The most commonly used cannabinoids, such as Δ^9-THC and WIN55,212-2, as well as synthetic cannabinoids such as AM-2201 and ACEA, all produced significant and dose-dependent reductions in oICSS maintained by stimulation of VTA DA neurons. This finding is consistent with our recent report that Δ^9-THC also dose-dependently inhibits oICSS maintained by stimulation of VTA glutamate neurons in VgluT2-Cre mice.[32] Similarly, beta-caryophyllene (BCP), a dietary terpenoid with CB_2R agonist profile,[67] inhibits oICSS maintained by optical stimulation of VTA DA neurons.[68] The reduction in oICSS is unlikely due to non-specific locomotor impairment because the same drug doses did not produce sedation or locomotor inhibition based on pilot observations and previous reports.[27] In addition, Δ^9-THC or WIN55,212-2, at doses equal to or higher than those affecting oICSS, failed to alter lever responding for sucrose reward. Together, these findings suggest that the most commonly used cannabinoids (Δ^9-THC and WIN55,212-2) and the synthetic cannabinoids ACEA and AM-2201 are not rewarding, but reward attenuating or aversive in experimental animals.

In contrast to the above findings, XLR-11 is the only cannabinoid tested herein that caused cocaine-like reward-enhancing effects in the oICSS procedure. While the effects of XLR-11 on oICSS were modest, they suggest that this new synthetic cannabinoid may have higher abuse potential than other cannabinoid compounds. XLR-11 was first identified as a constituent in herbal smoking mixtures that were sold under a variety of brand names.[69] Previous studies show that XLR-11 displays similar or greater potency than Δ^9-THC in rats and mice.[38,44] *In vivo*, XLR-11 also produces Δ^9-THC-like effects in rodents that were attenuated by rimonabant, a CB_1R antagonist.[44] The available epidemiological evidence also suggests that XLR-11 displays abuse liability in humans.[69] As such, XLR-11 has been banned in many countries and has been listed as a controlled substance (Schedule I) in the United States since 2013.

Notably, CBD and 5F-AMB did not show significant effects on oICSS responding. These findings support the increasingly accepted view that CBD is neither rewarding/reward enhancing nor aversive or reward attenuating in both humans and experimental animals.[41,70] 5F-AMB is a designer CB_1R agonist sold recently for recreational use in humans.[71] 5F-AMB has a much higher potency for CB_1R (K_i = 8.71 ± 0.04 nM) and CB_2R (7.99 ± 0.13 nM) than Δ^9-THC.[37,45] In humans, inhalation of 5F-AMB causes adverse effects such as impaired memory, loss of consciousness, and catalepsy with muscle rigidity.[72] In the present study, we found that 5F-AMB is neither reward enhancing nor reward attenuating at the doses of 0.01–0.03 mg/kg, though higher doses could not be tested because they caused significant locomotor impairment.

The neural mechanisms through which cannabinoids enhance or inhibit DA-dependent oICSS are not fully understood. There are several possible explanations. First, the rewarding *versus* aversive effects of cannabinoids may depend on drug doses, such that low doses are rewarding while high doses are aversive, as sometimes reported for eICSS.[27] However, the present findings do not support this hypothesis since we did not observe such biphasic effects on oICSS for any compound tested herein. A second possibility is that the reward-enhancing *versus* reward-attenuating effects of cannabinoids may be related to their binding affinities or efficacies at CB_1R and CB_2R. This possibility seems unlikely, because there appears to be no correlation between the *in vivo* effects in oICSS and *in vitro* binding and efficacy data (Table 1). For example, Δ^9-THC is a partial agonist at CB_1R and CB_2R with K_i values of 35.3–39.5 nM for rat CB_1R and 3.9–40 nM for rat CB_2R,[40,73] while WIN55,212-2 is a full agonist at CB_1R (K_i = 9.94 nM) and CB_2R (16.2 nM).[40] Yet, Δ^9-THC appears to be more effective than WIN55,212-2 in attenuating oICSS. Similarly, AM-2201 is a potent full agonist at CB_1R and CB_2R (with K_i = 1.0 nM at CB_1R and K_i = 2.6 nM at CB_2R),[46,74] while ACEA is a potent and selective CB_1R agonist (with K_i = 1.4 nM for CB_1R and $K_i \geq$ 2,000 nM for CB_2R; see Table 1). AM-2201 is not more potent than ACEA in suppressing oICSS responding.

A third possible explanation is that the rewarding or reward-enhancing *versus* aversive or reward-attenuating effects of cannabinoids could depend on cannabinoid actions at multiple neuronal subtypes or in different neural circuits. We have previously reported that CB_1Rs expressed in glutamate neurons are involved in Δ^9-THC-induced aversion,[32] and CB_2Rs are mainly expressed in VTA DA neurons.[48,75] In the present study, we did not detect obvious CB_1 mRNA expression in VTA DA neurons but detected significant CB_1 mRNA expression in ~60% VTA GABA, while CB_2Rs are detected in ~90% VTA DA neurons, but not in VTA GABA or glutamate neurons. Given that VTA DA neurons receive both excitatory glutamatergic and inhibitory GABAergic inputs,[34,41] we hypothesized that cannabinoid binding to CB_1R and CB_2R on midbrain DA, glutamate, and GABA neurons may together underlie cannabinoid action observed in the present study (Figure 7). More specifically, CB_1R activation on VTA GABA neurons could mediate cannabinoid reward via GABA-mediated DA neuronal disinhibition.[5,6] By contrast, activation of CB_1R on VTA glutamate neurons and CB_2R on VTA DA neurons could produce reward attenuation (or aversion) by decreased glutamatergic inputs to DA neurons and inhibition of DA neurons, respectively.[32,41,48,76] Thus, the unique behavioral effects produced by each cannabinoid may depend on the balance of both opposite actions mediated by distinct cell types (Figure

7). The discovery of CB_1R expression in glutamate neurons and CB_2R expression in VTA DA neurons may help explain why most of the cannabinoids we tested are not rewarding, but aversive. Namely, activation of both receptors in DA and glutamate neurons should theoretically inhibit VTA DA neurons and therefore suppress DA-mediated BSR. We note that this hypothesis may not explain well why XLR-11 was oICSS enhancing, because XLR-11 displays higher affinity to CB2Rs. One possible explanation is that XLR-11 may have other off-targets that regulate the mesolimbic DA system.

In conclusion, we used a new animal procedure of oICSS to systematically re-evaluate the rewarding *versus* aversive effects of multiple phytocannabinoids and synthetic cannabinoids on responding maintained by stimulation of VTA DA neurons. We found that most cannabinoids (including Δ^9-THC, WIN55212-2, AM-2201, and ACEA) are not reward enhancing, but aversive or reward attenuating in experimental animals. Although the receptor mechanisms underlying this effect require further study, it is likely that cannabinoid reward *versus* aversion is mediated by combined actions at CB_1R and CB_2R in distinct neuronal populations with different phenotypes, including DA, glutamate, and GABA neurons in the VTA. Importantly, oICSS reliably predicts the rewarding effects of cocaine. As such, the oICSS procedure may be a valuable tool for screening novel compounds for their abuse potential, to address the current surge in new synthetic psychostimulants, opioids, and cannabinoids in recreational drug markets worldwide.

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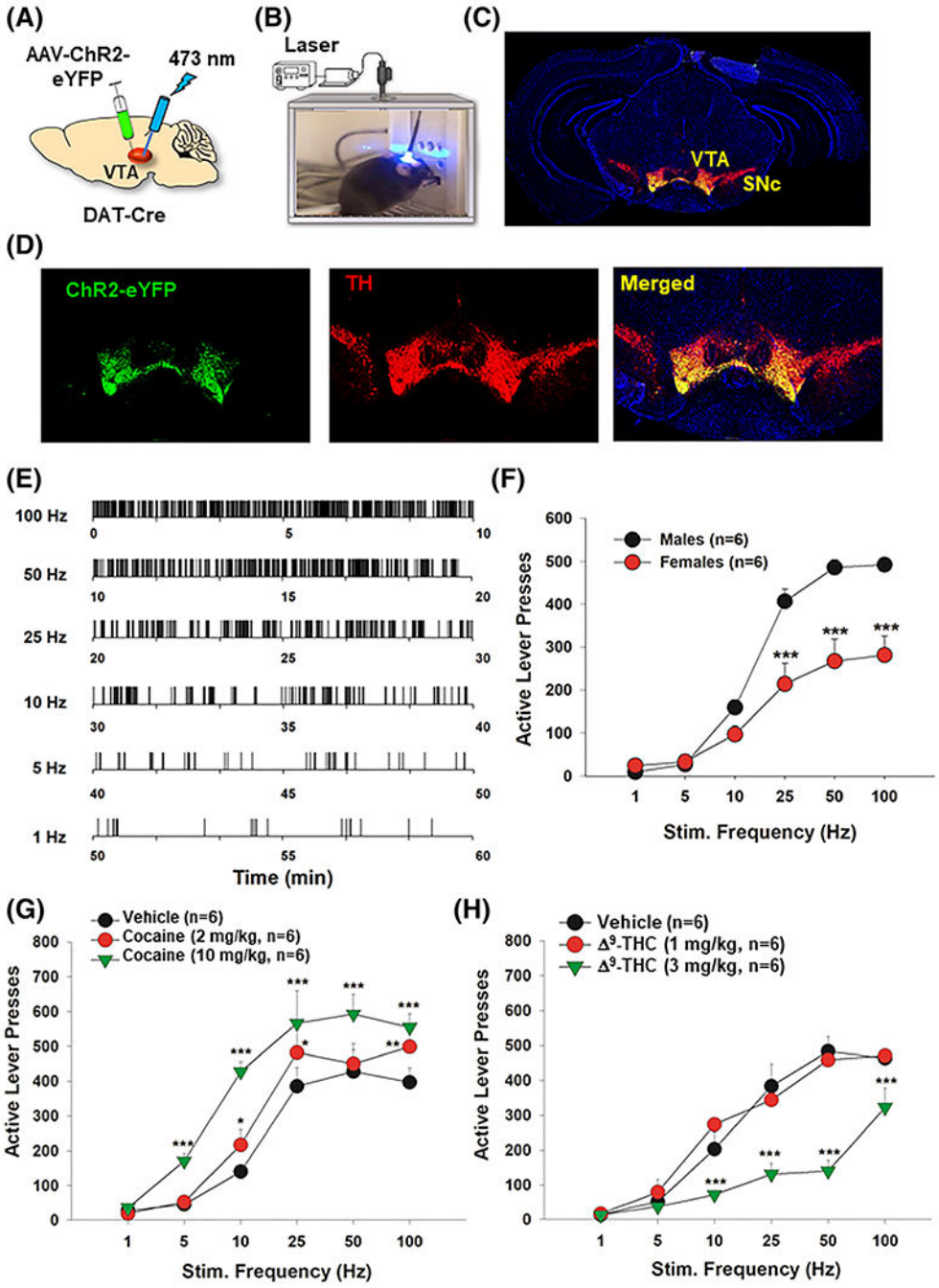

FIGURE 1.

Optogenetic intracranial self-stimulation (oICSS) experiment and the effects of cocaine and Δ^9-tetrahydrocannabinol (Δ^9-THC) on oICSS in DAT-Cre mice. A, A schematic diagram of the AAV-ChR2-eYFP microinjection and intracranial optical fiber implantation within the ventral tegmental area (VTA) in dopamine (DA) transporter (DAT)-Cre mice. B, Image of the setup of the oICSS experiment. C, Immunostaining of whole brain slice indicating the placement of the AAV-ChR2-EYFP expression in the VTA. D, 20× magnification of the VTA showing ChR2-EYFP expression in VTA TH-positive DA neurons. E, Representative

lever responding to different frequencies of laser stimulation in a single session from a single mouse. F, Graph of the lever responding over different frequencies of laser stimulation illustrating the stimulation–response curve in male and female mice. G, Cocaine (10 mg/kg, intraperitoneal [ip]) dose-dependently shifted the oICSS curve upward when compared with vehicle control. H, Δ^9-THC dose-dependently shifted the oICSS curve downward. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the vehicle control group

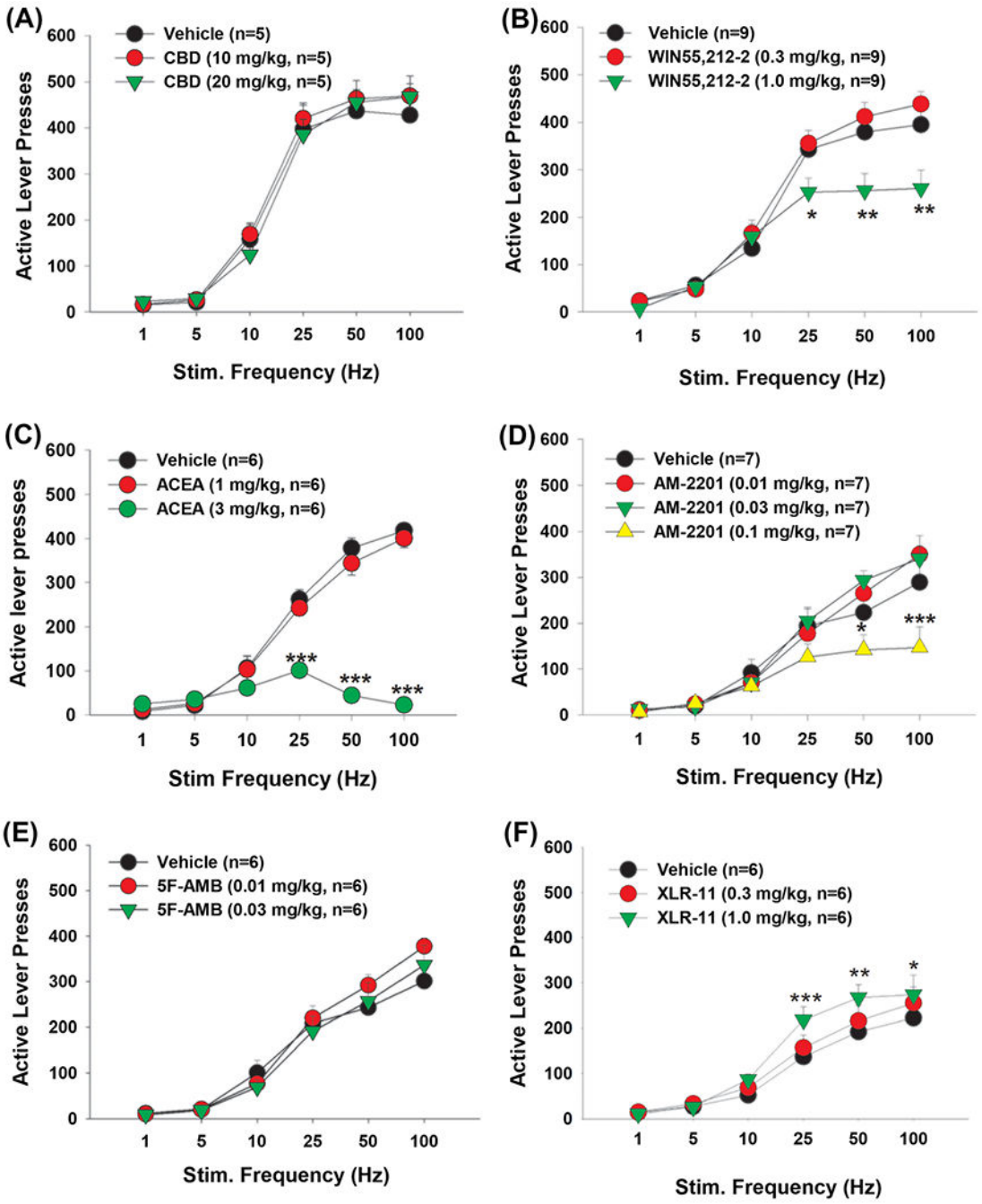

FIGURE 2.

Effects of cannabinoids on optogenetic intracranial self-stimulation (oICSS) in dopamine transporter (DAT)-Cre mice. A, Cannabidiol (CBD) did not show significant effects on oICSS responding. B–D, WIN55,212-2, ACEA, and AM-2201 (respectively) dose-dependently shifted the oICSS curve downward. E, 5F-AMB did not show any significant effects at the current doses. F, XLR-11, at 1.0 mg/kg, significantly shifted the oICSS curve upward. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the vehicle control group

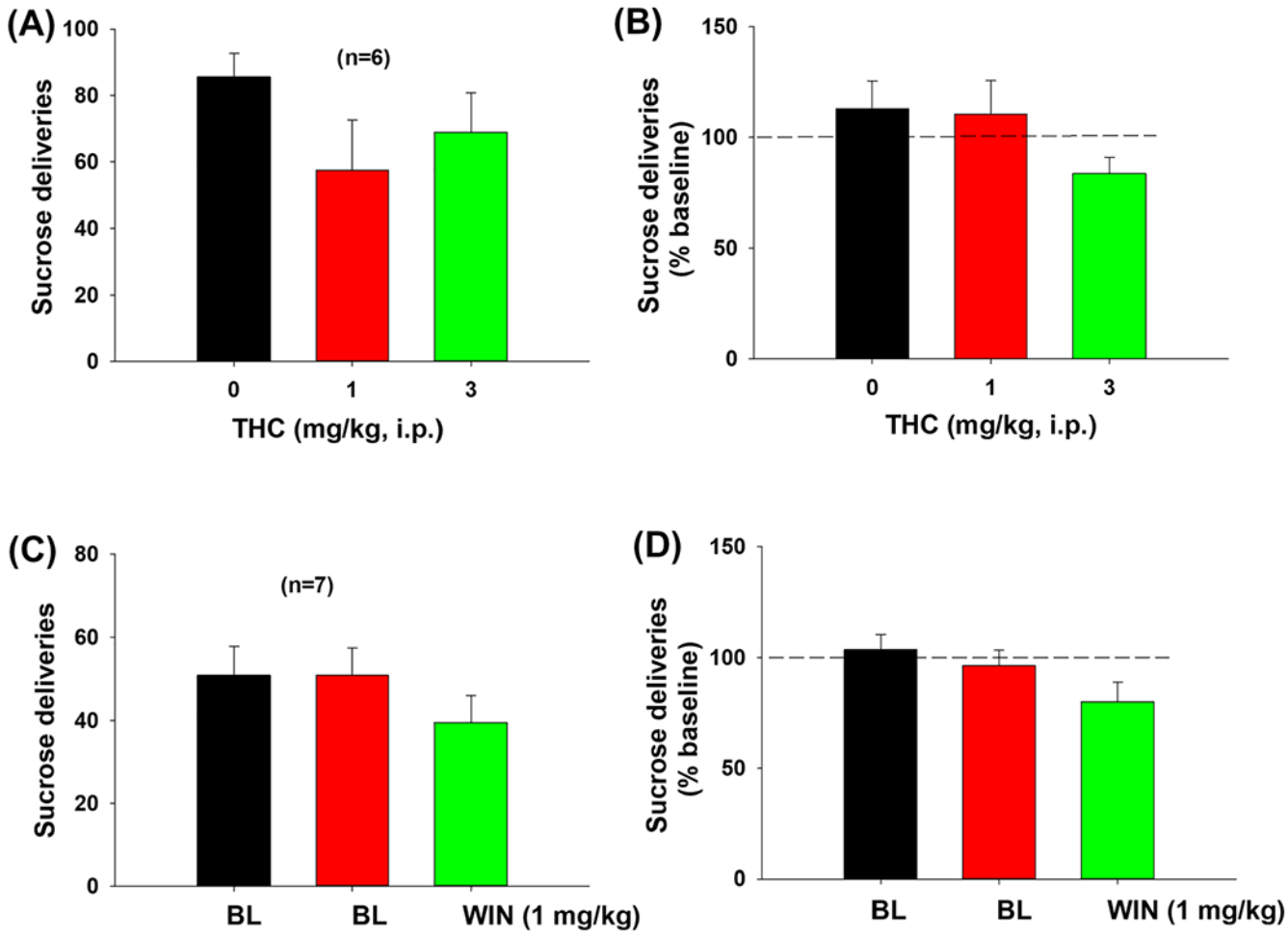

FIGURE 3.

Impact of Δ^9-tetrahydrocannabinol (Δ^9-THC) and WIN55,212-2 on oral sucrose self-administration in mice. Pretreatment with Δ^9-THC A,B, or WIN55,212-2 C,D, did not alter oral sucrose self-administration

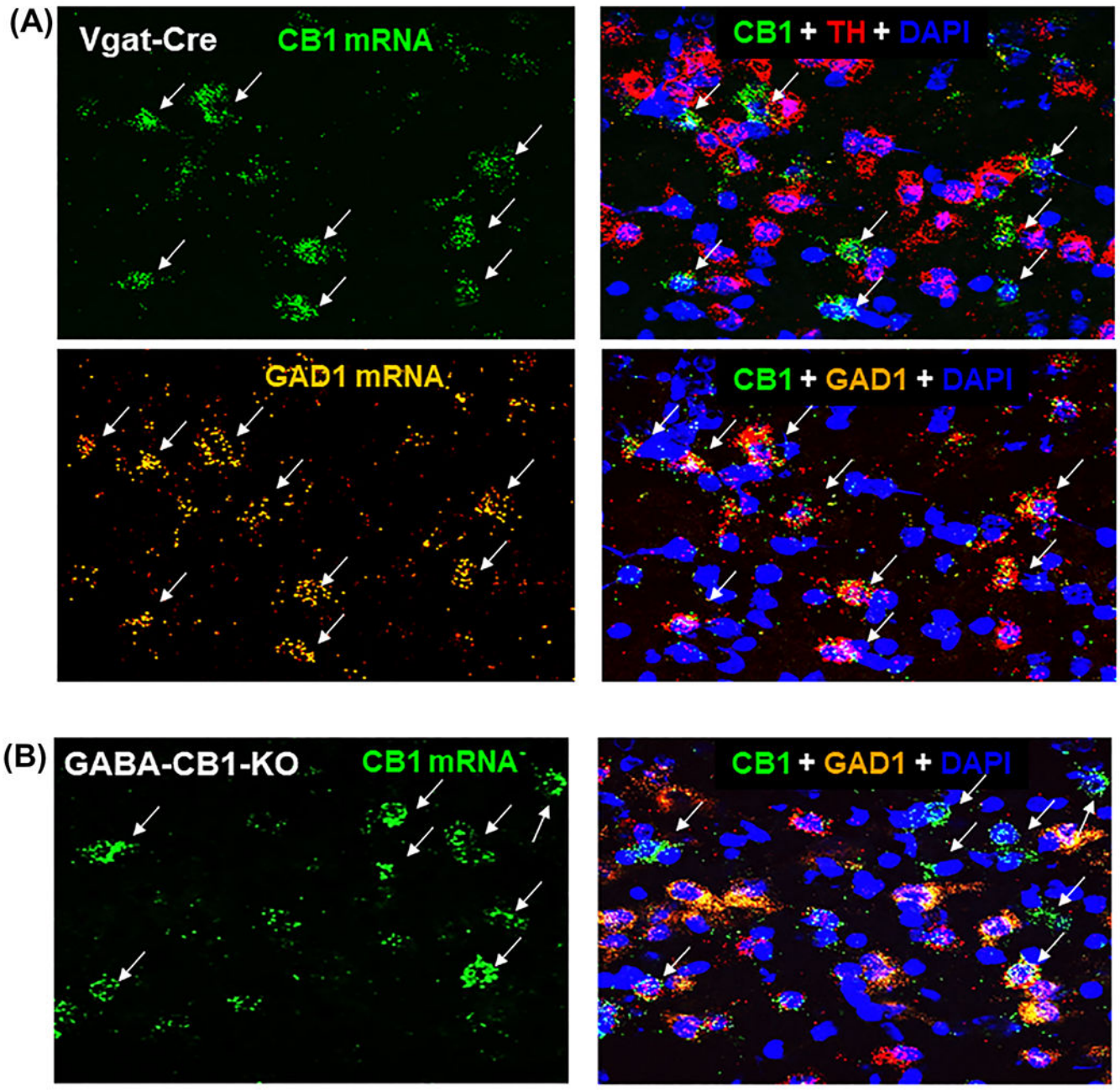

FIGURE 4.

The cellular distributions of CB_1 mRNA in the ventral tegmental area (VTA) by RNAscope *in situ* hybridization (ISH) assays. Triple-staining for CB1, TH, and GAD1 mRNA indicates that high densities of CB_1R mRNA (green, arrows) were not co-localized with TH mRNA in VTA DA neurons (red), but co-localized with GAD_1 mRNA in GABA neuron (orange, arrows) in Vgat-Cre mice A. Selective deletion of CB_1 receptors from GABA neurons abolished CB1 mRNA-staining in the VTA of GABA-CB_1-KO mice B. TH, Tyrosine hydroxylase; GAD1, glutamic acid decarboxylase 1; DAPI, 4′,6-diamidino-2-phenylindole, a fluorescent dye that binds to DNA as a marker of cell nuclei

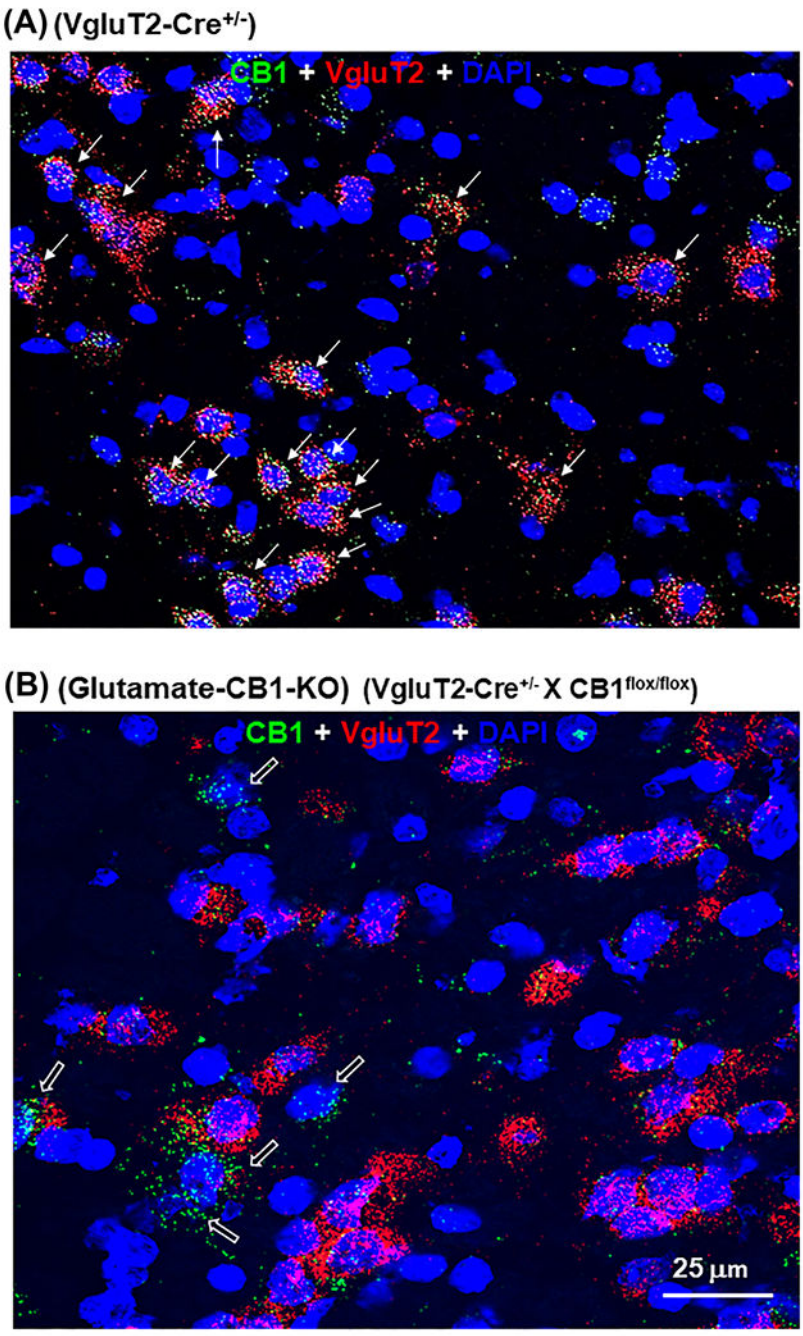

FIGURE 5.

CB1 mRNA expression in glutamate neurons in the ventral tegmental area (VTA) by RNAscope *in situ* hybridization (ISH) assays. CB_1R mRNA (green, arrows) was co-localized with VgluT2 mRNA (red) in glutamate neurons (red, arrows) in VgluT2-Cre mice A, but not in glutamate-CB1-KO mice B. CB1 mRNA was still detectable in other non-glutamate (VgluT2-negative, open arrows) neurons in the VTA in glutamate-CB1-KO mice B

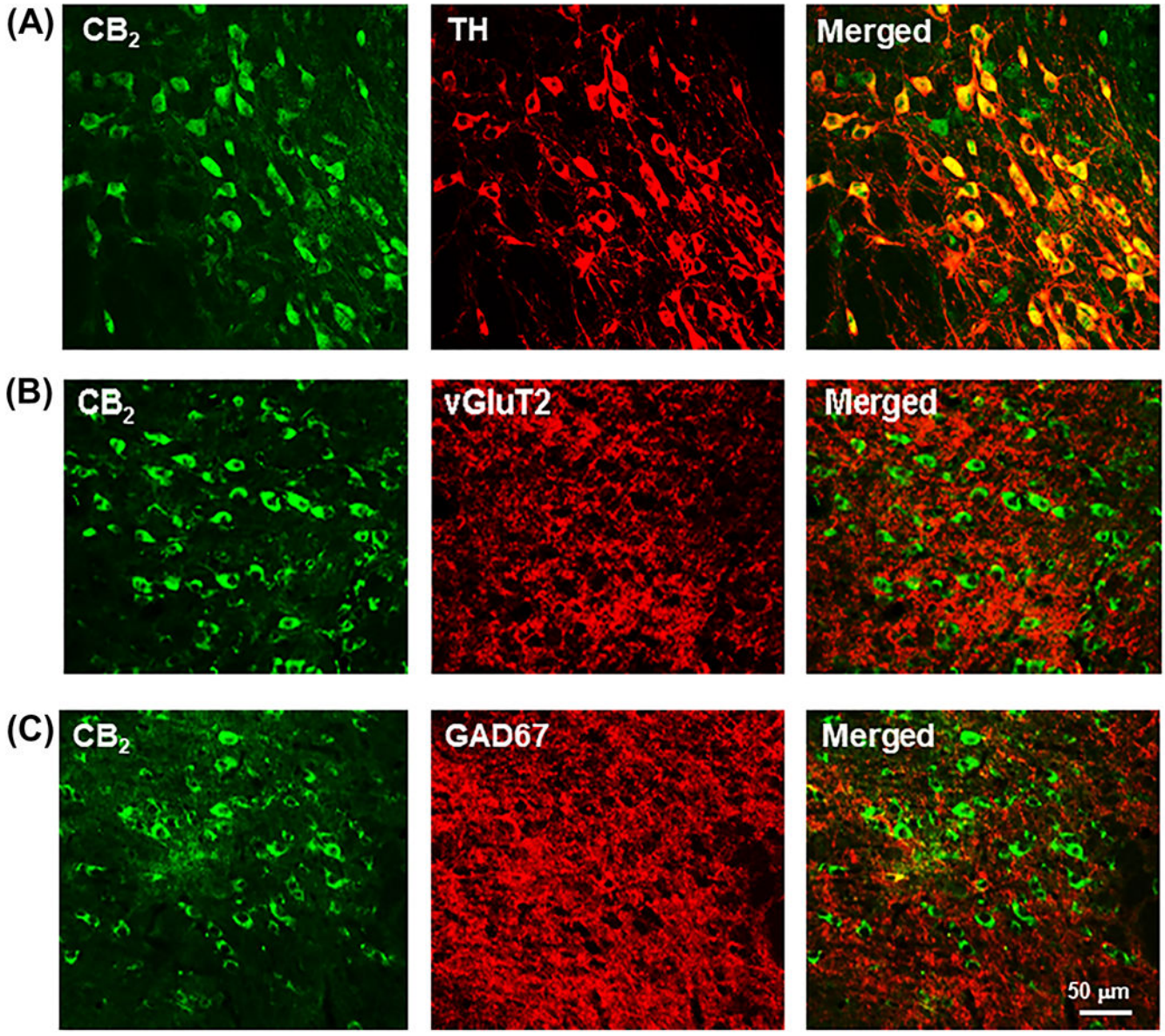

FIGURE 6.

CB_2R-immunostaining in different phenotypes of neurons in the ventral tegmental area (VTA), illustrating CB_2R-immunostaining in VTA TH-positive DA neurons A, but not in VTA vGluT2-positive glutamate neurons B or GAD67-positive GABA neurons C. BL, baseline responding in the absence of drug treatment

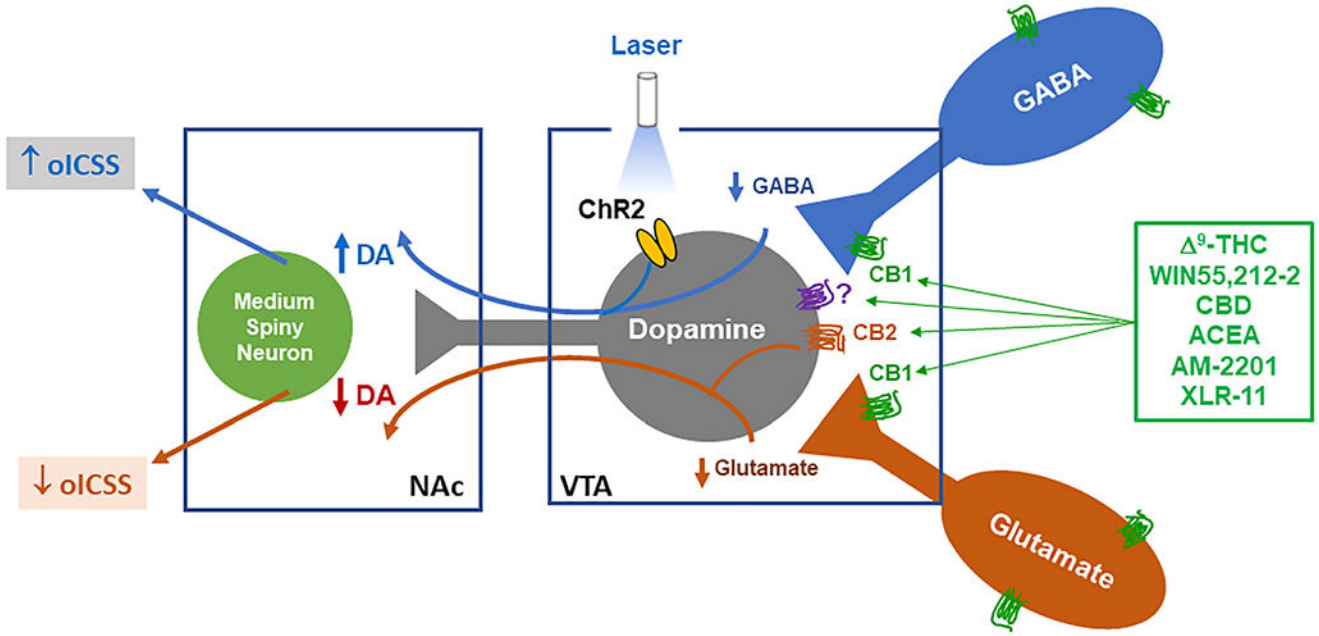

FIGURE 7.

Diagram summarizing CB_1R and CB_2R expression in VTA dopaminergic (DA), glutamatergic, and GABAergic neurons. Cannabinoids may bind to CB_1R in GABAergic neurons, producing rewarding or reward-enhancing effects. Conversely, cannabinoids may also bind to CB_1R on VTA glutamate neurons or glutamatergic afferents, or to CB_2R on DA neurons, producing aversive or reward-attenuating effects. The final subjective effect depends on the balance of both opposite actions. NAc, nucleus accumbens; oICSS, optogenetic intracranial self-stimulation; VTA, ventral tegmental area

TABLE 1

Cannabinoid receptor binding affinities of cannabinoids tested in this study

Compound	CB_1R K_i (nM)	CB_2R K_i (nM)	CB_1R/CB_2R	Reference	Half life ($T_{1/2}$)	oICSS effect[a]
Δ^9-THC	35.3	3.9	9.05	40	~92–108 min[67]	↓
	39.5	40	0.99	41		
Cannabidiol	4,350	2,860	1.52	42	~120 min [68]	No change
ACEA	1.4	>2,000	>0.0007	43	N/A	↓
WIN55,212-2	9.94	16.2	0.613	40	24-36 h [69]	↓
XLR-11	24	2.1	11.4	44	N/A	↑
5F-AMB	8.71	7.99	1.09	45	N/A	No change
AM-2201	1.0	2.6	0.38	46	4-6 h [70]	↓

Abbreviations: N/A, not available; oICSS, optogenetic intracranial self-stimulation.

[a]The findings in the present study.

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TABLE 2

Experimental groups and the drug treatments in each group of mice

Group #	Treatment	Drug dose (mg/kg)
1 (*n* = 6)	Cocaine (*n* = 6, males)	0, 2, 10
	Δ^9-THC (*n* = 6, males)	0.3, 1
	ACEA (*n* = 6, males)	1, 3
2 (*n* = 9)	WIN55,212-2 (*n* = 9, 6 males + 3 females)	0, 0.3, 1
	CBD (*n* = 5, 3 males + 2 females)	10, 20
	AM-2201 (*n* = 7, 5 males + 2 females)	0.01, 0.03, 0.1
	5F-AMB (*n* = 6, 4 males + 2 females)	0.01, 0.03
3 (*n* = 6)	XLR-11 (*n* = 6, males)	0, 0.3, 1

Abbreviations: CBD, cannabidiol; Δ^9-THC, Δ^9-tetrahydrocannabinol.