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Acta Pharmacologica Sinica

ARTICLE

# Repeated cocaine administration upregulates $CB_2$ receptor expression in striatal medium-spiny neurons that express dopamine $D_1$ receptors in mice

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Cannabinoid $CB_2$ receptors ($CB_2R$) are importantly involved in drug reward and addiction. However, the cellular mechanisms underlying $CB_2R$ action remain unclear. We have previously reported that cocaine self-administration upregulates $CB_2R$ expression in midbrain dopamine (DA) neurons. In the present study, we investigated whether cocaine or heroin also alters $CB_2R$ expression in striatal medium-spiny neurons that express dopamine $D_1$ or $D_2$ receptors ($D_1$-MSNs, $D_2$-MSNs) and microglia. Due to the concern of $CB_2R$ antibody specificity, we developed three mouse $CB_2$-specific probes to detect $CB_2R$ mRNA using quantitative RT-PCR and RNAscope in situ hybridization (ISH) assays. We found that a single injection of cocaine failed to alter, while repeated cocaine injections or self-administration dose-dependently upregulated $CB_2R$ gene expression in both brain (cortex and striatum) and periphery (spleen). In contrast, repeated administration of heroin produced a dose-dependent reduction in striatal $CB_2$ mRNA expression. RNAscope ISH assays detected $CB_2R$ mRNA in striatal $D_1$- and $D_2$-MSNs, not in microglia. We then used transgenic $CX3CR1^{eGFP/+}$ microglia reporter mice and $D_1$- or $D_2$-Cre-RiboTag mice to purify striatal microglia or ribosome-associated mRNAs from $CX3CR1^{eGFP/+}$, $D_1$-MSNs, or $D_2$-MSNs, respectively. We found that $CB_2R$ upregulation occurred mainly in $D_1$-MSNs, not in $D_2$-MSNs or microglia, in the nucleus accumbens rather than the dorsal striatum. These findings indicate that repeated cocaine exposure may upregulate $CB_2R$ expression in both brain and spleen, with regional and cell type-specific profiles. In the striatum, $CB_2R$ upregulation occurs mainly in $D_1$-MSNs in the nucleus accumbens. Given the important role of $D_1$-MSNs in brain reward function, the present findings provide new insight into mechanisms by which brain $CB_2Rs$ modulate cocaine action.

**Keywords:** cocaine; cannabinoid; $CB_2$ receptor; microglia; self-administration; $D_1$-MSNs

*Acta Pharmacologica Sinica* (2022) 43:876–888; https://doi.org/10.1038/s41401-021-00712-6

## INTRODUCTION

The endocannabinoid system (ECS) is importantly involved in the development of dependence to many drugs of abuse, such as cannabis, cocaine, nicotine, opiates, and alcohol [1–5]. The ECS consists of cannabinoid receptors, endogenous ligands, and the enzymes involved in the synthesis and degradation of endocannabinoids. Two major subtypes of cannabinoid receptors, $CB_1$ receptor ($CB_1R$) and $CB_2$ receptor ($CB_2R$), have been cloned and characterized [6]. Because $CB_1Rs$ are highly expressed in the brain, while $CB_2Rs$ were initially found in peripheral immune cells, it has been generally believed that the psychoactive effects of cannabis are mediated by activation of brain $CB_1Rs$ instead of $CB_2Rs$ [7].

However, $CB_2Rs$ are found in the brains of healthy subjects, albeit at very low levels compared to brain $CB_1Rs$ [7–12]. Anatomically, $CB_2Rs$ are found in activated microglia during neuroinflammation and in many other CNS disorders [13] as well as in subpopulations of neurons in the brain of normal healthy subjects [14–17]. Functionally, brain $CB_2Rs$ have been shown to be involved in multiple dopamine (DA)-related CNS disorders, such as Parkinson's disease [18, 19], schizophrenia [20], anxiety [21], depression [22], Huntington's disease [23], and substance use disorders [8, 24]. In light of these findings, it has been proposed that brain $CB_2R$ expression is dynamic and can be upregulated in response to various insults as stated above.

In addition, we have recently reported that cocaine self-administration upregulates $CB_2R$ expression in midbrain DA neurons [11], indicating that inducible expression of $CB_2Rs$ is not restricted to microglia within the CNS. This $CB_2R$ upregulation may in part explain the antagonism of $CB_2R$ agonists against cocaine self-administration [9–11, 25–27], and of cocaine- or nicotine-induced hyperactivity and place preference [8, 24, 28–30], as activation of $CB_2Rs$ inhibits midbrain DA neurons and DA release in the nucleus accumbens (NAc) [26, 31]. These findings indicate that dynamic changes in $CB_2R$ expression outside microglia can critically shape behavior. Similarly, $CB_2R$ upregulation has also been reported in the NAc (shell) in rats after cocaine self-

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Received: 31 January 2021 Accepted: 2 June 2021
Published online: 27 July 2021



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administration, followed by a reduction in $CB_2R$ expression in the prefrontal cortex (PFC) and NAc (core) after extinction from cocaine self-administration [32]. However, little is known about the cellular distributions of $CB_2Rs$ in the striatum and which type(s) of cells displaying $CB_2R$ upregulation after cocaine administration.

In the present study, we used multiple transgenic approaches to address these questions. We first used two custom-designed mouse $CB_2$ mRNA-specific probes to examine whether acute or chronic cocaine or heroin administration alters $CB_2$ mRNA expression in the PFC, striatum, and spleen by quantitative real-time polymerase chain reaction (qRT-PCR). We then used RNAscope in situ hybridization to examine the cellular distributions of $CB_2R$ mRNA in the ventral striatum (also called nucleus accumbens, NAc). Lastly, we used fluorescence-activated cell sorting (FACS) and transgenic CX3CR1$^{eGFP/+}$ microglia reporter mice to isolate striatal microglia and $D_1$- or $D_2$-Cre-RiboTag mice to isolate ribosome-associated mRNAs from $D_1$ or $D_2$ receptor-expressing medium-spiny neurons ($D_1$- or $D_2$-MSNs) to examine $CB_2$ mRNA expression in these specific cell populations. These data provide the first analysis of cell type-specific changes in $CB_2Rs$ in the striatum following cocaine or heroin exposure and reveal $D_1$-MSNs as a key cell population in which dynamic changes in $CB_2R$ expression may be relevant for regulation of circuit function and behavior following drug exposure.

## MATERIALS AND METHODS

### Animals

Male wild-type (WT) and $CB_2R$-knockout ($CB_2^{-/-}$) mice with C57BL/6J genetic backgrounds were used and bred at the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP). This strain of $CB_2^{-/-}$ mice is C-terminal knocked out —only the last 341 base pairs on exon 3 are deleted that encode part of intracellular and extracellular third loops, transmembrane regions 6 and 7, and intracellular C-terminus region [33, 34]. This strain of $CB_2^{-/-}$ mice was used as controls to determine the specificity of detected $CB_2$ mRNA signals. Genotyping was performed in our laboratory according to protocol from Charles River Laboratories International Inc. (Wilmington, MA, USA). CX3CR1$^{eGFP/+}$ breeders were originally obtained from Jackson labs (Stock # 005582) and bred at the NIDA, IRP. In these mice, eGFP is knocked into the CX3CR1 locus and eGFP expression in brain of these mice is specific to microglial cells [35]. All mice used for experiments were heterozygous (CX3CR1$^{eGFP/+}$). $D_1$-Cre hemizygote (line FK150) and $D_2$-Cre hemizygote (line ER44) BAC transgenic mice with C57BL/6J background were purchased from GENSAT (www.gensat.org). Homozygous RiboTag mice with C57BL/6J background expressing Cre-inducible HA-Rpl22 were purchased from Jackson Laboratory [36–38]. These RiboTag mice were crossed to $D_1$-Cre or $D_2$-Cre mouse line to generate $D_1$-Cre-RiboTag or $D_2$-Cre-RiboTag transgenic mice for isolation of ribosome-associated mRNAs from cell type-specific $D_1$-MSNs or $D_2$-MSNs.

All mice used in the present experiments were matched for age (8–14 weeks) and weight (25–35 g). They were housed individually in a climate-controlled animal colony room on a reversed light-dark cycle (lights on at 7:00 PM, lights off at 7:00 AM) with free access to food and water. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Academy of Sciences, and were approved by the Animal Care and Use Committee of the NIDA of the US National Institutes of Health and the University of Maryland School of Medicine.

### Single-dose cocaine treatment

To determine whether a single dose of cocaine injection alters brain $CB_2$ gene expression, 28 drug naïve mice (WT) were randomly divided into four dose groups (10, 20, 30 mg/kg, i.p.) or vehicle (saline) ($n = 7$ per group). At 1 h after cocaine injection, all mice were deeply anaesthetized with 100 mg/kg pentobarbital and transcardially perfused with saline to remove all blood from the brain. Whole brains were then rapidly removed, and the PFC and striatum tissues were dissected on dry ice for qRT-PCR assays.

### Repeated cocaine or heroin treatment

To determine whether repeated cocaine or heroin administration alters brain $CB_2$ expression, additional 20 drug naïve WT mice were divided into five groups—repeated saline vs. repeated cocaine (10, 20 mg/kg, i.p., $n = 4–8$ per group) or heroin (2, 8 mg/kg, s.c.) ($n = 4$ each group). Before the drug injection, each animal was placed in a locomotor detection chamber (Accuscan Instruments, Inc., Columbus, OH, USA) for 3 days (3 h per day) for environmental habituation. Then, each animal received 2 days of saline injection before cocaine administration. On each test day, mice were placed in the chamber for 1 h of habituation, and then removed and given either saline (0.1 mL saline, i.p.), one dose of cocaine or heroin. Animals were then placed back into the locomotor chambers for 2 h to assess the effects of cocaine or heroin on locomotion. Each mouse received one injection per day for a total of 7 successive days. Data were collected in 10 min intervals using the VersaMax data analysis system (Accuscan Instruments, Inc., Columbus, OH, USA). Total distance traveled was used to compare saline- vs. the drug (cocaine or heroin)-induced changes in locomotion. At 24 h after the last injection, all mice were deeply anaesthetized with 100 mg/kg pentobarbital and transcardially perfused with saline to remove all blood from the brain. Whole brains were then rapidly removed, and the striatum, PFC or spleen tissues were dissected on dry ice for qRT-PCR assays.

### Prolonged cocaine self-administration

We also examined whether prolonged cocaine self-administration alters $CB_2$ expression in the brain. Intravenous (i.v.) catheterization surgery and cocaine self-administration procedures were identical to those we reported previously [26]. Briefly, after 5–7 days of recovery from surgery, each mouse was placed into a standard operant chamber and allowed to lever press for i.v. cocaine self-administration (1 mg/kg/infusion) under an FR1 reinforcement schedule. After 7 days of initial cocaine self-administration, the cocaine dose was changed from 1 mg/kg/infusion to 0.5 mg/kg/infusion with maximal 50 infusions for additional 3 weeks until stable self-administration was established. Stable self-administration was defined as: (1) at least 20 infusions per 3-h session; (2) less than 20% variability in daily cocaine infusions across two consecutive sessions; and (3) higher than 2:1 active/inactive lever press ratio [26, 39]. Methohexital sodium (Brevital) (an extremely short-acting barbiturate) was used to verify the patency of i.v. catheters during the cocaine self-administration experiment when necessary. At 24 h after the last cocaine self-administration session, animals were transcardially perfused with saline and the whole brain was either removed for RNAscope ISH assays or PFC and striatum were dissected for qRT-PCR.

### Oral sucrose self-administration

To determine whether the changes in brain $CB_2$ mRNA expression were cocaine-specific, we also examined brain $CB_2$ mRNA expression in mice after the same duration of oral sucrose self-administration. The procedure for oral sucrose self-administration was identical to the comparable procedure for cocaine except, 1) no surgery was performed on the animals in the sucrose experiment; and 2) active lever press led to the delivery of 0.02 mL of 5% sucrose solution into a liquid food tray on the operant chamber wall. At 24 h after the last session of sucrose self-administration, animals were sacrificed for qRT-PCR and RNAscope ISH assays.

### RNAscope in situ hybridization assay

To determine which type of cells in the striatum displayed $CB_2R$ upregulation, we used RNAscope ISH assays to examine $CB_2$ mRNA expression in the striatum in naïve WT mice. After brain perfusion, whole brains were taken out and frozen on dry ice. The fresh frozen tissue sections (12 µm thick) were mounted on positively charged microscopic glass slides (Fisherbrand Superfrost Plus; Fisher Scientific, Pittsburgh, PA). A mouse $CB_2$-specific RNA probe (RNAscope probe: Mm-*Cnr2*-$O_2$, cat# 436091) that targets coding DNA sequence (291–719 bp) of the Mus Cnr2 mRNA sequence (NM_009924.3) (Fig. 4a) was designed and provided by the manufacturer (Advanced Cell Diagnostics division of Bio-Techne Corporation, Minneapolis, MN, USA) and well evaluated in a previous study [40]. RNAscope probes used to label microglia (Mm-*Itgam*-C2, cat # 311491-C2, targeting CD11b mRNA), D1-MSNs (Mm-*Drd1a*-C2, cat # 406491-C2, targeting Drd1a mRNA), or D2-MSN (Mm-*Drd2*-C3, cat # 406501-C3, targeting Drd2 mRNA) were provided by the manufacturer (Advanced Cell Diagnostics division of Bio-Techne Corporation, Minneapolis, MN, USA). RNAscope ISH assays for $CB_2$ mRNA expression in each cell type were performed according to the manufacturer's instructions [41]. Briefly, the slides were post-fixed at 4 °C for 15 min in pre-chilled 10% neutral buffered formalin (NBF, 23-245-684; Fisher Scientific International, Inc., Pittsburgh, PA, USA) and hybridized sequentially with target probes at 40 °C for 2 h, amplifier 1 at 40 °C for 30 min, amplifier 2 at 40 °C for 15 min, amplifier 3 at 40 °C for 40 min, amplifier 4 at 40 °C for 15 min, and counterstained with 4′,6-diamidino-2-phenylindole at room temperature for 10 s. Stained slides were coverslipped with fluorescent mounting medium (ProLong Gold Antifade Reagent P36930; Invitrogen division of Thermo Fisher Scientific Corporation, Waltham, MA, USA) and scanned into digital images using a Carl Zeiss microscope (Thornwood, NY, USA) at ×40 magnification using manufacturer-provided software. From each brain sample, three adjacent sections were stained using mouse $CB_2$, ubiquitin C (UBC), and bacterial gene (dapB) probes, respectively. UBC was used as an endogenous positive control to assess RNA probe integrity. The dapB served as a negative control to assess background staining. The probe designs for UBC and dapB were as previously described [41]. ImageJ software (NIH) was used to quantify $CB_2$ mRNA signals in each individual cell. Only cells with clear boundaries were subjected to quantification analysis.

### Microdissection of nucleus accumbens and dorsal striatal tissues

Heterozygous CX3CR1$^{eGFP/+}$ reporter mice were used for the separation of microglia ($GFP^+$) and non-microglia ($GFP^-$) cell populations by FACS technologies. $D_1$-Cre-RiboTag and $D_2$-Cre-RiboTag mice were used to isolate ribosome-associated mRNAs from $D_1$- or $D_2$-MSNs. Before sacrificing, each phenotype of reporter mice was given either saline or cocaine (20 mg/kg, i.p., 7 days). At 24 h after the last injection, all mice were anesthetized with Euthasol (sodium pentobarbital 150 mg/kg and sodium phenytoin 19.2 mg/kg, Virbac) and perfused transcardially with 10 mL of oxygenated, ice-cold N-methyl-d-glucamine (NMDG)-based solution [42]. Brains were then rapidly dissected and coronal forebrain sections (300 µm thick) were prepared using a vibratome in ice-cold NMDG-based cutting solution bubbled continuously with 95% $O_2$/5% $CO_2$. NAc and DST were microdissected from striatal coronal sections (Bregma: 1.60–0.70 mm) using fine tipped forceps. Microdissected tissue was minced using a scalpel under the stereoscope before being transferred to Eppendorf tubes containing 1 mL Hibernate A solution (Brain Bits) stored on ice.

### Microglia separation by FACS

Microdissected striatal tissues from WT and CX3CR1$^{eGFP/+}$ mice were gently dissociated in Hibernate A solution using sequential trituration with fire-polished glass pipettes with openings of decreasing diameter (final pipette ~ 0.4 mm diameter opening). Resulting cell suspensions were spun down, re-suspended in 300 µL 1× PBS and filtered through a 40 µm mesh filter. Throughout the experiment, samples were kept at 4 °C on ice. Samples were sorted using a FACS Aria I cell sorter (BD Biosciences). The population of cells could be readily identified based on forward scattering (FSC) and side scattering (SSC) properties. A gating strategy based on FSC and SSC width and height was used to select only single cells. Microglial cells and non-microglia cells were then identified and sorted according to GFP expression. Cell suspensions from WT tissue were used as a GFP-negative control for the establishment of GFP-negative gates. GFP-positive and GFP-negative cells were collected from DST and NAc of CX3CR1$^{eGFP/+}$ mice. The average collecting cells for each group are around 1000 [40, 42].

### RNA extraction

*RNA extraction from dissected tissues from WT mice*. Total RNA-was extracted using a RNeasy Mini kit (QIAGEN, Valencia, CA, USA), according to the kit instructions. Briefly, total less than 30 mg of brain tissue was disrupted and homogenized in 600 µL RLT buffer with 6 µL β-mercaptoethanol, and then centrifuged at 12,000 r/min for 10 min at room temperature. Following centrifugation, the supernatant was transferred to a new microcentrifuge tube, and then an equal amount of 70% ethanol was added and mixed by pipetting. The mixed sample was transferred to a RNeasy spin column and centrifuged for 30 s at 12,000 r/min. The RNA bound on the column membrane was washed out with RW1 buffer and RPE buffer. The RNA was then dissolved in 40 µL diethylpyrocarbonate-treated water. Purity and integrity of each extract were determined by absorbance at 260 nm using the Eppendorf BioPhotometer Plus (Eppendorf AG, Hamburg, Germany). The RNA was kept at −80 °C until assay.

*RNA extraction from sorted microglial cell*. Total RNA from sorted cells was isolated using the PicoPure RNA isolation kit (Arcturus Bioscience). All cells were sorted by FACS directly into Eppendorf tubes containing 50 µL PicoPure RNA extraction buffer and, following the sort, samples were incubated at 42 °C for 30 min and stored in RNase-free tubes at −80 °C until further processing. Column filtration, washing, and elution of RNA from the columns were performed according to the manufacturer's instructions in section C of the PicoPure RNA isolation protocol. RNA samples were quantitated using the Agilent Bioanalyzer High Sensitivity kit (Agilent, Santa Clara, CA).

*RNA extraction from RiboTag mice*. NAc samples were pooled from four male and female $D_1$-Cre-RiboTag and $D_2$-Cre-RiboTag mice and RNA was isolated from immunoprecipitated polyribosomes using primary mouse anti-HA antibody (Covance Research Products, Cat# MMS101R) and secondary antibody coated magnetic Dynabeads (Dynabeads protein G, Invitrogen) to pull-down the MSN-specific RNA. Immunoprecipitated polyribosomes and non-immunoprecipitated input were prepared according to our previous studies [36, 37]. RNA from polyribosome immunoprecipitated samples or input was subsequently extracted using the Total RNA Kit (Omega) according to the manufacturer's instructions.

### cDNA synthesis and qRT-PCR assays

Single strand cDNAs were synthesized with Superscript III first strand cDNA synthesis kit (Invitrogen, Life Technologies), according to the manufacturer's protocol. Procedures for qRT-PCR for detection of $mCB_2$ mRNA were the same as we reported previously [10, 16]. In brief, a $mCB_{2A}$ probe and specific $mCB_2$-KO Taqman probe ($CB_2$-KO probe) were used, which targets the

upstream sequence of intact gene or gene-deleted region in the exon 3 of $mCB_2$ gene, respectively (Fig. 1a). Mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-mRNA was used as an endogenous control. All Taqman probe and primers were purchased from Applied Biosystems (Foster City, CA, USA). The qRT-PCR reactions were performed in a 7500 Fast qRT-PCR system using the program: 95 °C hold for 20 s followed by 40 cycles of 95 °C denaturation for 3 s and 60 °C annealing and extension for 30 s. qRT-PCR analyses of $CB_2$-mRNA levels were performed using the $2^{-\Delta\Delta Ct}$ method [43]. In the present study, data are presented as the fold change in $mCB_2$ gene expression normalized first to the internal GAPDH gene or S18 gene and then relative to each vehicle group in each sample. The cycle threshold (Ct) was defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceeds background level). ΔCt was determined as [mean of the triplicate Ct values for the $mCB_2$ gene] − [mean of the triplicate Ct values for GAPDH or S18]. ΔΔCt represented the difference between the paired tissue samples, as calculated by the formula ΔΔCt = [ΔCt of $mCB_2$ in treated group tissue − ΔCt of $mCB_2$ in vehicle group tissue]. The N-fold differential expression of the $mCB_2$ gene in treated group tissues compared with vehicle group tissue was expressed as $2^{-\Delta\Delta Ct}$ [10, 16, 43].

*qRT-PCR from sorted microglia cells.* The PreAmplification-RT-PCR method was used for FACS sorted samples [44]. TaqMan PreAmp Master Mix Kit was used for cDNA preamplification (Cat# 4391128; Applied Biosystems, Life Technologies) using pooled primer mixes of 20× dilution of TaqMan Gene Expression Assay. cDNAs were pre-amplified in an ABI 9700 Thermal Cycler using the program: 95 °C hold for 10 min, 14 cycles of 90 °C denaturation for 15 s, and 60 °C annealing and extension for 4 min. Pre-amplification PCR products were immediately diluted five times with molecular biology grade water and stored at −20 °C or immediately processed for RT-PCR. Duplex RT-PCR assays were performed on technical duplicates using a FAM-labeled probe for each target gene and a Vic-labeled probe for the endogenous control gene (gapdh) by using TaqMan Advanced Fast PCR Master Mix (Cat# 4444963; Life Technologies). RT-PCR reactions were run in a 7500 Fast qRT-PCR system using the program: 95 °C hold for 20 s followed by 40 cycles of 95 °C denaturation for 3 s and 60 °C annealing and extension for 30 s. qRT-PCR analyses of $CB_2$-mRNA levels were also performed using the $2^{-\Delta\Delta Ct}$ method as described above.

*qRT-PCR from D1 or D2-MSNs.* For cDNA synthesis, qRT-PCR and analysis we follow the steps as described previously [36, 37]. The list of primers used in this study is $CB_2$-forward primer 5′-AGCTCGGATGCGGCTAGAC-3′, $CB_2$-reverse 5′-AGGCTGTGGCCCAT-GAGA-3′ [10], Gapdh-forward 5′-AGGTCGGTGTGAACGGATTTG-3′ and Gapdh-Reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′ [36, 37]. The template cDNA sequence of $CB_2Rs$ was obtained from GenBank (ncbi.nlm.nih.gov/). Samples were run blinded to the condition. mRNA expression changes were measured using quantitative polymerase chain reaction with PerfeCTa SYBR Green FastMix (Quanta). Quantification of mRNA changes was performed using the −ΔΔCt method, using GAPDH as a house-keeping gene and immunoprecipitated samples were then normalized to the averaged input from the respective MSN subtype.

### Data analysis

All the RT-PCR data were presented as means (±SEM). One-way analysis of variance (ANOVA) was used to analyze the difference in fold change of $CB_1$ and $CB_2$ mRNA among different groups. Individual group comparisons were carried out using the Student–Newman–Keuls method.

## RESULTS

### Acute cocaine administration failed to alter $CB_2$ mRNA expression

To explore whether cocaine exposure induces dynamic changes in $CB_2R$ expression, we designed two probes—one targets an exon junction of the $CB_2R$ gene ($mCB_{2A}$ probe) and another one targets the portion of exon 3 that is disrupted in $CB_2R$-knockout mice ($mCB_2$-KO probe) (Fig. 1a). In qRT-PCR analysis in WT mice, both $mCB_{2A}$ and $mCB_2$-KO probes detected similar levels of $CB_2$ mRNA signal in the striatum and spleen (Fig. 1b, c). Consistent with our previous findings [10, 16], we found that $CB_2$ mRNA level is much higher (20–25-fold) in spleen than in striatum. In contrast, the $mCB_2$-KO probe detected $CB_2$ mRNA signal only in WT, not in $CB_2$-KO mice, indicating that the detected signal is highly specific to the $CB_2R$ gene. We then used both probes to detect cocaine-induced changes in $CB_2$ expression. We found that a single injection of cocaine at varying doses (10, 20, 30 mg/kg, i.p.) had no effect on $CB_2$ mRNA expression in either the striatum or PFC at 1 h (Fig. 1d, e) or 3 h (data not shown) post injection.

### Repeated cocaine administration increases $CB_2$ mRNA expression in striatum and spleen

We then assessed whether repeated daily injections of cocaine similarly alter brain $CB_2$ mRNA expression. Repeated injections of cocaine (10 or 20 mg/kg, i.p.) for 7 consecutive days produced robust locomotor sensitization (Fig. 2a), indicating circuit and behavior-level changes in response to drug exposure. At 24 h after the last cocaine injection, we measured $CB_1$ and $CB_2$ mRNA levels in striatum (Fig. 2b), PFC (Fig. 2c), and spleen (Fig. 2d) via qRT-PCR. We found that $CB_2$ mRNA, as detected by $mCB_2$-KO probe, was significantly increased in both the striatum (Fig. 2b, $F_{2,11} = 7.03$, $P < 0.05$) and spleen (Fig. 2d, $F_{2,11} = 6.96$, $P < 0.05$) in WT mice after repeated cocaine administration. In contrast, there was no change in $CB_2$ mRNA expression in the PFC (Fig. 2c, $F_{2,11} = 2.27$, $P > 0.05$). Repeated cocaine injections also failed to significantly alter $CB_1$ mRNA expression (Fig. 2b, d), with the exception of a small reduction in $CB_1$ mRNA in the PFC following 10 mg/kg cocaine injections (Fig. 2c), suggesting that chronic cocaine treatment selectively upregulates $CB_2$ mRNA expression in both the brain and periphery.

### Repeated heroin administration downregulates $CB_2$ mRNA expression in striatum

To determine whether this effect can be generalized to other drugs of abuse, we also observed the effects of repeated heroin exposure on $CB_2$ mRNA expression in the striatum. Figure 2e, f shows that repeated administration of heroin (2, 8 mg/kg, s.c., once daily for 7 days) produced robust locomotor sensitization in mice in a dose-dependent manner. Unexpectedly, a significant downregulation of $CB_2$ mRNA expression was detected in the striatum as assessed by quantitative RT-PCR when measured at 24 h after the last heroin administration (Fig. 2f, $F_{2,11} = 4.88$, $P < 0.05$).

### Cocaine self-administration upregulates $CB_2$ mRNA expression

Molecular and circuit level changes following drug exposure can exhibit key differences depending on whether the drug is investigator administered or voluntarily consumed [45]. For this reason, we then examined whether chronic cocaine self-administration also upregulates $CB_2$ mRNA expression in the brain. We compared these results of dynamic changes in $CB_2R$ expression following self-administration of palatable food (sucrose solution). Figure 3a shows the time courses of i.v. cocaine or oral sucrose self-administration; the majority of the animals reached the maximally allowed 50 cocaine infusions or 100 sucrose deliveries per session after ~1 week of self-administration training. At 24 h after the last cocaine self-administration, qRT-PCR analysis showed that $CB_2$ mRNA levels were significantly increased in both the striatum and PFC (Fig. 3b, c) when compared with drug naïve


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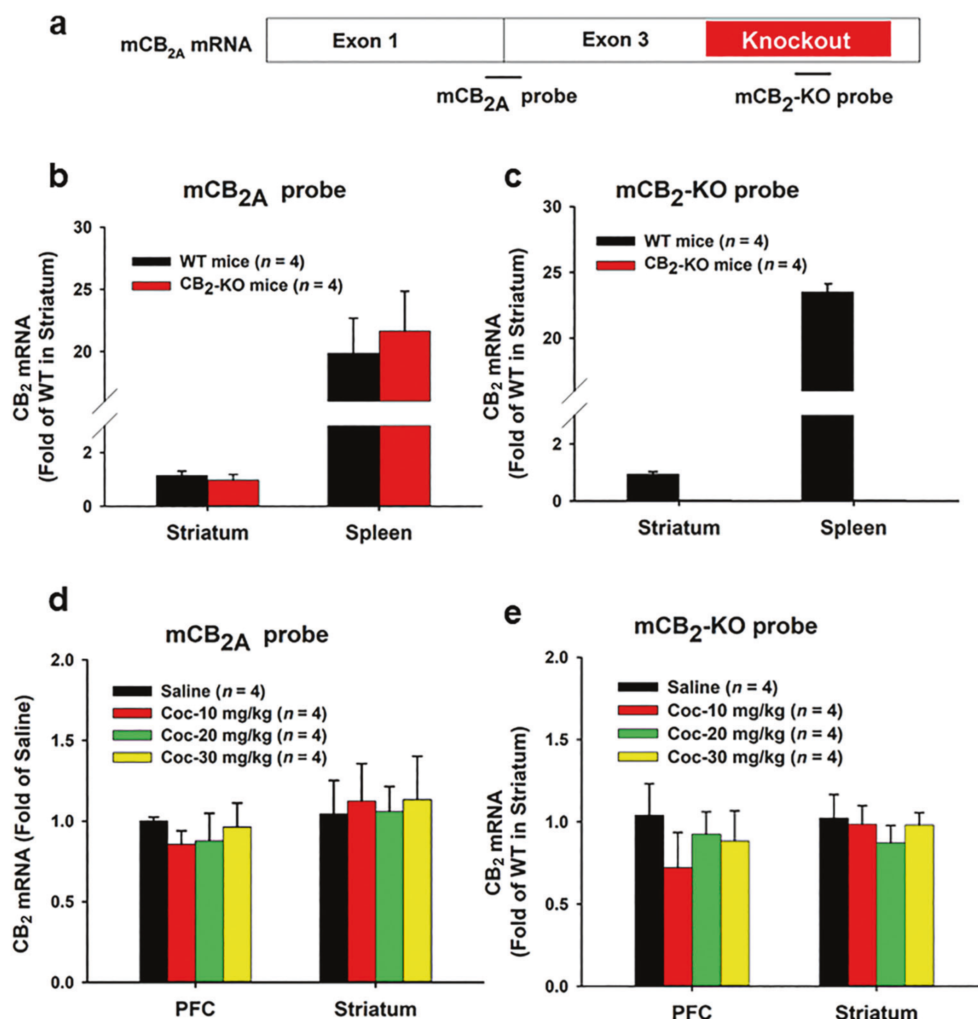


**Fig. 1 Effects of acute cocaine administration on $CB_2$ mRNA expression in the brain and spleen. a** A diagram showing the transcript structure of $CB_{2A}$ isoform and two Taqman probes that target either the upstream region ($mCB_{2A}$ probe) or the gene-deleted region ($mCB_2$-KO probe) in the $CB_2$-KO mice used in the present study. **b**, **c** qRT-PCR results, indicating that both the $mCB_{2A}$ and $mCB_2$-KO probes detected similar levels of $CB_2$ mRNA in the striatum and spleen in WT mice. While the $mCB_{2A}$ probe also detected similar levels of $CB_2$ mRNA in $CB_2$-KO mice, the $mCB_2$-KO probe detected $CB_2$ mRNA only in WT, not in $CB_2$-KO mice. **d**, **e** qRT-PCR results: indicating that a single injection of cocaine (10, 20, 30 mg/kg, i.p.) did not alter $CB_2$ mRNA expression, measured by both the $mCB_2$ probes, in either the PFC or striatum in WT mice as measured 1 h after acute cocaine injection.

mice or sucrose self-administration mice. One-way ANOVA revealed a statistically significant effect (Fig. 3b: PFC, $F_{2,11} = 8.98$, $P < 0.01$; ST: $F_{2,11} = 16.35$, $P < 0.001$; Fig. 3c: PFC, $F_{2,13} = 7.14$, $P < 0.01$; ST: $F_{2,13} = 6.24$, $P < 0.05$). In contrast, oral sucrose self-administration failed to alter brain $CB_2$ mRNA expression when compared with drug naïve mice ($P > 0.05$).

### Cellular distributions of $CB_2$ mRNA in the striatum

To determine which type of cells displays $CB_2$ upregulation after cocaine exposure, we then used RNAscope ISH assays to examine $CB_2$ mRNA expression in microglia, D1-MSNs, and D2-MSNs in the NAc. In this assay, we used another mouse $CB_2$-specific RNA probe that targets the gene-deleted region in the $CB_2$-KO mice that we used as control in this study (Fig. 4a). We detected low-to-moderate levels of $CB_2$ mRNA in the NAc (Fig. 4b, left panel). However, when using a microglial-specific CD11b RNA probe, we failed to detect clear microglial cells (Fig. 4b, middle panel) nor co-localization of $CB_2$ and CD11b (Fig, 4b, right panel). However, when using Drd1 or Drd2 RNA probes, we detected clear $CB_2$ mRNA in ~50% Drd1-labled D1-MSNs (Fig. 4c) or Drd2-labeled D2-MSNs (Fig. 4d) in the NAc.

### Repeated cocaine does not alter $CB_2$ mRNA expression in purified microglia

Since $CB_2$R-expressing D1-MSNs and D2-MSNs are mixedly distributed in the striatum, it is technically difficult for us to use RNAscope ISH methods to quantitatively measure $CB_2$ mRNA density in each phenotype of cells. In addition, RNAscope ISH assays also failed to detect clear microglial cells in the NAc, which may be related to their extremely small cell sizes in normal healthy mice. Thus, to further determine which type of cells (neurons or microglia) in the NAc displays $CB_2$ upregulation after cocaine exposure, we alternatively used three thansgenic mice and FACS technology to purify or isolate different phenotypes of cells. We first used $CX3CR1^{eGFP/+}$ reporter mice to separate $GFP^+$ microglia and $GFP^-$ non-microglial cells in the dorsal striatum (DST) and NAc (Fig. 5a). Surprisingly, repeated cocaine injection (20 mg/kg per day for 7 days) failed to alter $CB_2$ mRNA expression in $GFP^+$ microglia, but significantly increased $CB_2$ mRNA expression in the $GFP^-$ non-microglial cell population (Fig. 5b, left panel, $t = 2.24$, $P < 0.05$) in the NAc. In contrast, no significant change in $CB_2$ mRNA was observed in either $GFP^+$ microglia or $GFP^-$ non-microglial cells in the DST after cocaine administration (Fig. 5b, right panels).

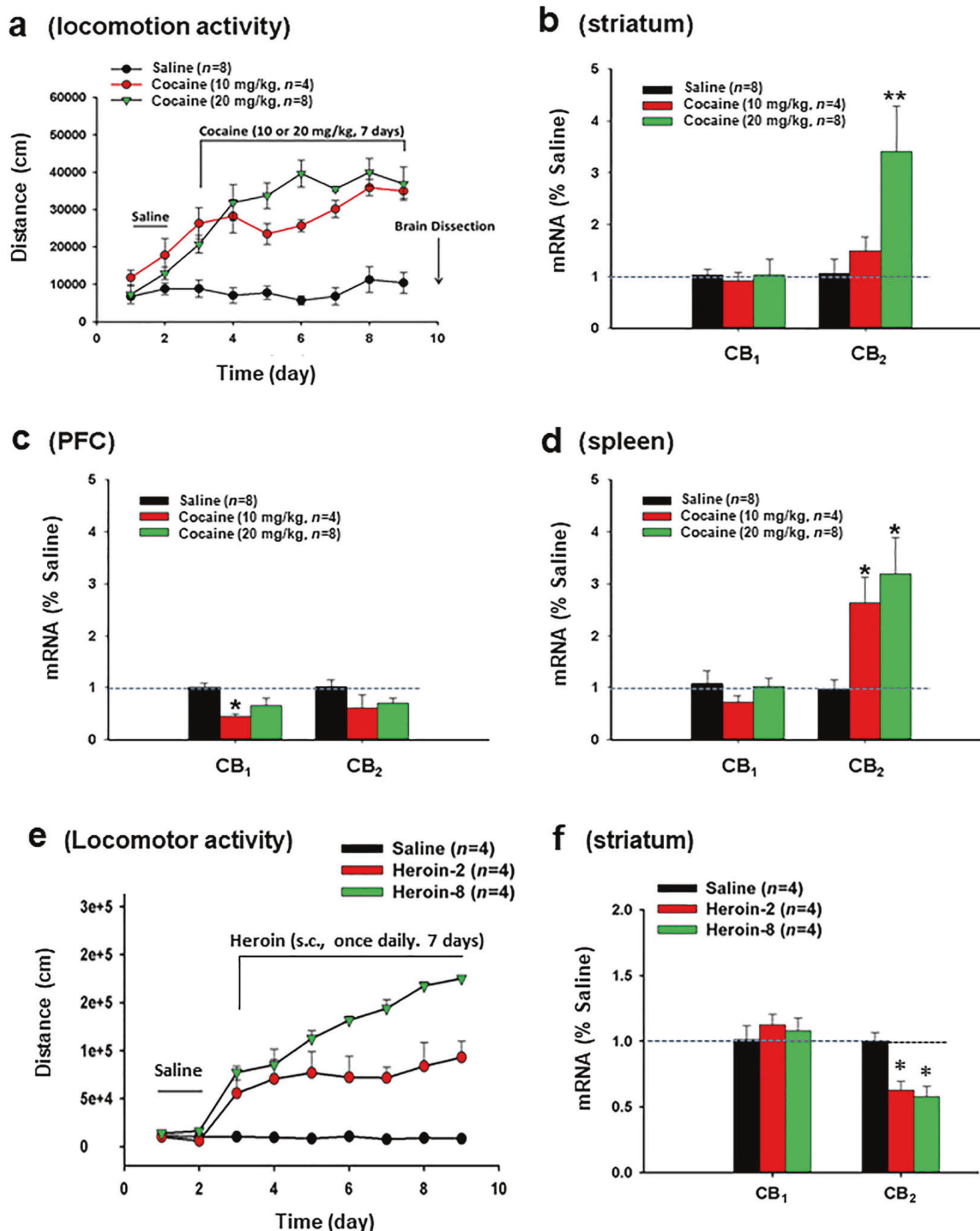


**Fig. 2 Effects of repeated cocaine administration on $CB_1$ and $CB_2$ mRNA expression as measured by qRT-PCR. a** Repeated cocaine administration produced robust locomotor sensitization at both 10 and 20 mg/kg doses. **b** Cocaine, at 20 mg/kg only, significantly increased $CB_2$ mRNA in the striatum as measured with the $mCB_2$-KO probe; **c** cocaine, at 10 or 20 mg/kg, failed to alter $CB_2$ mRNA expression in the PFC. **d** Cocaine, at both doses, significantly increased $CB_2$ mRNA expression in spleen as measured at 24 h after the last cocaine injection. In contrast, repeated cocaine failed to alter $CB_1$ mRNA expression except that cocaine, at 10 mg/kg, tended to decrease $CB_1$ mRNA expression in the PFC (**c**). **e** Repeated heroin administration also produced robust locomotor sensitization in a dose-dependent manner. **f** Repeated heroin-treated mice displayed a reduction in $CB_2$ mRNA expression in the striatum. $^*P < 0.05$, compared to saline group.

Similarly, repeated cocaine administration did not alter $CB_1$ mRNA expression in either cell population in the DST or NAc (Fig. 5b).

## Repeated cocaine upregulates $CB_2$ mRNA expression in $D_1$-MSNs in the NAc

Given that cocaine upregulates $CB_2$ mRNA expression in non-microglial cells within the NAc, we then sought to more clearly identify the cell population responsible for this dynamic change in $CB_2R$ expression. There are two major types of neurons in the striatum: $D_1$- and $D_2$-MSNs, which are differentially involved in motivated drug-taking and drug-seeking behavior [46, 47]. We then used Drd1a-Cre-RiboTag and Drd2-Cre-RiboTag mouse lines to isolate ribosome-associated RNAs from $D_1$-MSNs and $D_2$-MSNs, respectively. In drug naïve mice, $CB_2$ mRNA levels in $D_1$-MSNs or $D_2$-MSNs were significantly lower than those in other RNA samples isolated from all the cells (Fig. 6a, left panel, $F = 4.01$, $P < 0.05$). However, when comparing $D_1$- and $D_2$-MSNs to one another, $D_2$-MSNs displayed 3–4 folds higher basal levels of $CB_2$ mRNA than $D_1$-MSNs (Fig. 6a right two panels, $t = 4.54$, $P < 0.05$). Following


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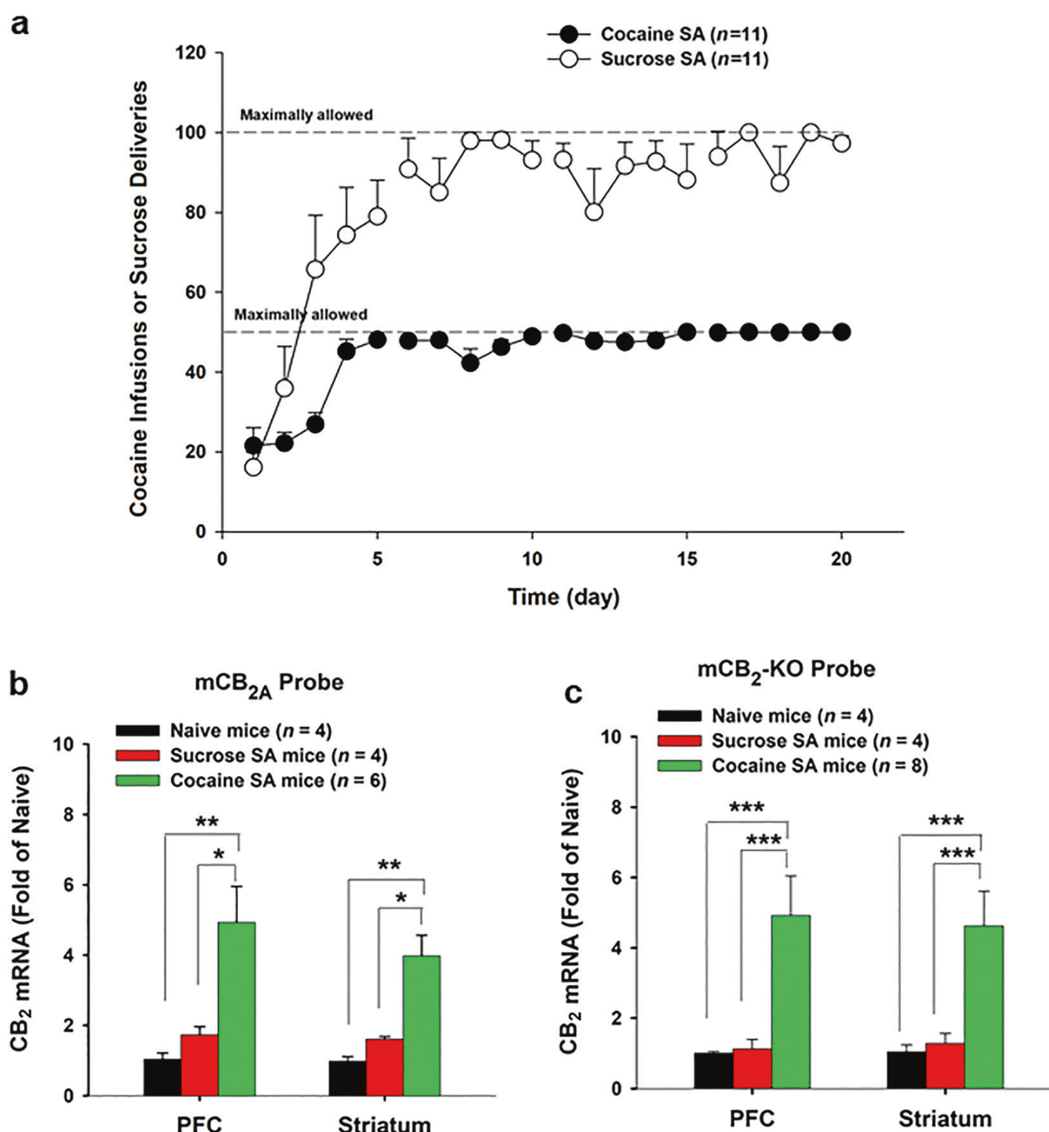


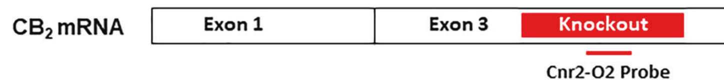

**Fig. 3 Effects of cocaine self-administration on $CB_1$ and $CB_2$ mRNA expression (by qRT-PCR). a** Mean numbers of cocaine self-administration (infusions) or oral sucrose deliveries during the 4 weeks of self-administration training; cocaine, but not sucrose, self-administration significantly upregulated $CB_2$ mRNA expression in both the PFC and striatum in WT mice measured with the $mCB_{2A}$ (**b**) and $mCB_2$-KO (**c**) probes, respectively. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to drug naïve mice or sucrose self-administration mice.

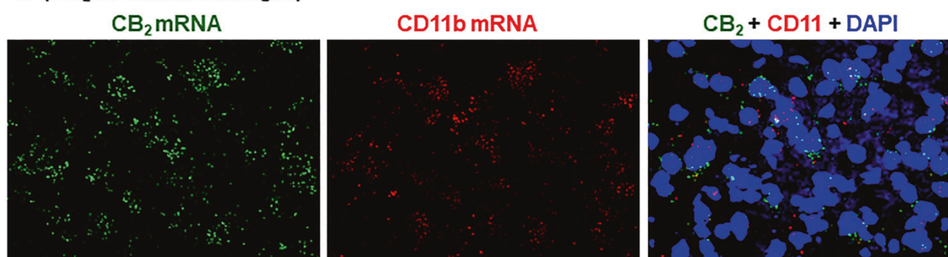
repeated cocaine administration, $D_1$-MSNs displayed significantly upregulated $CB_2$ mRNA expression (Fig. 6b, right panel, $t = 2.53$, $P < 0.05$; left panel, $t = 6.81$, $P < 0.05$), while $D_2$-MSNs did not (Fig. 6b, $P < 0.05$). $CB_1$ mRNA expression was not altered by repeated cocaine administration in either $D_1$-MSNs or $D_2$-MSNs (Fig. 6c).

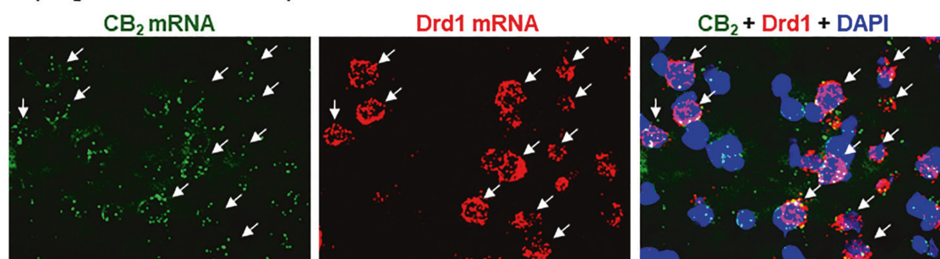
## DISCUSSION

In this study, we used three mouse $CB_2R$-specific probes and multiple (4) transgenic mouse lines to examine the impact of acute vs. repeated cocaine administration on brain $CB_2R$ expression. We found that a single injection of cocaine failed to alter $CB_2R$ expression, but repeated cocaine administration or self-administration caused a significant increase in $CB_2$ mRNA expression in the striatum and spleen tissues. Unexpectedly, this $CB_2$ upregulation does not occur in microglial cells, but occurs mainly in $D_1$-MSNs in the NAc. In a series of parallel studies, we did not observe significant changes in $CB_1$ mRNA expression. These findings suggest that neuronal $CB_2Rs$ are inducible and responsive to recurring cocaine use and abuse. The present finding that NAc $CB_2$ was upregulated in $D_1$-MSNs, combined with our previous finding that $CB_2$ was upregulated in VTA DA neurons [11], at least in part explain how $CB_2R$ agonists attenuate cocaine self-administration and other addiction-related behaviors in rats and mice [25, 26, 48].

The expression of functional $CB_2Rs$ in the brain, particularly in neurons, has been the subject of debate for many years [7, 49]. Concerns have been raised regarding the $CB_2R$ signal specificity when using antibodies to detect brain $CB_2R$ by immunostaining. Therefore, in the present study, we chose to detect $CB_2$ gene expression using three custom-designed mouse $CB_2$-specific probes ($mCB_{2A}$, $mCB_2$-KO, Cnr2-$O_2$) based on the $CB_2$ gene structures in WT and $CB_2$-KO mice, combined with the use of $CB_2$-rich spleen tissues and $CB_2$-KO mice as positive and negative controls. These probes have been confirmed to be mouse $CB_2$-specific as assessed by the findings in $CB_2$-KO mice in the present study (Fig. 1) and also in our previous studies [9, 10, 40]. Using qRT-PCR assays, we detected low levels of $CB_2$ mRNA expression in the PFC and striatum in healthy, drug naïve mice.



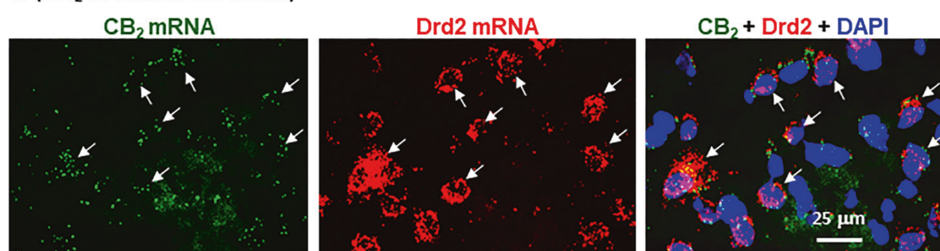




**Fig. 4 Cellular distributions of $CB_2$ mRNA in the NAc as assessed by RNAscope in situ hybridization.** **a** Diagram showing the transcript structure of the $CB_2A$ isoform and of another $mCB_2$-specific probe (*Cnr2*-$O_2$ probe) that targets the gene-deleted region in the $CB_2$-KO mice. **b** CD11b mRNA is barely detectable in the NAc and no $CB_2$ and CD11b co-localization was detected. $CB_2$ mRNA was detected in Drd1-positive D1-MSNs (**c**) and Drd2-positive D2-MSNs (**d**).

An important finding in the present study was that a single injection of cocaine at a wide range of doses (10, 20, 30 mg/kg) failed to alter brain $CB_2$ mRNA expression when measured at 1 or 3 h after cocaine injection. In contrast, repeated administration of high doses of cocaine (10, 20 mg/kg) for 7 consecutive days or cocaine self-administration (for 4 weeks) significantly upregulated $CB_2$ mRNA expression in the striatum and the spleen. In addition, cocaine self-administration, but not repeated cocaine exposure for 7 days, also upregulated $CB_2$ mRNA expression in the PFC, suggesting that prolonged cocaine exposure is required to alter $CB_2$ expression in the cortex. It may also suggest that voluntary consumption of cocaine or the associative learning associated with self-administration is necessary to induce $CB_2$ upregulation in the PFC. These findings are consistent with, but expand, previous findings that brain $CB_2Rs$ can be upregulated under chronic neuroinflammation as stated above.

Another important finding is that peripheral $CB_2R$ expression appears more sensitive to cocaine exposure than that in the brain since recurrent exposure to 10 or 20 mg/kg cocaine upregulates $CB_2$ mRNA expression in spleen, while 20 mg/kg cocaine is required to upregulate $CB_2$ expression in the brain. Cocaine is well known to suppress immune function and cause changes in phagocytic activity of macrophages and production of immunoregulatory cytokines [50–53] and cannabinoids are known to have anti-inflammatory and immunomodulatory properties by activation of $CB_2Rs$ [54, 55]. Thus, the present finding suggests that chronic exposure to cocaine may produce immunosuppressive effects by upregulation of $CB_2R$ expression in peripheral immune cells.

An unexpected finding is that repeated administration of opioids (heroin) produced an opposite reduction in NAc $CB_2$ mRNA expression. This is consistent with a recent report indicating that repeated morphine administration caused a significant reduction in $CB_2$, not $CB_1$, mRNA expression in VTA [56], but is not fully consistent with an early report that repeated administration of morphine produces conditioned place preference, which is associated with a significant reduction in $CB_2$ mRNA expression in brain stem and a significant increase in $CB_2$ mRNA expression in the cerebral cortex and peripheral spleen and blood immune cells in rats [57]. These findings suggest that chronic administration of opioids may also differentially alter $CB_2$ mRNA expression in different tissues or brain regions. Given that

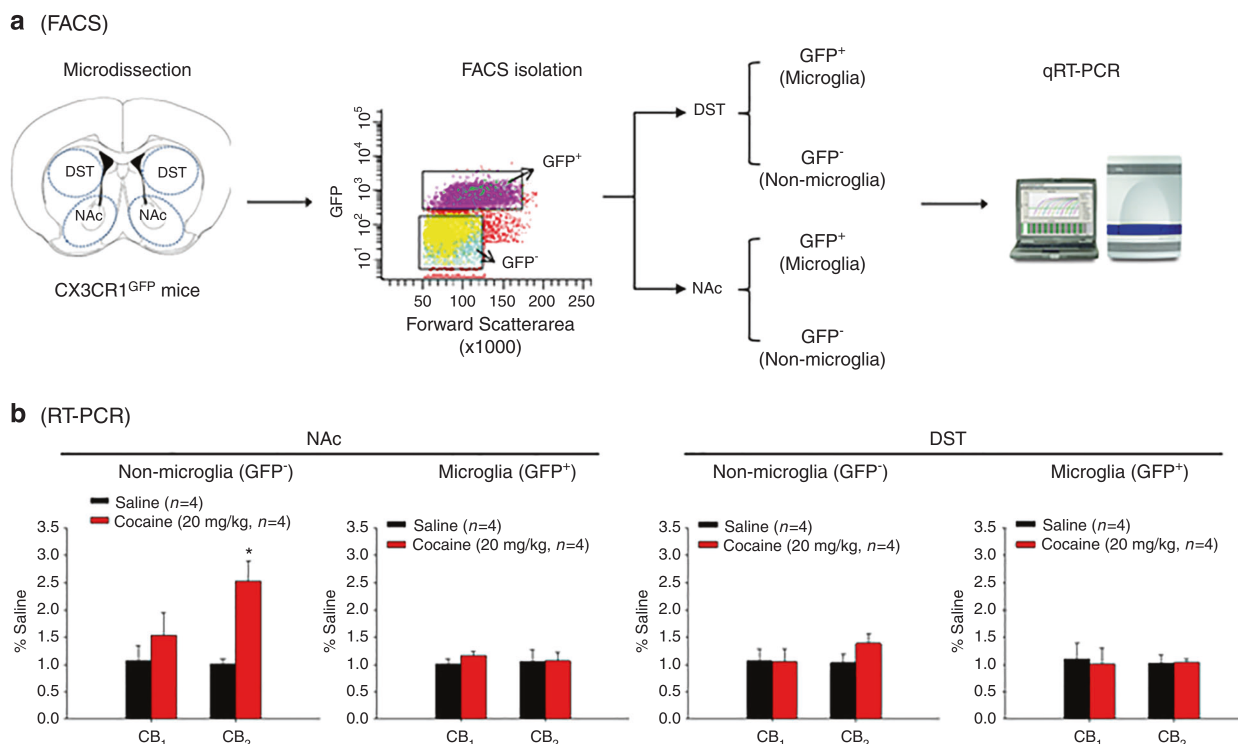


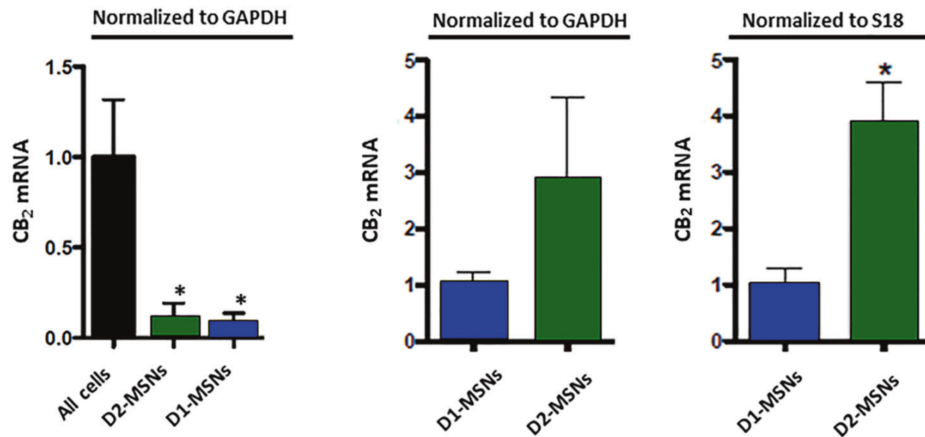

**Fig. 5 Effects of repeated cocaine administration on microglial $CB_2$ mRNA expression in the striatum. a** Flowchart of FACS and qRT-PCR techniques for separating $GFP^+$ microglia and $GFP^-$ non-microglial cells from $CX3CR1^{eGFP/+}$ mice. **b** qRT-PCR results, indicating that repeated cocaine administration (20 mg/kg, i.p. for 7 days) significantly increased $CB_2$ mRNA expression in $GFP^-$ non-microglial cells, but not in $GFP^+$ microglia, in the NAc. There was no change in $CB_2$ mRNA expression in either cell population in the dorsal striatum (DST).

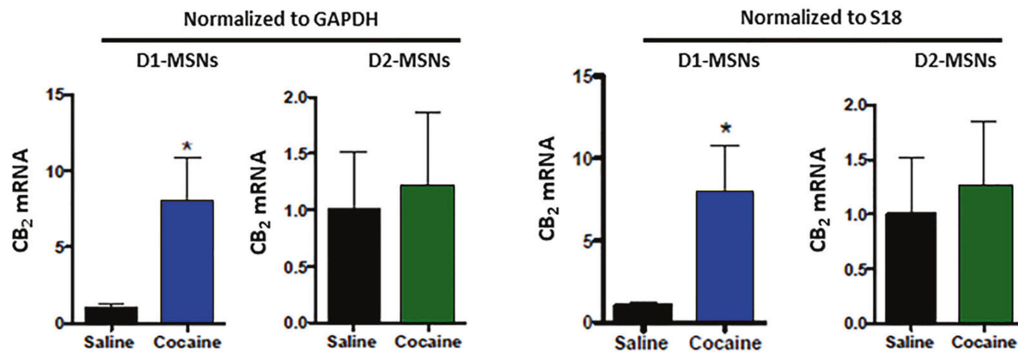
activation of $CB_2Rs$ is protective against various insults in the brain and periphery [6, 13, 58], the present finding suggests that $CB_2R$ upregulation may be similarly protective against chronic drug-induced changes or damage in the brain and periphery.

Another unexpected finding is that recurring cocaine use did not alter microglial $CB_2$ mRNA expression. This finding is conflicted with a well-accepted view that brain $CB_2$ upregulation occurs mainly or exclusively in microglial cells during chronic neuroinflammation [13]. This conclusion is largely based on early antibody-based findings that $CB_2$-like immunostaining was found in neuritic plaque-associated astrocytes and microglia in hippocampus and entorhinal cortex in post-mortem brain tissues from patients with severe Alzeimor's Disease [59], in CD3- or CD68-positive perivascular (microglial) cells in non-human primates with simian immunodeficiency virus-induced encephalitis [60], or in activated microglia and astrocytes in the spinal cord of mice with chronic neuropathic pain [61]. This is supported by the finding that $CB_2R$-dependent-eGFP signal was also detected in microglia in areas of intense inflammation and amyloid deposition in the brain in transgenic $CB_2$-Cre-reporter ($CB_2^{EGFP/f/f}$) mice co-expressing five familial Alzheimer's disease mutations (5XFAD) [62]. However, there are two major concerns in these studies. First, it is unknown whether those $CB_2$ antibodies are $CB_2$-specific, and second, the $CB_2$-like immunostaining was seen not only in microglia, but also in astrocytes [63]. In contrast to these findings, we have previously reported that cocaine self-administration upregulates $CB_2R$ expression in VTA DA neurons, not in glial cells [11]. Systemic administration of lipopolysaccharide (LPS), a bacterial endotoxin, caused significant microglial and astrocytic proliferation in the VTA, but failed to cause $CB_2R$ upregulation in microglia or astrocytes [10]. Together, these findings suggest that $CB_2R$ upregulation may occur in different phenotypes of cells, including microglia, astrocytes, or neurons depending upon different experimental or pathological conditions, which strongly argue the current prevailing view (dogma) that brain $CB_2R$ upregulation occurs mainly or exclusively in microglia.

Perhaps the most important finding in this study is the discovery of $CB_2R$ upregulation in NAc $D_1$-MSNs in mice after recurring cocaine use. Interestingly, $D_1$-MSNs displayed lower basal level of $CB_2$ mRNA expression than $D_2$-MSNs, but displayed a higher $CB_2R$ response to repeated cocaine in the NAc, rather than in the DST. These findings suggest that neuronal $CB_2R$ upregulation in the striatum displays subregional- and cell type-specific profiles. As mentioned above, growing evidence indicates that brain $CB_2Rs$ modulate pharmacological action produced by cocaine [9, 10, 25, 26, 48], morphine [64, 65], nicotine [28], and alcohol [66] in a number of addiction- and pain-related behavioral models. However, the neural mechanisms underlying this $CB_2R$-mediated action are poorly understood. First, it is unlikely that microglia $CB_2R$ are involved since there is little evidence supporting $CB_2$ expression in microglia in normal healthy or in LPS-treated mice [10] or microglial $CB_2$ upregulation in rats with cocaine self-administration history [11]. Second, as stated above, cocaine self-administration upregulates $CB_2$ gene expression in VTA DA neurons [11], suggesting that a presynaptic $CB_2R$ mechanism in the mesolimbic DA system may be involved (Fig. 7). Lastly, in the present report, we found that chronic cocaine administration also upregulates $CB_2R$ expression in NAc $D_1$-MSNs, suggesting that a postsynaptic $CB_2R$ mechanism may also play an important role in mediating $CB_2R$ modulation of cocaine action (Fig. 7).

It is unknown precisely how $CB_2Rs$ in $D_1$-MSNs modulate cocaine action. Recent optogenetic studies indicate that activation of $D_1$-MSNs in the NAc is critically associated with positive reinforcement, while activation of $D_2$-MSNs are mostly associated with aversion [67, 68], although not all evidence supports this



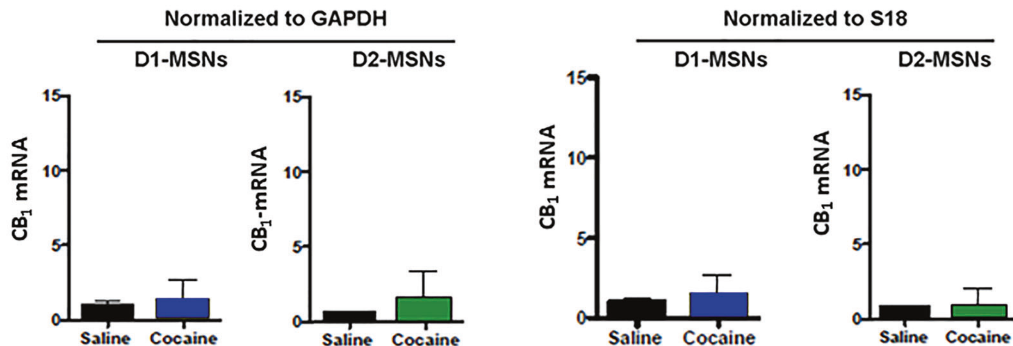




**Fig. 6 Effects of repeated cocaine administration on $CB_2$ mRNA expression in striatal $D_1$-MSNs vs. $D_2$-MSNs.** **a** qRT-PCR results, indicating the relative levels of $CB_2$ mRNA in striatal $D_1$-MSNs, $D_2$-MSNs, and All (unsorted) cells, prepared by FACS from D1-Cre-RiboTag and D2-Cre-RiboTag mice. $D_2$-MSNs displayed higher basal level of $CB_2$ mRNA than D1-MSNs when normalized to the house-keep genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or S18 protein; **b** repeated cocaine administration (20 mg/kg, i.p. for 7 days) selectively increased $CB_2$ expression in $D_1$-MSNs, not in $D_2$-MSNs; **c** repeated cocaine administration failed to alter $CB_1R$ expression in the same samples. $*P < 0.05$, compared to "All cells" (**a** left panel), $D_1$-MSNs (**a** right panel), or saline control mice (**b**).

dichotomy [69, 70]. Accordingly, it is hypothesized that the acute rewarding effects of cocaine are most likely mediated by activation of $D_1$-MSNs via Gs-coupled $D_1$ receptors and inhibition of $D_2$-MSNs via Gi-coupled $D_2$ receptors [67, 71, 72] (Fig. 7). Since $CB_2Rs$ are Gi-coupled receptors, we further hypothesized that $CB_2R$ upregulation in VTA DA neurons or NAc $D_1$-MSNs would increase neuronal response to endocannabinoids or $CB_2R$ agonists, producing enhanced inhibitory effects on both presynaptic DA neurons and postsynaptic $D_1$-MSNs, which subsequently counteracts cocaine-induced increase in extracellular DA and cocaine's action in postsynaptic $D_1$-MSNs (Fig. 7). Thus, the present finding of $CB_2$ upregulation in striatal $D_1$-MSNs provides new mechanistic insight on how brain $CB_2Rs$ modulate pharmacological action produced not only by cocaine, but also by other drugs of abuse, such as opioids, nicotine and alcohol.

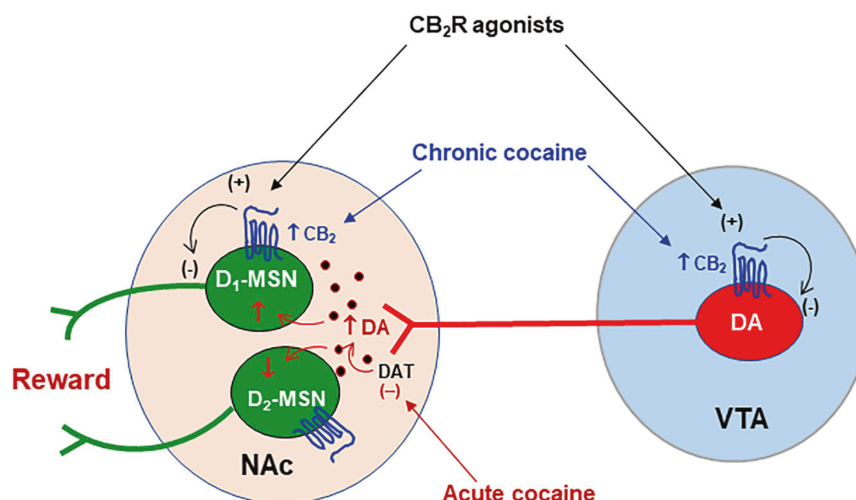



**Fig. 7 Schematic diagram showing the effects of cocaine on CB$_2$R expression in the mesolimbic DA reward system.** This system originates from DA neurons in the VTA and projects to the NAc. Acute cocaine exposure blocks DA transporter (DAT) and elevates extracellular DA level in the NAc, which subsequently activates D$_1$-MSNs via Gs-coupled D$_1$Rs and inhibits D$_2$-MSNs via Gi-coupled D$_2$Rs, producing rewarding effects. Chronic cocaine administration increases CB$_2$R expression in VTA DA neurons as we reported previously [11] and also in NAc D$_1$-MSNs as shown in the present study. Accordingly, activation of CB$_2$Rs by CB$_2$R agonists would inhibit presynaptic VTA DA neurons and postsynaptic D$_1$-MSNs in the NAc, producing therapeutic anti-cocaine effects. VTA ventral tegmental area, NAc nucleus accumbens, (+) activation, (−) inhibition.

In conclusion, brain CB$_2$R involvement has been recently reported in several DA-regulated CNS disorders, including substance abuse and addiction. The present findings of neuronal CB$_2$R upregulation in NAc D1-MSNs not only challenges the well-accepted view that CB$_2$R upregulation is mainly or exclusively expressed in activated microglia, but also provide additional evidence indicating that chronic cocaine use and abuse may upregulate CB$_2$R expression in brain neurons and peripheral immune cells. These new findings suggest that CB$_2$R upregulation may represent a general defense or protective mechanism in response to various insults including chronic psychostimulant abuse and addiction. Thus, brain CB$_2$Rs may constitute a new therapeutic target in medication development for the treatment of cocaine use disorders as well as many other CNS disorders.

## ACKNOWLEDGEMENTS

This research was supported by the Intramural Research Program (IRP) of the National Institute on Drug Abuse (NIDA) (DA000633-01), National Institutes of Health (NIH), and grant support for MKL in UMB (NIH grant R01DA038613).

## AUTHOR CONTRIBUTIONS

HYZ, ELG, MKL, and ZXX designed the experiments. HYZ, LDB, RC, HS, QRL, and MKL conducted the experiments. HYZ, LDB, RC, HS, MKL, and ZXX performed data analyses. HYZ and ZXX wrote the manuscript. LDB, ELG, and MKL revised the manuscript. All authors have approved the final version of this article.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41401-021-00712-6.

**Competing interests:** The authors declare no competing interests.

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