Cannabinoid CB₂ receptors modulate midbrain dopamine neuronal activity and dopamine-related behavior in mice

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Cannabinoid CB₂ receptors (CB₂Rs) have been recently reported to modulate brain dopamine (DA)-related behaviors; however, the cellular mechanisms underlying these actions are unclear. Here we report that CB₂Rs are expressed in ventral tegmental area (VTA) DA neurons and functionally modulate DA neuronal excitability and DA-related behavior. In situ hybridization and immunohistochemical assays detected CB₂ mRNA and CB₂R immunostaining in VTA DA neurons. Electrophysiological studies demonstrated that activation of CB₂Rs by JWH133 or other CB₂R agonists inhibited VTA neuronal firing in vivo and ex vivo, whereas microinjections of JWH133 into the VTA inhibited cocaine self-administration. Importantly, all of the above findings observed in WT or CB₂−/− mice are blocked by CB₂R antagonist and absent in CB₂−/− mice. These data suggest that CB₂R-mediated reduction of VTA DA neuronal activity may underlie JWH133’s modulation of DA-regulated behaviors.

The presence of functional cannabinoid CB₂ receptors (CB₂Rs) in the brain has been controversial. When CB₂Rs were first cloned, in situ hybridization (ISH) failed to detect CB₂ mRNA in brain (1). Similarly, Northern blot and polymerase chain reaction (PCR) assays failed to detect CB₂ mRNA in brain (2–5). Therefore, CB₂Rs were considered “peripheral cannabinoid receptors” (1, 6).

In contrast, other studies using ISH and radioligand binding assays detected CB₂ mRNA and receptor binding in rat retina (7), mouse cerebral cortex (8), and hippocampus and striatum of nonhuman primates (9). More recent studies using RT-PCR also detected CB₂ mRNA in the cortex, striatum, hippocampus, amygdala, and brainstem (9–14). Immunoblot and immunohistochemistry (IHC) assays detected CB₂R immunoreactivity or immunostaining in various brain regions (13, 15–20). The specificities of the detected CB₂R protein and CB₂-mRNA remain questionable, however, owing to a lack of controls using CB₂−/− and CB₂−/− mice in most previous studies (21). A currently accepted view is that brain CB₂Rs are expressed predominantly in astrocytes and microglia during neuroinflammation, whereas brain neurons, except for a very small number in the brainstem, lack CB₂R expression (21).

On the other hand, we recently reported that brain CB₂Rs modulate cocaine self-administration and cocaine-induced increases in locomotion and extracellular dopamine (DA) in the nucleus accumbens in mice (22). This finding is supported by recent studies demonstrating that systemic administration of the CB₂R agonist O-1966 inhibited cocaine-induced conditioned place preference in WT mice, but not in CB₂−/− mice (23), and that increased CB₂R expression in mouse brain attenuates cocaine self-administration and cocaine-enhanced locomotion (19). In addition, brain CB₂Rs may be involved in several DA-related CNS disorders, such as Parkinson’s disease (24), schizophrenia (25), anxiety (26), and depression (27). The cellular mechanisms underlying CB₂R modulation of DA-related behaviors and diseases are unclear, however. Given that midbrain DA neurons of the ventral tegmental area (VTA) play an important role in mediating the reinforcing and addictive effects of drugs of abuse (28, 29), we hypothesized that brain CB₂Rs, similar to other G protein-coupled receptors, are expressed in VTA DA neurons, where they modulate DA neuronal function and DA-related behaviors.

In the present study, we tested this hypothesis using multiple approaches. We first assayed for CB₂ mRNA and protein expression in brain and in VTA DA neurons using quantitative RT-PCR (qRT-PCR), ISH, and double-label IHC techniques. We then examined the effects of the selective CB₂R agonist JWH133 and several other CB₂R agonists on VTA DA neuronal firing in both ex vivo and in vivo preparations using electrophysiological methods. Finally, we observed the effects of microinjections of JWH133 into the VTA on intravenous cocaine self-administration to study whether activation of VTA CB₂Rs modulates DA-dependent behavior. This multidisciplinary approach has provided evidence of functional CB₂Rs in VTA DA neurons. Importantly, all findings observed in WT or CB₂−/− mice are consistent with the hypothesis that CB₂Rs modulate DA neuronal activity and DA-regulated behaviors. Thus, brain CB₂Rs may constitute a new therapeutic target in medication development for treatment of a number of CNS disorders.


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were blocked by a CB2R antagonist and/or absent in CB2−/− mice, suggesting that CB2Rs expressed in VTA DA neurons play an important role in modulating DA neuronal activity and DA-related functions.

**Results**

**CB2 mRNA Is Expressed in the Brain.** We first used qRT-PCR to examine CB2 mRNA expression in the brain and spleen of WT, CB1−/−, and CB2−/− mice using three different TaqMan probes targeting different gene sequences (Fig. 1 and Table S1). Fig. 1 shows the gene structure, two mouse CB2-R transcripts (CB2A and CB2B), the CB2 gene-deleted region in CB2−/− mice (6), and three probes used to detect CB2 mRNA in WT, CB1−/−, and CB2−/− mice. When using the probes that target the conjunction region of exons 1 and 3 (CB2A probe) or exons 2 and 3 (CB2B probe), we detected similar or even higher levels of CB2A and CB2B mRNA in prefrontal cortex, striatum, midbrain, and spleen in CB2−/− mice compared with WT mice (Fig. 1C, Left and Middle); however, when using the CB2-KO probe that targets the deleted gene sequence in CB2−/− mice (Fig. 1B), we detected CB2 mRNA in WT and CB1−/− mice, but not in CB2−/− mice (Fig. 1C, Right). Further quantitative assays indicated that CB2A mRNA was ~60 times lower in cortex than in spleen (Fig. 1C, Left), and that CB2B mRNA was ~500 times lower in cortex than in spleen (Fig. 1C, Middle).

**CB2 mRNA Is Expressed in VTA DA Neurons.** Based on the foregoing findings, we successfully developed a CB2-specific riboprobe targeting the gene-deleted regions in the presently used CB2−/− mice to examine CB2 mRNA expression in VTA DA neurons. In this experiment, we first used traditional ISH methods (SI Experimental Procedures) to examine CB2 mRNA in VTA neurons, and then performed IHC assays with tyrosine hydroxylase (TH) antibody to identify the phenotype(s) of CB2 mRNA-positive neurons. We found that the antisense riboprobe detected CB2 mRNA signaling in the midbrain of WT and CB1−/− mice, but not CB2−/− mice (Fig. 2A, b), and that the corresponding CB2 mRNA sense (control) probe did not detect any mRNA signal (Fig. 2A, b).

To confirm this finding, we used another highly-sensitive RNAscope ISH method, which allowed us to simultaneously detect TH mRNA and CB2 mRNA in VTA neurons. In this experiment, we initially designed two RNAscope probes that target the deleted gene sequences in CB2−/− mice and the 3’ untranslated region (UTR) of the CB2 gene, respectively. We found only one probe targeting the 3’ UTR (Fig. 2 B, a) that worked well. Fig. 2 B, b and c shows CB2 mRNA staining in VTA DA (TH mRNA-positive) neurons in WT and CB2−/− mice using this RNAscope probe. This probe apparently detected CB2 mRNA in CB2−/− mice, because it detected the intact 3’ UTR region located downstream of the gene-deleted sequence (Fig. 2 B, a). Fig. S1 shows representative confocal images under high magnification, illustrating colocalization of CB2 mRNA with TH mRNA in VTA DA neurons of WT and CB2−/− mice. In addition, CB2 mRNA is also expressed in TH-negative VTA non-DA neurons (Fig. S1). Taken together, the

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** CB2 mRNA expression in WT, CB1−/−, and CB2−/− mice. (A) Mouse CB2 genomic structure and transcripts (mRNAs), illustrating that the CB2 gene contains three exons with two separate promoters (P1 and P2). (B) CB2A and CB2B transcripts and the binding sites of three TaqMan probes used to detect CB2 mRNA by RT-PCR. The CB2A and CB2B probes target the 5’ UTR, whereas the CB2-KO probe targets the CB2-deleted gene sequence in the Zimmer strain of CB2−/− mice. (C) CB2 mRNA was detectable in WT, CB2−/−, and CB1−/− mice when using the CB2A or CB2B probe, but was not detectable in CB2−/− mice when using the CB2-KO probe. The CB2 mRNA levels in each brain or spleen tissue are the relative levels (folds) compared with those in cortex of WT mice (defined as 1). All quantified data are normalized to control (cortex). Error bars indicate ±SEM. *P < 0.05, compared with WT mice. NM_009924.2 and AK036658.1 are the GenBank cDNA codes.
mRNA staining in VTA DA neurons, but not in CB mice, mRNA in midbrain (Zimmer strain). These findings suggest that (i) the presently used CB strain is a partial CB knockout mouse, (ii) both the NIH-5633 and Alomone mCB2 antibodies display a significant degree of (but not absolute) mCB2-R specificity, and (iii) the Abcam rCB2 antibody specificity is unknown, because N terminal-containing CB2-R fragment(s) may be present in this strain of CB2−/− mice.

CB2-R Immunostaining Is Detected in Splenocytes, but Is Barely Detectable in Glial Cells. We also used the same antibodies to detect CB2 immunostaining in CB2-rich splenocytes. Fig. S4 shows that both the NIH-5633 and Alomone antibodies detected high densities of CB2 immunostaining in splenocytes of WT mice, but very low densities in CB2−/− mice. Finally, we used the two different ISH assays with two probes targeting different gene sequences detected similar CB2 mRNA staining in VTA DA neurons, suggesting that the CB2−/− mice used in this study (Zimmer strain) are partial knockouts, and that the majority of the upstream and downstream gene sequences from the gene-deleted region are still present in this strain of CB2−/− mice.

CB2-R Immunostaining in VTA DA Neurons. After detecting CB2 mRNA, we assayed for CB2-R protein expression in VTA DA neurons using IHC techniques. Fig. S2A shows the mouse CB2-R structures in WT and CB2−/− mice, the deleted receptor region in CB2−/− mice, and the binding sites (epitopes) of three antibodies on mouse CB2-Rs. Fig. S2B shows representative mouse CB2-R immunostaining with the NIH-5633 mouse CB2 (mCB2) antibody under various magnifications (4x, 10x, 20x, and 40x) in the midbrain of WT mice, illustrating CB2-R immunostaining in VTA DA neurons (labeled by TH; yellow in merged images), as well as in VTA non-DA cells (green cells in merged images). Fig. S3 shows representative CB2 immunostaining with the Abcam rCB2 Ab that recognizes the intact N terminal of CB2 Rs in CB2−/− mice, illustrating similar densities of CB2 immunostaining in VTA DA neurons in WT, CB1−/−, and CB2−/− mice.

Fig. 3 A and B presents representative confocal images of mCB2-R immunostaining using the NIH-5633 and Alomone mCB2 antibodies that target the deleted intracellular third loop or the deleted C terminal of CB2-Rs in CB2−/− mice, illustrating that CB2-R staining was detected in VTA DA neurons in WT and CB1−/− mice, but barely detectable in CB2−/− mice. Pre-absorption of the antibody with specific immune peptide blocked CB2-R immunostaining in VTA DA neurons, suggesting that both antibodies are mCB2-specific. Fig. 3 C and D shows mean densities of CB2-R immunostaining per DA neuron over hundreds of TH-positive DA neurons from two to five WT, CB1−/−, or CB2−/− mice, illustrating a significant reduction in the density of CB2-R immunostaining in DA neurons in CB2−/− mice compared with WT mice (Fig. 3C: F2,1067 = 142.42, P < 0.001; Fig. 3D: F2,669 = 235.50, P < 0.001). These findings suggest that (i) the presently used CB1−/− strain is a partial CB1 knockout mouse, (ii) both the NIH-5633 and Alomone mCB2 antibodies display a significant degree of (but not absolute) mCB2-R specificity, and (iii) the Abcam rCB2 antibody specificity is unknown, because N terminal-containing CB2-R fragment(s) may be present in this strain of CB2−/− mice.

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same antibodies to determine whether CB₂ receptors are expressed in glial cells. Fig. S5 shows that the NIH-5633 antibody detected a very low density of CB₂ immunostaining in GFAP-labeled astrocytes, but not in CD11b-labeled microglia, in mice treated with vehicle (saline) or lipopolysaccharide (LPS), a bacterial endotoxin. These data suggest that CB₂Rs are expressed mainly in DA neurons rather than in glial cells in the VTA.

CB₂Rs Modulate Neuronal Firing in Single Dissociated VTA DA Neurons. None of the foregoing tested antibodies was completely mCB₂-specific; thus, we next used electrophysiological methods to examine whether CB₂Rs in VTA DA neurons are functionally responsive to CB₂R ligands. We first examined the effects of the selective CB₂R agonist JWH133 on DA neuronal firing using perforated patch-clamp recording in single dissociated VTA DA neurons. Identification of DA neurons was based on three criteria (30, 31): electrophysiology [DA neurons exhibit low spontaneous firing rates (FRs; 1–3 Hz) with long action potential (AP) duration and a distinctive H-current], pharmacology [DA neuronal firing is inhibited by DA or by a D₂ receptor agonist (e.g., quinpirole)], and IHC staining (recorded neurons are TH-positive) (Fig. S6).

Fig. 4 shows a representative dissociated TH-positive VTA DA neuron (Fig. 4A) and characteristic low DA neuronal FR (~1 Hz) (Fig. 4B). JWH133 significantly reduced VTA DA neuronal firing in WT mice in a dose-dependent manner (Fig. 4B and C, a; ***P < 0.01, paired t test, compared with pre-JWH133 baseline). This effect was blocked by coadministration of the selective CB₂R antagonist AM630 (1 μM) and was absent in CB₂−/− mice (Fig. 4C, a). AM630 alone (1 μM) did not affect neuronal firing. In addition, we also tested the effects of four additional CB₂R agonists [GW405833 (GW), SER601, CB65, and HU308] on VTA DA neuronal firing, and found that all tested CB₂R agonists produced similar inhibitory effects on DA neuronal firing (Fig. 4C, b) (*P < 0.05, paired t tests).

Fig. 4. Activation of CB₂Rs reduces VTA DA neuronal firing ex vivo. (A) Phase-photo image showing a dissociated VTA DA neuron. (B and C) Representative recording and summarized data illustrating that JWH133 and additional four CB₂R agonists (GW405833, SER601, CB65, and HU308) inhibited VTA DA neuronal firing similarly in WT mice, but not in Zimmer CB₂−/− mice. This inhibitory effect was blocked by coadministration of AM630 (1 μM). (D and E) Representative AP traces and summarized group data illustrating that JWH133 altered membrane potential (MP), AP firing rate, AP initiation, AP duration, and AHP in WT mice. (F and G) Representative depolarizing current-induced AP firing and summarized data illustrating that JWH133 decreased VTA DA neuronal excitability. (H and I) Representative records and summarized data illustrating that JWH133 or GW405833 (GW) inhibited VTA DA neuronal firing in brain slices in a concentration-dependent manner. This effect was blocked by coadministration of AM630 and was absent in CB₂−/− mice. All quantified data are normalized to control (predrug baseline). Error bars indicate ± SEM. *P < 0.05; **P < 0.01, compared with predrug controls. Also see Fig. S6.
Further analysis of neuronal firing patterns revealed that JWH133 significantly attenuated the AP FR (Fig. 4 D, a), hyperpolarized the membrane potential (Fig. 4 D, b), prolonged AP initiation latency (Fig. 4 D, b), shortened AP duration (Fig. 4 D, c), and increased the afterhyperpolarization potential (AHP) (Fig. 4 D, d). Fig. 4E shows pooled data, illustrating that JWH133-induced changes in each AP parameter were statistically significant compared with pre-JWH133 baselines (*P < 0.05; **P < 0.01, paired t tests). The actual values of each AP parameter before and after JWH133 administration are presented in Table S2. Fig. 4 F and G shows AP firing induced by injections of different intensities of current (10–50 pA) via the recording electrode, illustrating that JWH133 (1 μM) significantly shifted the input–output relationship curve to the right (Fig. 4G; two-way ANOVA for repeated measures over injection current, F2,15 = 3.95, P < 0.05), suggesting reduced neuronal excitability of VTA DA neurons after JWH133 application.

CB2Rs Modulate DA Neuronal Activity in ex Vivo VTA Slices. We also tested the effects of JWH133 on VTA DA neuronal firing in VTA slice preparations using cell-attached patch-clamp recording techniques. In this recording mode, the intracellular environment of the recorded neurons is not interrupted. Fig. 4H shows representative records of VTA DA neuronal firing, illustrating that JWH133 significantly reduced VTA DA neuronal firing in VTA slices. Fig. 4I shows pooled data, illustrating that JWH133 and GW405833 significantly inhibited neuronal firing in a dose-dependent manner in WT mice (**P < 0.01, paired t test). This effect was blocked by AM630 (1 μM) and was absent in CB2−/− mice.

We also examined the effects of JWH133 on GABA neuronal firing in VTA slices prepared from mice expressing GFP under the control of the GAD67 promoter (GAD67-GFP knock-in mice) (32) (Fig. S6). We found that, unlike VTA DA neurons, VTA GABA neurons (labeled by GFP) were insensitive to JWH133 (1, 10 μM) under the same experimental conditions (Fig. S6 D, c; F3,36 = 0.85, P > 0.05), suggesting that CB2Rs predominantly modulate VTA DA, but not GABA, neuronal function.

CB2Rs Modulate VTA DA Neuronal Firing in Vivo. To determine whether the effects observed in ex vivo cells and brain slices can be seen in vivo, we examined the effects of systemic administration of JWH133 on VTA DA neuronal firing in anesthetized mice using single-unit recording techniques. The criteria for identification of DA neurons in vivo were the same as we reported previously (33, 34). Fig. 5A shows the recording sites as revealed by histological examination after completion of electrophysiological recording. Fig. 5B shows a representative extracellular recording, illustrating that systemic administration of JWH133 (10 or 20 mg/kg i.p.) dose-dependently inhibited the basal FR and burst firing (BS) rate (defined as the number of burst spikes/s) in VTA DA neurons. This effect was reversed by systemic administration of the D2R antagonist, 0.1 mg/kg. All quantified data are normalized to control. Error bars indicate SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared with predrug controls.

Fig. 5. CB2R activation inhibits VTA DA neuronal firing in vivo. (A) Brain section image illustrating the track of a recording electrode and tips (recording sites) of the electrodes in the brain, and a characteristic action potential in a VTA DA neuron. (B) Representative extracellular single unit recording illustrating that JWH133 (10 or 20 mg/kg i.p.) dose-dependently inhibited basal FR and BS of VTA DA neurons in an anesthetized WT mouse. This effect was reversed by AM630 (10 mg/kg) administered 10 min after JWH133 injection. (C) Normalized FRs over the pre-JWH133 baseline, illustrating that JWH133 dose-dependently inhibited VTA DA neuronal firing in WT and CB2−/− mice, but not in CB2−/− mice. (D and E) Representative single-unit recording and summarized data illustrating that AM630 (10 mg/kg) alone failed to alter basal FR or ISI CV, but slightly potentiated BS. AM630 pretreatment prevented 20 mg/kg JWH133-induced inhibition of neuronal firing. Subsequent administration of quinpirole (a DA D2R agonist, 0.1 mg/kg) inhibited VTA DA neuronal firing, which was reversed by haloperidol (a D2R antagonist, 0.1 mg/kg). All quantified data are normalized to control. Error bars indicate ±SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared with predrug controls.
of 10 mg/kg AM630. Fig. 5C shows normalized FRs over pre-
JWH133 baselines, illustrating that JWH133 significantly inhibited
VTA DA neuronal firing in WT and CB2−/− mice, but not in CB2−/+
mice. One-way ANOVA for repeated measures over JWH133
doses revealed a significant JWH133 treatment main effect on
basal FR and BS in WT mice (F2,27 = 5.07, P < 0.001 and F3,27 =
6.65, P < 0.01, respectively) and in CB2−/− mice (F2,12 = 6.35, P <
0.05 and F2,12 = 6.87, P < 0.05), but not in CB2+/− mice (Fig. 5
C, a and b). In addition, JWH133 also significantly decreased
the interspike interval coefficient of variation (ISI CV) in WT
mice (from pre-JWH133 baseline of 1.0 ± 0 to 0.84 ± 0.04 after
10 mg/kg JWH133 or to 0.83 ± 0.03 after 20 mg/kg JWH133;
F3,36 = 4.03, P < 0.05), suggesting a significant alteration in the
burst firing pattern of VTA DA neurons after JWH133 administra-
tion (35).

We note that the inhibitory effect produced by JWH133 on
burst firing was not completely reversed by AM630 administered
after JWH133 (Fig. 5 C, b). Therefore, we further examined the
effects of AM630 pretreatment on JWH133’s action in VTA DA
neurons. We found that pretreatment with AM630 (10 mg/kg)
completely prevented JWH133-induced reduction in basal FR,
burst firing, and ISI CV (Fig. 5 D and E), suggesting CB2-
mediated effects. In addition, AM630 alone slightly enhanced
burst firing (Fig. 5E), but this enhancement was not statistically
significant compared with the pre-AM630 baseline (F2,21 = 0.50,
P > 0.05).

CB2Rs in the VTA Modulate Cocaine Self-Administration. Finally, we
investigated whether activation of CB2-Rs in the VTA alters DA-
regulated behavior. VTA DA neurons play a critical role in the
rewarding effects of cocaine (28); thus, we examined whether
microinjection of JWH133 into the VTA alters i.v. cocaine self-
administration. In this experiment, 12 WT and 12 CB2−/− mice
were allowed daily cocaine self-administration. After 3–4 wk of
daily access to cocaine (0.5 mg/kg for 3 h), most of the WT (9 of
12) and CB2−/− mice (8 of 12) had acquired stable cocaine self-
administration, defined as (i) at least 20 infusions per 3-h ses-
tion, (ii) <20% variability in daily cocaine infusions across two
consecutive sessions, and (iii) an active/inactive operant response
ratio exceeding 2:1 (22, 36).

We found that bilateral microinjections of JWH133 (1 or 3 μg/1
μl/side) into the VTA (Fig. 6 A, a) significantly reduced cocaine
self-administration in WT mice, but not in CB2+/+ mice (Fig. 6 A, b
and c). This effect was blocked by coadministration of AM630 and
JWH133, whereas microinjections of AM630 alone had no effect
on cocaine self-administration. One-way ANOVA for re-
peated measures over dose revealed a significant JWH133
treatment main effect in WT mice (F3,16 = 4.83, P < 0.05), but not
in CB2−/− mice (F2,12 = 0.81, P > 0.05) (Fig. 6 A, c). In contrast,
bilateral microinjections of the same doses of JWH133 into the
VTA failed to alter oral sucrose self-administration (Fig. 6 A, d).
Furthermore, bilateral microinjections of the same doses of
JWH133 into a brain region adjacent to but outside and
dorsal to the VTA (Fig. 6 B, a) had no effect on cocaine self-administration (Fig. 6 B, b). These results suggest that activation
of VTA CB2-Rs selectively modulates cocaine, but not food,
self-administration behavior.

Discussion

The major findings of the present study can be summarized as
follows: (i) qRT-PCR detected low levels of CB2 mRNA in
several brain regions; (ii) ISH and IHC assays detected CB2
mRNA and receptor expression in VTA DA neurons; (iii) activa-
tion of CB2-Rs by JWH133 or other CB2 agonists inhibited
VTA DA neuronal firing in single dissociated neurons, VTA
slices, and anesthetized animals; (iv) microinjections of JWH133
into the VTA inhibited cocaine self-administration; and (v) all of
the foregoing effects of JWH133 were blocked by CB2-R antagon
or absent in CB2−/− mice. Taken together, these findings from
genes to behavior provide convincing evidence that brain CB2-Rs
are expressed in VTA DA neurons, where they modulate DA
neuronal function and DA-regulated behavior.

CB2 mRNA Is Expressed in Mouse Brain. Although growing evidence
now suggests the presence of CB2-Rs in brain, conclusive
evidence has been lacking owing to a lack of CB2−/− mice and other
necessary controls. Thus, whether functional CB2-Rs are expressed
in VTA DA neurons has been unclear. To fully address these
issues, we used multiple experimental approaches to study CB2-R
expression and function in VTA DA neurons. We first used qRT-
PCR to detect CB2 mRNA expression in the brains of WT, CB2−/−,
and CB2−/− mice. We found that three different probes (CB2A,
CB2B, and CB2-KO probes) detected CB2 mRNA in the brains of
WT mice. The findings in CB2−/− mice depended on the probes
used. When we used probes targeted at the CB2-R gene sequences
upstream and downstream from the region, the CB2 mRNA signal
was consistently detectable, whereas when we used a probe tar-
getting the gene-deleted region, no CB2 mRNA signal was detected.
These findings suggest that the CB₂/−/− mice used in this study are actually partial, not full, knockouts in gene structure. Although these animals lack functional CB₂Rs, the majority of the CB₂ gene sequence is still present; thus, extreme caution is required when addressing CB₂ signaling specificity using such CB₂/−/− mice as controls.

We note that brain CB₁s, and CB₂s mRNA levels are very low compared with that in CB₂-rich spleen (~60-fold lower for CB₂ mRNA and ~500-fold lower for CB₂ mRNA in cortex than in spleen). This may explain why earlier ISH and RT-PCR studies failed to detect brain CB₂ mRNA, given that the experimental conditions used to detect high-density CB₂ mRNA in spleen might not be optimal for detecting low levels of CB₂ mRNA in the brain. Low densities of CB₂ mRNA in brain do not necessarily mean low levels of CB₂R expression, however. For example, brain opioid receptor mRNA levels are generally very low, particularly in the cerebral cortex, olfactory bulb, and spinal cord (37, 38); however, high densities of opioid receptors are expressed in those brain regions (37, 39). Because the CB₂-KO probe targeting the gene-deleted region in CB₂/−/− mice detected CB₂ mRNA in WT and CB₂/−/− mice, but not in CB₂/−/− mice, we posit that the mRNA signaling detected by this probe is mCB₂-specific.

**CB₂ mRNA Is Expressed in VTA DA Neurons.** The foregoing findings with the CB₂-KO probe led us to successfully develop a mCB₂-specific riboprobe that allowed us to use the CB₂/−/− mice as a negative control for studying CB₂ gene expression in VTA DA neurons. Using this probe, which also targets the gene-deleted region in CB₂/−/− mice, we found that CB₂ mRNA is expressed in VTA neurons in WT and CB₂/−/− mice, but not in CB₂/−/− mice. This is consistent with the foregoing findings from qRT-PCR. Furthermore, double-label CB₂ mRNA assays (by ISH) and TH assays (by IHC) detected a low-to-moderate density of CB₂ mRNA staining in TH-positive VTA DA neurons in WT mice, but not in CB₂/−/− mice, suggesting that CB₂R mRNA is natively expressed in mouse VTA DA neurons.

To further explore these findings, we successfully developed another riboprobe (CB₂; RNAseq probe) that allowed us to readily detect very low levels of CB₂ mRNA in the VTA and other brain loci. This probe is mCB₂-specific because it is a long riboprobe with 943 base pairs (SI Experimental Procedures) and targets a 3′ UTR sequence of the mCB₂ gene that shows no homology with CB₁ or other genes in the nucleotide sequence (Advanced Cell Diagnostics). In addition, RNAseq ISH uses a unique double RNA-specific oligonucleotide probe design strategy; thus, the chance of such Z-Z probes binding nonspecifically next to each other is very low (40). Furthermore, the RNAseq technology uses a unique probe design strategy that allows simultaneous amplification of multiple target mRNA signals and suppression of background noise signals (40). Therefore, it is highly sensitive to very low levels of mRNA, even a single molecule, and thus is particularly suitable for detecting CB₂ mRNA expression in the brain. Using such a probe, we detected low-to-moderate CB₂ mRNA in VTA DA neurons. This is congruent with our findings from qRT-PCR (Fig. 1) and from ISH combined with IHC (Fig. 24). This probe also apparently detected CB₂ mRNA signaling in CB₂/−/− mice (Fig. 2B), targeting the downstream 3′ UTR as distinct from the upstream gene-deleted region. These congruent findings using two different ISH assays with two different probes strongly suggest that CB₂ mRNA is expressed in VTA DA neurons.

**CB₂R Protein Is Expressed in VTA DA Neurons.** To determine whether CB₂R proteins are expressed in VTA DA neurons, we used three strains of mice (WT, CB₁/−/−, and CB₂/−/−) and three different CB₂ antibodies (Abcam, Alomone, and NIH-5633) for CB₂ immunostaining. Similar patterns and densities of CB₂R immunostaining in VTA DA neurons were detected in WT and CB₁/−/− mice; however, the findings in CB₂/−/− mice depended on the epitope of the antibody. Both the NIH5633 and Alomone mCB₂ antibodies with epitope at the receptor-deleted region in CB₂/−/− mice detected much higher densities of CB₂-R immunostaining in both VTA DA neurons and spinoocytes in WT mice than in CB₂/−/− mice, whereas the Abcam rCB₂ antibody with epitope at the intact N terminal of CB₂-Rs detected similar densities of CB₂ immunostaining, supporting mCB₂-specificity of the detected signal. We note that both the NIH5633 and Alomone antibodies should not detect CB-R immunostaining in CB₂/−/− mice, because they target the receptor-deleted region; however, both antibodies detected weak immunostaining in both VTA and splen cells in CB₂/−/− mice, suggesting that they are not absolutely mCB₂-R-specific.

It has been reported that CB₂Rs are expressed and up-regulated in microglia during neuroinflammation in humans (21, 41). Our experiments did not reveal obvious CB₂R immunostaining in glial cells in the VTA. To confirm this, we used a GFAP antibody and a CD11b antibody to label VTA astrocytes and microglia, respectively, and used LPS to stimulate glial cell proliferation. We observed only weak CB₂ immunostaining in GFAP-labeled astrocytes and no CB₂ immunostaining in CD11b-labeled microglia. These findings are consistent with previous reports that microglia and astrocytes may not express CB₂Rs in healthy rats and mice (14, 41–43). More studies are required to address it.

**CB₂R Activation Inhibits VTA DA Neuronal Firing.** To determine whether CB₂Rs expressed in VTA DA neurons are functional, we used electrophysiological methods to study the response(s) of VTA DA neurons to selective CB₂ ligands. We explored this at three levels: single dissociated DA neurons, DA neurons in VTA slices, and DA neurons in intact anesthetized mice. We found that systemic or local administration of JWH133 or any of the four other CB₂ agonists significantly inhibited VTA DA neuronal firing in a dose- or concentration-dependent manner in WT and CB₁/−/− mice. This inhibitory effect was reversed by pharmacologic blockade of CB₂-Rs (AM630) or was absent in CB₂/−/− mice, suggesting a CB₂-R-mediated effect. This finding is consistent with previous reports in which JWH133 or other CB₂ agonists inhibited spontaneous and evoked neuronal firing to noxious stimuli in spinal cord and thalamus (44–47) and inhibited excitatory neuronal firing in the prefrontal cortex (48). Thus, our electrophysiological data provide direct evidence demonstrating that the expressed CB₂-Rs in VTA DA neurons are functional, and that activation of these receptors inhibits VTA DA neuronal firing and decreases VTA DA neuronal excitability.

Furthermore, we found little effect of JWH133 on neuronal firing in identified VTA GABA neurons using GAD67-GFP transgenic mice, apparently contrary to a previous report that CB₂-R agonists inhibit GABAergic synaptic transmission in cerebral cortex (49). This finding may be related to different cellular distributions of CB₂-Rs in different brain regions.

As noted above, the burst activity of VTA DA neurons has been associated with increased vulnerability to cocaine self-administration (50). Consequently, we investigated whether VTA CB₂-R activation inhibits cocaine self-administration behavior. We found that microinjections of JWH133 into the VTA selectively decreased cocaine, but not sucrose, self-administration in WT and CB₁/−/− mice, but not in CB₂/−/− mice. In contrast, microinjections of the same doses of JWH133 into a dorsal brain region adjacent to the VTA failed to alter cocaine self-administration, suggesting an effect produced by activation of CB₂-Rs in the VTA, not in adjacent structures. This is consistent with our previous findings that systemic or local administration of JWH133 into the nucleus accumbens inhibits cocaine self-administration (22), and that transgenic up-regulation of brain CB₂-Rs attenuates cocaine self-administration and cocaine-induced increased locomotion (19). The present findings are also congruent with recent
reports that brain CB2Rs have important roles in other DA-related functions and/or CNS disorders (12, 25, 51, 52).

In conclusion, the present study demonstrates that CB2Rs are expressed in VTA DA neurons and functionally modulate DA neuronal excitability and DA-related behavior. Considering that midbrain DA neurons play important roles in brain reward, locomotion, cognition, motivation, and various goal-directed behaviors, our findings provide direct evidence supporting an important role for VTA CB2Rs in these actions, as well as in DA-related neurologic disorders, such as drug addiction. Thus, brain CB2Rs may constitute a new therapeutic target for treatment of such CNS disorders.

Experimental Procedures

Animals. Male WT, CB1 receptor knockout (CB1−/−) (53), and CB2−/− mice (6, 54) with C57BL/6J genetic backgrounds were bred at the National Institute on Drug Abuse. In some experiments, glutamate decarboxylase-67 (GAD67)-GFP knock-in mice on a CD-1 background (55) were used for recording VTA GABA neuronal firing. All experimental procedures were conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals (56), and were approved by the National Institute on Drug Abuse’s Animal Care and Use Committee. The animals used for the electrophysiology experiments were transferred from the National Institute on Drug Abuse and bred at the Barrow Neurological Institute.

Experimental procedures carried out at the Barrow Neurological Institute were conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals (56) and the Barrow Neurological Institute’s Institutional Animal Care and Use Committee guidelines.

qRT-PCR. The qRT-PCR assay of brain CB2, mRNA levels was performed as described previously (15). Because immune cells in blood contain a high density of CB2Rs, all mice used for qRT-PCR were perfused transcardially with 30–50 mL 0.9% saline under deep anesthesia, to prevent contamination of brain tissue by blood cells. Then brain and spleen were removed, and the prefrontal cortex, striatum, and midbrain were dissected. Three specific CB2 probes were used: a CB2A probe that recognizes the conjuncture region of exons 1 and 3, a CB2B probe that recognizes the conjuncture region of exons 2 and 3, and a CB2-KO probe that targets the gene-deleted region close to the 3′ end of exon 3 in CB2−/− mice. Mouse β-actin mRNA served as an endogenous control. The specific base pair sequences of the minor groove binder (MGB)- TaqMan probes and the primers used to detect CB2 and β-actin mRNAs are listed in Table S1.

ISH. CB3, ISH and TH IHC Assays. Total RNA was isolated from C57BL/6J mouse brain samples using the Qiagen RNeasy Mini Kit according to the manufacturer’s protocol. cDNA was synthesized from total RNA using the Bio-Rad iScript cDNA Synthesis Kit. For CB2-mRNA riboprobe synthesis, oligonucleotide primers were designed specifically to detect the deleted mCB2 mRNA region in CB2−/− mice: forward primer 5′-AGCTGGATGCGGTAGAAC-3′ and reverse primer 5′-AGGCTGGCCCCATGAGA-3′. The template cDNA sequence of CB2Rs were obtained from GenBank (accession number: NM_009924.2). RNase A ISH Assays. Both the CB2 and TH RNA probes were designed and provided by Advanced Cell Diagnostics (Hayward, CA). The CB2-specific RNA probe was designed to detect the 3′ UTR (1877–2820 bp) of the Mus Cnr2 mRNA sequence (NM_009377.1, C1 channel) (Fig. 2B, a). The TH RNA probe was designed to detect 483–1,603 bp of Mus musculus TH mRNA sequence (NM_009924.3, C2 channel). Complete experimental methods for CB2-ISH assays, along with TH-ISH and RNase A ISH assays, are described in SI Experimental Procedures.

IHC Assays. Three CB3R antibodies were used to detect CB3R expression in VTA DA neurons in WT, CB1−/−, and CB2−/− mice. Complete experimental methods for IHC assays are described in SI Experimental Procedures.

Electrophysiology Studies. Standard electrophysiological methods were used to record VTA DA neuronal responses to selective CB3R ligands in single dissociated VTA DA neurons (using perforeted patch-clamp recording), in VTA slices (using cell-attached patch-clamp recording), and in anesthetized mice (using extracellular single-unit recording). Complete electrophysiological methods are described in SI Experimental Procedures.

Intravenous Cocaine Self-Administration. Animal surgery, cocaine or sucrose self-administration, and intracranial microinjection procedures were as described previously (22). In brief, after stable cocaine or sucrose self-administration was achieved, subjects randomly received one microinjected dose of intra-VTA JWH133 (1 or 3 μg/side), AM630 (3 μg/side), a mixed solution of AM630 (3 μg/side) and JWH113 (3 μg/side), or vehicle (Tocrisolve 100) 30 min before cocaine or sucrose self-administration. After each test, animals underwent an additional 3–5 d of self-administration until the baseline response rate was reestablished before the next dose was tested. Cannula placements were verified after completion of the experiments by standard histological and anatomic localization techniques.

Data Analyses. All data are presented as means ± SEM. One-way or two-way ANOVA (SigmaStat software) was used to analyze the significance of the effects of JWH113 or other drugs on neuronal firing and cocaine self-administration. Individual group comparisons were carried out using the Student Newman–Keuls method. In addition, paired t tests were used to analyze some electrophysiological data, as described previously (30, 31). F ratios and burst firing were determined every 10 s, as described previously (57, 58).

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56. Committee on Care and Use of Laboratory Animals (1996) Guide for the Care and Use of Laboratory Animals (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.  