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ARTICLE

CB_2 receptor antibody signal specificity: correlations with the use of partial CB_2-knockout mice and anti-rat CB_2 receptor antibodies

Hai-ying Zhang[1], Hui Shen[2], Chloe J. Jordan[1], Qing-rong Liu[3], Eliot L. Gardner[1], Antonello Bonci[2] and Zheng-xiong Xi[1]

Cannabinoid CB_1 receptors are highly expressed in the brain and functionally modulate presynaptic neurotransmitter release, while cannabinoid CB_2 receptors (CB_2Rs) were initially identified in the spleen and regarded as peripheral cannabinoid receptors. Recently, growing evidence indicates the presence of functional CB_2Rs in the brain. However, this finding is disputed because of the specificity of CB_2R antibody signals. We used two strains of currently available partial CB_2-knockout (CB_2-KO) mice as controls, four anti-rat or anti-mouse CB_2R antibodies, and mRNA quantification to further address this issue. Western blot assays using the four antibodies detected a CB_2R-like band at ~40 kD in both the brain and spleen. Notably, more bands were detected in the brain than in the spleen, and specific immune peptides blocked band detection. Immunohistochemical assays also detected CB_2-like immunostaining in mouse midbrain dopamine neurons. CB_2R deletion in CB_2-KO mice may reduce or leave CB_2R-like immunoreactivity unaltered depending on antibody epitope. Antibodies with epitopes at the receptor-deleted region detected a significant reduction in CB_2R band density and immunostaining in N-terminal-deleted Deltagen and C-terminal-deleted Zimmer strain CB_2-KO mice. Other antibodies with epitopes at the predicted receptor-undeleted regions detected similar band densities and immunostaining in wild-type and CB_2-KO mice. Quantitative RT-PCR assays detected CB_2 mRNA expression using probes that targeted upstream or downstream gene sequences but not the probe that targeted the gene-deleted sequence in Deltagen or Zimmer CB_2-KO mice. These findings suggest that none of the tested four polyclonal antibodies are highly mouse CB_2R-specific. Non-specific binding may be related to the expression of mutant or truncated CB_2R-like proteins in partial CB_2-KO mice and the use of anti-rat CB_2 antibodies because the epitopes are different between rat and mouse CB_2Rs.

Keywords: cannabinoid; CB_2 gene; CB_2 receptors; CB_2 receptor antibody; specificity; CB_2-KO mice; midbrain dopamine neurons; spleen; specific immune peptides

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INTRODUCTION

The endogenous cannabinoid system consists of endocannabinoids (e.g., anandamide and 2-arachidonoylglycerol), the enzymes responsible for their synthesis and degradation, and cannabinoid receptors and transporters [1, 2]. Two types of cannabinoid receptors (CB_1Rs and CB_2Rs) have been identified. CB_1Rs are highly expressed in the brain and functionally modulate presynaptic neurotransmitter release [1, 2]. In contrast, CB_2Rs were initially identified in the spleen and regarded as peripheral cannabinoid receptors [3, 4]. This view has been challenged by recent findings that CB_2R and its mRNA are expressed in the brains of rats and mice [5–9]. In addition, Western blot (WB) assays and immunohistochemistry (IHC) consistently detected CB_2R signaling in multiple brain regions and neuronal phenotypes [5, 6, 10–16]. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis has also identified CB_2 mRNA and its isoforms in several regions of the central nervous system, including the retina [17, 18], cortex [8, 19–21], striatum [20, 21], hippocampus [21, 22], amygdala [19, 20], brainstem [5], and cerebellum [23]. In situ hybridization (ISH) assays revealed CB_2R mRNA expression in several neuronal phenotypes, including glutamatergic neurons in the cortex and hippocampus [22, 24, 25] and dopaminergic (DA) neurons in the midbrain [9, 26–29]. Electrophysiological assays confirmed the presence of functional CB_2Rs in brain glutamatergic neurons [22, 30–32], GABAergic neurons [33], and DA neurons [9, 22, 26, 29]. However, the specificity of the detected CB_2R signals was questioned because CB_2-knockout (CB_2-KO) mice were not used as controls in many early studies [34]. Recent findings that CB_2R antibody signals were detected in wild-type (WT) and CB_2-KO mice support this skepticism [35, 36]. A C-terminal-deleted partial CB_2-KO strain was used in those studies, and the expression of mutant or truncated CB_2-like proteins may have partially contributed to the observed "non-specific" binding. Anti-rat or anti-human CB_2R antibodies were used in those studies [35, 36], and species differences in antibody epitopes and CB_2R structures [8, 28] may also confound interpretations of antibody signal

[1]Addiction Biology Unit, Molecular Targets and Medications Discovery Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD 21224, USA; [2]Synaptic Plasticity Section, Cellular Neurobiology Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD 21224, USA and [3]Laboratory of Clinical Investigation, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA
Correspondence: Zheng-xiong Xi (zxi@intra.nida.nih.gov)

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specificity. Methodological limitations may be a valid reason for skepticism regarding CB_2R expression in the brain. Therefore, determinations of CB_2R antibody signal specificity are urgently needed to understand the presence and function of CB_2Rs in the brain and the potential utility of CB_2R ligands in the treatment of various neuropsychiatric disorders.

The present study used multiple approaches to investigate CB_2R signal specificity. We used WB assays and four antibodies that targeted the receptor-deleted or -undeleted regions in CB_2-KO mice. We used double-label fluorescent IHC assays and the same antibodies to examine CB_2R immunostaining in midbrain DA neurons in different mouse genotypes. Midbrain DA neurons were chosen because functional CB_2Rs were found in this region [7, 9]. We used qRT-PCR and three TaqMan probes to examine and compare CB_2R gene (mRNA) expression in different mouse genotypes. All of these assays included multiple positive and negative controls, including immune peptides, CB_2-rich spleen tissue, CB_1-KO mice, and two strains of currently available CB_2-KO mice (i.e., the N-terminal-deleted Deltagen strain and the C-terminal-deleted Zimmer strain) to determine the specificity of detected CB_2R signals.

MATERIALS AND METHODS

Animals

Male WT, CB_1-KO [37], and two strains of CB_2-KO mice [4, 30] with C57BL/6J genetic backgrounds were bred at the National Institute on Drug Abuse (NIDA). Genotyping was performed in our laboratory prior to experimentation. All animals used in the present experiments were matched for age (8–14 weeks) and weight (25–35 g). Mice were housed individually in a climate-controlled animal colony room on a reversed light–dark cycle (lights on at 7:00 P.M., lights off at 7:00 A.M.) with free access to food and water. The animals were maintained in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experimental procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the U.S. National Research Council, and the Animal Care and Use Committee of the NIDA of the U.S. National Institutes of Health approved all procedures.

Western immunoblotting assays

Four CB_2 antibodies were used. (1) The Abcam rat CB_2 (rCB_2) polyclonal antibody was purchased from Abcam (ab-3561, Abcam PLC, Cambridge, MA, USA) with epitope (amino acids 1–32) at the rCB_2R N-terminal. The epitopes of rat and mouse CB_2 (mCB_2) receptors differ by 5 amino acid residues. (2) The Alomone rCB_2 polyclonal antibody was purchased from Alomone (ACR-002, Alomone Labs, Jerusalem, Israel), which recognizes the third intracellular loop. The epitope of the Alomone antibody amino acids (228–242 amino acids) is identical between rCB_2Rs and mCB_2Rs. (3) The Mackie rCB_2 antibody was provided by Dr. Ken Mackie at Indiana University. The epitope (amino acids 326–342) of the Mackie antibody differs by 3 amino acids between rCB_2Rs and mCB_2Rs. (4) A mCB_2 polyclonal antibody (NIH5633, Baltimore, MD, USA) was custom designed. The epitope (amino acids 326–340) is located at the mCB_2R C-terminal. The NIH5633 mCB_2 antibody was produced by Genemed Synthesis, Inc. (San Antonio, TX, USA). Table 1 details the epitopes of these CB_2 antibodies. The immune peptide for each individual antibody stated above was purchased from the same antibody provider and the amino acid sequences (epitopes) of the immune peptides are highlighted in Table 1.

All mice for WB assays were perfused transcardially with 0.9% saline under deep anesthesia to prevent contamination of brain tissue with CB_2-rich immune cells in blood. Whole striatum and the spleen were dissected. Tissues were homogenized in RIPA lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA), and the protein concentration of each sample was quantified using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). A total of 30 µg spleen proteins and 50 µg striatal proteins were used for Western immunoblot assays. Membranes were incubated with a rabbit anti-CB_2 antibody (Abcam, 1:2000; Alomone, 1:250; NIH5633, 1:1000; Mackie, 1:500) and mouse anti-β-actin (1:2500) (Sigma-Aldrich, St. Louis, MO, USA) and incubated with secondary antibodies, goat anti-mouse IgG for β-actin (IRDye 680CW), and goat anti-rabbit IgG for CB_2 (IRDye 800CW) (LI-COR Bioneurosciences, Lincoln, NE, USA). Membranes were scanned using a LI-COR Odyssey Image System (LI-COR Biosciences, Lincoln, NE, USA). Band density was measured using the Image J software (http://rsb.info.nih.gov/ij/).

IHC assays

The IHC procedures were performed as reported previously [9]. Briefly, mice were deeply anesthetized and transcardially perfused with cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brain tissues were transferred to 20% sucrose in phosphate buffer at 4 °C overnight. Coronal sections were cut at 10 µm on a cryostat (CM3050S, Leica Microsystems Nussloch GmbH, Nussloch, Germany). Tissue sections containing the ventral tegmental area (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 one of the above-listed CB_2 antibodies (Abcam, 1:1000; Alomone, 1:250; NIH5633, 1:500; Mackie, 1:500) 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_2 receptors, and goat anti-mouse Alexa 568 for TH (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. Densitometric analysis was used to quantify CB_2 immunostaining density on individual VTA DA neurons and determine whether deletion of CB_2Rs abolished or attenuated the expression of CB_2Rs in VTA DA neurons [23]. Each CB_2-positive DA neuron was outlined manually, and CB_2 fluorescence intensity was measured using the Image J software. The background signal was defined as the mean background from 5 to 10 regions outside of DA neurons in each slice. The background signal was subtracted, and the ratio F/A was used to define the mean fluorescence of individual DA cells (F) normalized to total cellular surface (A). Quantification was performed on >100 cells from 2 to 5 animals of each strain.

qRT-PCR assays

Immune cells in blood contain a high density of CB_2 receptors. Therefore, all mice used for qRT-PCR were perfused transcardially with 30–50 mL 0.9% saline under ketamine and xylazine anesthesia to prevent contamination of brain tissue. Brains and spleen were removed, and the prefrontal cortex, striatum, and midbrain were dissected. Total RNA was extracted using a RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. The purity and integrity of each extract were determined using absorbance at 260 nm in an Eppendorf BioPhotometer Plus (Eppendorf AG, Hamburg, Germany).

qRT-PCR procedures for the detection of mCB_2 mRNA were performed as reported previously [8, 9]. Briefly, three specific mCB_2 probes were used: a mCB_{2A} probe that recognizes the conjunction region of encoding exons 1 and 3 (aligns with base pair positions 89–182 of the cDNA NM_009924.2); a mCB_2-Zimmer-ko probe (for the Zimmer CB_2-KO strain) that targets a region close to the 3′ end of exon 3 (aligns with base pair positions 885-989 of NM_009924.2; the deleted exon 3 aligns with

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Table 1. The amino acid sequences of CB_2Rs in rats, mice, and two strains of CB_2-KO mice

```
                           (Abcam rCB2-Ab)
                     *    * *                    **             TM1
Rat CB2             MAGCRELELTNGSNGGLEFNPMKEYMILSDAQQIAVAVLCTLMGLLSALENVAVLYLILS 60
Mouse CB2           MEGCRETEVTNGSNGGLEFNPMKEYMILSSGQQIAVAVLCTLMGLLSALENMAVLYIILS 60
mCB2-KO(Zimmer)     MEGCRETEVTNGSNGGLEFNPMKEYMILSSGQQIAVAVLCTLMGLLSALENMAVLYIILS 60
mCB2-KO(Deltagen)   MEGCRETEVTNGSNGGLEFNPMKEY----------------------------------- 60

                                               TM2                        TM3
Rat CB2             SQRLRRKPSYLFIGSLAGADFLASVIFACNFVIFHVFHGVDSRNIFLLKIGSVTMTFTAS 120
Mouse CB2           SRRVRRKPSYLFISSLAGADFLASVIFACNFVIFHVFHGVDSNAIFLLKIGSVTMTFTAS 120
mCB2-KO(Zimmer)     SRRLRRKPSYLFISSLAGADFLASVIFACNFVIFHVFHGVDSNAIFLLKIGSVTMTFTAS 120
mCB2-KO(Deltagen)   ------------------------------------------------------------ 120

                                                         TM4
Rat CB2             VGSLLLTAVDRYLCLCYPPTYKALVTRGRALVALGVMWVLSALISYLPLMGWTCCPSPCS 180
Mouse CB2           VGSLLVTAVDRYLCLCYPPTYKALVTRGRALVALCVMWVLSALISYLPLMGWTCCPSPCS 180
mCB2-KO(Zimmer)     VGSLLLTAVDRYLCLCYPPTYKALVTRGRALVALCVMWVLSALISYLPLMGWTCCPSPCS 180
mCB2-KO(Deltagen)   -----------------PPTYKALVTRGRALVALCVMWVLSALISYLPLMGWTCCPSPCS 180

                                      TM5                          (Alomone rCB2-Ab)
Rat CB2             ELFPLIPNDYLLGWLLFIAILFSGIIYTYGYVLWKAHQHVASLTEHQDRQVPGIARMRLD 240
Mouse CB2           ELFPLIPNDYLLGWLLFIAILFSGIIYTYGYVLWKAHRHVATLAEHQDRQVPGIARMRLD 240
mCB2-KO(Zimmer)     ELFPLIPNDYLLGWLLFIAILFSGIIYTYGYVLWKA------------------------ 216
mCB2-KO(Deltagen)   ELFPLIPNDYLLGWLLFIAILFSGIIYTYGYVLWKAHRHVATLAEHQDRQVPGIARMRLD 240

                                      TM6                           TM7
Rat CB2             VRLAKTLGLVMAVLLICWFPALALMGHSLVTTLSDKVKEAFAFCSMLCLVNSMINPIIYA 300
Mouse CB2           VRLAKTLGLVLAVLLICWFPALALMGHSLVTTLSDQVKEAFAFCSMLCLVNSMVNPIIYA 300
mCB2-KO(Zimmer)     ------------------------------------------------------------ 300
mCB2-KO(Deltagen)   VRLAKTLGLVLAVLLICWFPALALMGHSLVTTLSDQVKEAFAFCSMLCLVNSMVNPIIYA 300

                                              (Mackie rCB2-Ab)
                                             *     * *
Rat CB2             LRSGEIRSAAQHCLTGWKKYLQGLGSEGKEEAPKSSVTETEAEVKTTTGPGSRTPGCSNC 360
Mouse CB2           LRSGEIRSAAQHCLIGWKKYLQGLGPEGKEEGPRSSVTETEADVKTT 347
mCB2-KO(Zimmer)     -----------------------------------------------
mCB2-KO(Deltagen)   LRSGEIRSAAQHCLIGWKKYLQGLGPEGKEEGPRSSVTETEADVKTT 347
                                           (NIH5633 mCB2-Ab)
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The epitopes (binding sites) of four CB_2R antibodies are highlighted in different colors, and the differing amino acid residues in the epitopes between mCB_2Rs and rCB_2Rs are labeled by the symbol asterisk (*)

base pair positions 887–1227 of NM_009924.2); and a mCB_2-Deltagen-ko probe (for the Deltagen CB_2-KO strain) that targets a region near the 5′ end of exon 3 (aligns with base pair positions 336-409 of cDNA NM_009924.2; the deleted exon 3 aligns with base pair positions 259–593 of cDNA NM_009924.2). Mouse β-actin mRNA was used as an endogenous control. The specific base pair sequences of the MGB-Taqman probes and the primers used to detect mCB_2 and β-actin mRNAs were reported previously. [9] All Taqman probes and primers were purchased from Applied Biosystems (Foster City, CA, USA).

qRT-PCR reactions were performed using a QIAGEN OneStep RT-PCR Kit (Catalog number 210212, QIAGEN Inc., Valencia, CA, USA). Each qRT-PCR assay was performed in triplicate in 96-well plates. The following thermal cycle conditions were used: reverse transcription at 50 °C for 30 min and an initial PCR activation step at 95 °C for 15 min followed by 40 PCR cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 30 s. Final extension was performed at 72 °C for 10 min. qRT-PCR analyses of CB_2-mRNA levels were performed using the $2^{-\Delta\Delta Ct}$ method [38]. Data in the present study are presented as the fold-change in mCB_2 gene expression normalized to the internal β-actin control gene and relative to normal cortex (control tissue) in WT mice. The cycle threshold (Ct) was defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceed the background level). ΔCt was determined as [mean of the triplicate Ct values for the mCB_2 gene]−[mean of the triplicate Ct values for β-actin]. ΔΔCt represents the difference between the paired tissue samples as calculated by the formula ΔΔCt = [ΔCt of mCB_2 in sample tissue−ΔCt of mCB_2 in control tissue]. The *N*-fold differential expression of the mCB_2 gene in spleen or brain tissues compared to control tissue (i.e., cortex in WT mice) is expressed as $2^{-\Delta\Delta Ct}$ [8].

Data analyses

All data are presented as the means (±S.E.M.). One-way analysis of variance was used to analyze differences in the density of CB_2

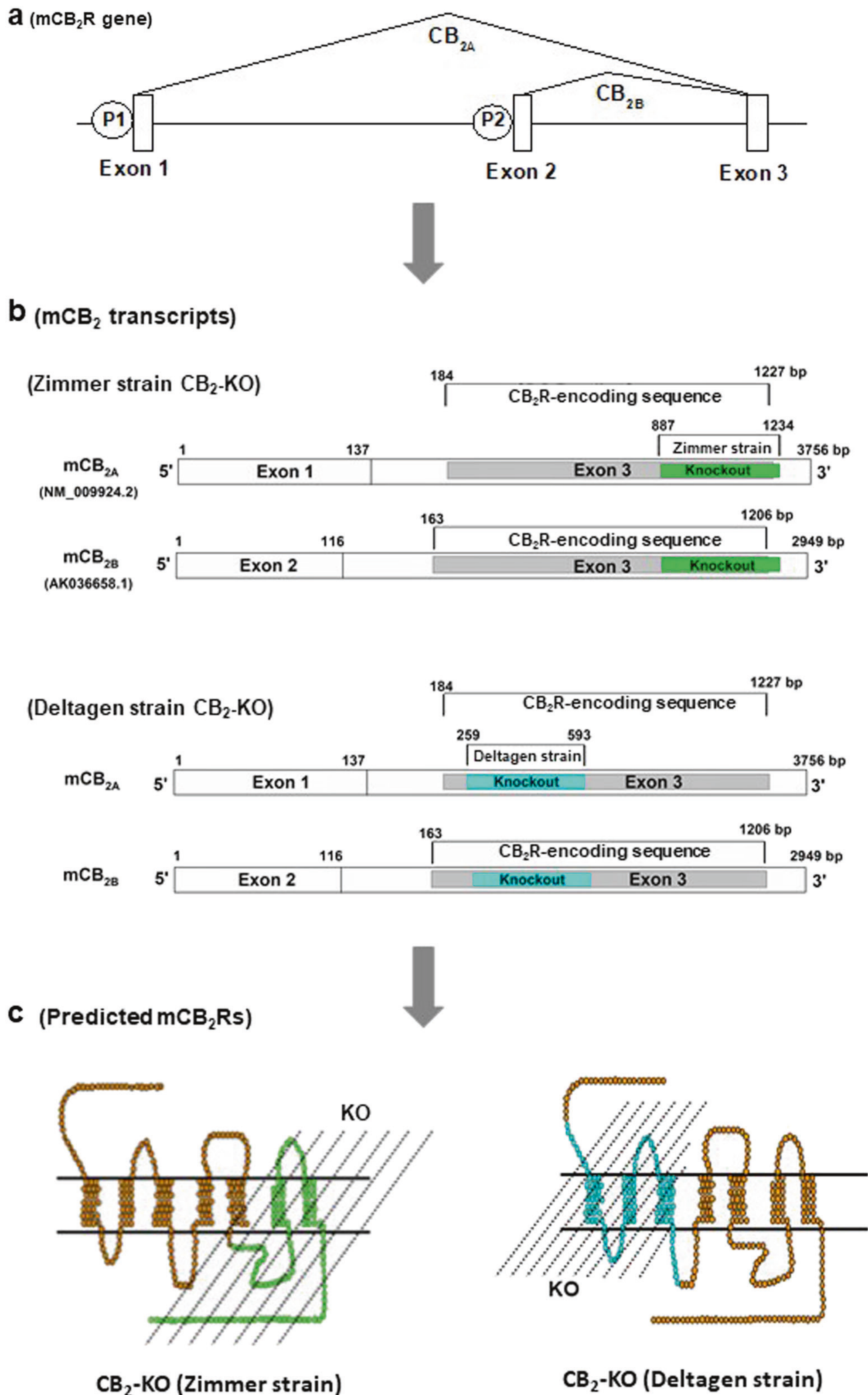
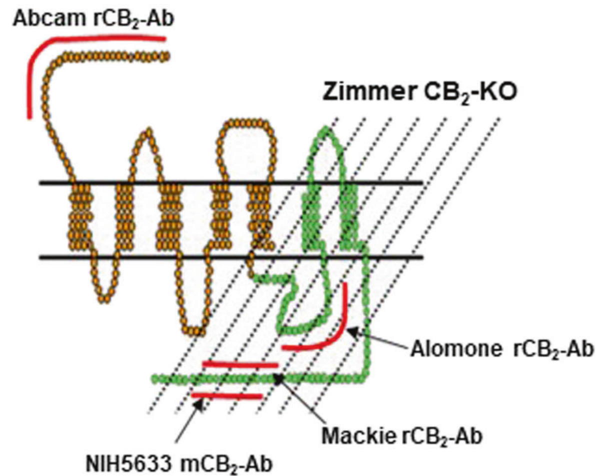
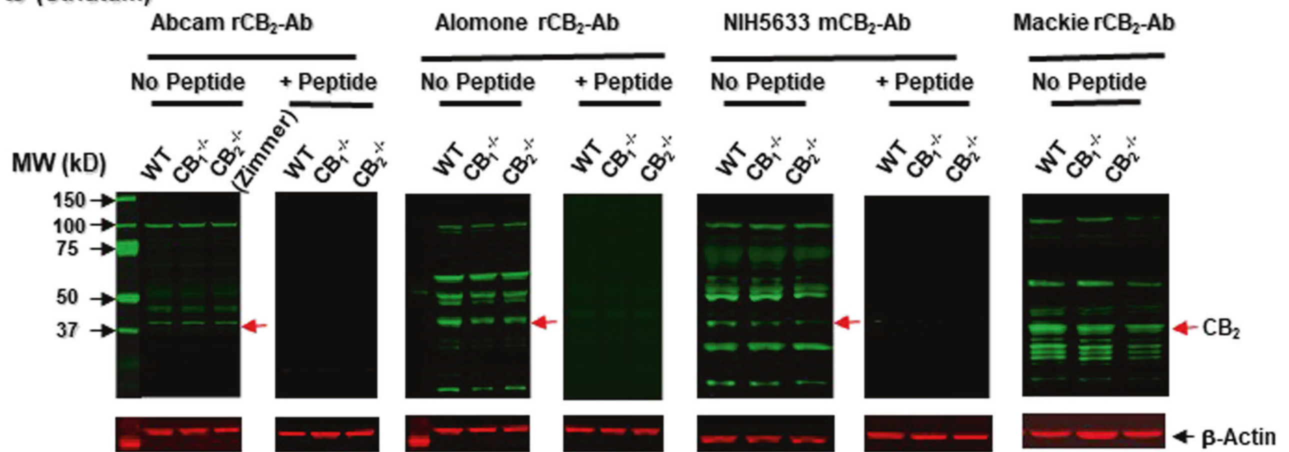


Fig. 1 Diagrams illustrating the structures of the mCB$_2$R gene (**a**), two transcripts (**b**), the gene-deleted regions, and the predicted CB$_2$Rs in two strains of CB$_2$R-KO mice (**c**)





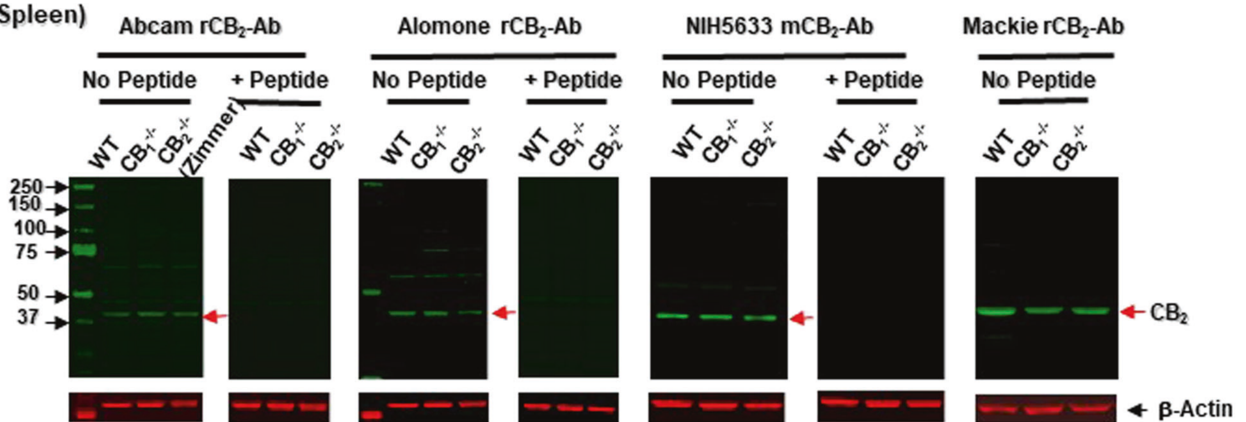
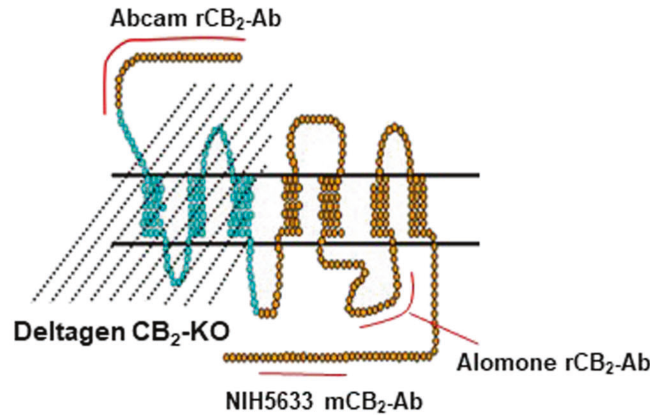


Fig. 2 Western blot results in WT, CB$_1$-KO, and Zimmer CB$_2$-KO mice using four different CB$_2$ antibodies. **a** A diagram showing the binding sites of the four antibodies used in WB assays. **b** The Abcam rCB$_2$-Ab detected an mCB$_2$-like band at ~40 kD (mCB$_2$ monomer, 39 kD) in the striatum of all three mouse genotypes, but the other three antibodies (Alomone, NIH5633, Mackie) detected relatively lower densities of an mCB$_2$-like band in Zimmer CB$_2$-KO mice. **c** CB$_2$-immunoreactive bands detected in the spleen using the same antibodies. More bands were detected in the striatum than the spleen by the multiple antibodies. Preabsorption of the CB$_2$ antibodies (Abcam, Alomone, NIH5633) with specific immune peptides blocked the immunoreactive bands



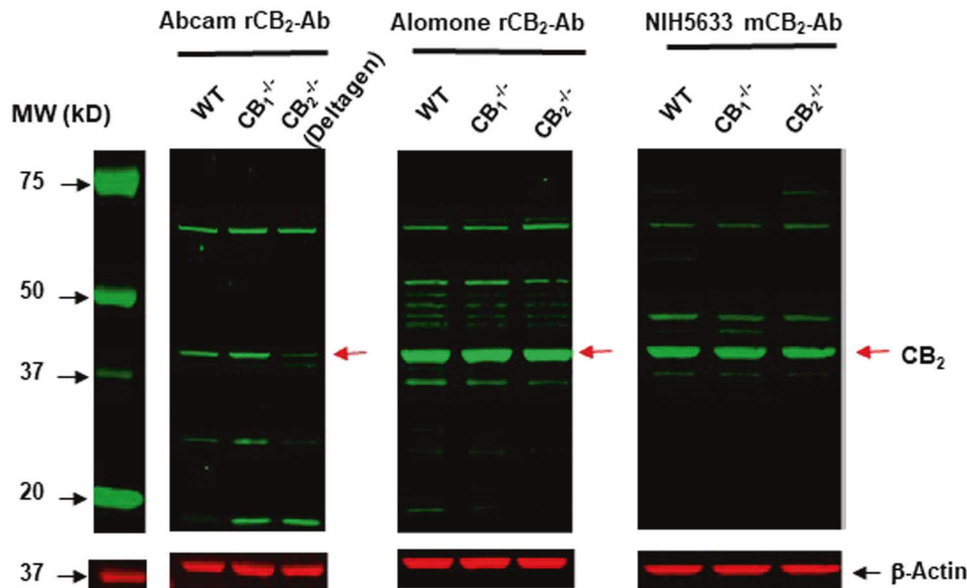


Fig. 3 Western blot results in WT, CB_1-KO, and Deltagen CB_2-KO mice using three different CB_2 antibodies. **a** A diagram showing the binding sites of the three antibodies used in WB assays. **b** The Abcam rCB_2 antibody detected a much lower density mCB_2-immunoreactive band in the Deltagen CB_2-KO mice than in WT mice, and the other two antibodies detected similar levels of the mCB_2-like band in all three mouse genotypes. *Ab* antibody

immunostaining between mouse strains. Individual group comparisons were performed using Student–Newman–Keuls post hoc test.

RESULTS

CB_2R gene structure, transcripts, and receptor proteins

The structures of the CB_2R gene, transcripts (mRNA) and receptor proteins must be known in detail to determine whether a detected signal is mCB_2R-specific. Figure 1 shows the mCB_2R gene structure, transcripts, and predicted receptor expression in WT mice and two strains of partial CB_2-KO mice. The mCB_2 gene consists of three exons with two separate promoters (P1 and P2) [8, 13, 28], which encode two transcripts (mCB_{2A} and mCB_{2B}) using exon 1 or exon 2 as different 5′ untranslated region sequences. The mCB_{2A} transcript contains exon 1 and exon 3, and the mCB_{2B} transcript contains exon 2 and exon 3. The CB_2R-encoding regions are located entirely on exon 3.

CB_2-KO mice used in the present study

The ideal negative control for determining CB_2R signal specificity would be the use of full CB_2-KO mice. However, such mice are not currently available. Therefore, we used the two available partial CB_2-KO strains, the Zimmer strain [4, 39] and the Deltagen strain (The Jackson Laboratory, *Cnr2*^{tm1Dgen}/J) [30, 40]. The Zimmer strain has a C-terminal 131 amino acid deletion. This mutation eliminates part of the intracellular and extracellular third loops, trans-membrane regions 6 and 7, and the intracellular C-terminus region (Fig. 1). The Deltagen strain has an N-terminal 112 amino acid deletion. This mutation causes loss of part of the extracellular N-terminal (from amino acid residues 26 to 137), trans-membrane regions 1–3, and intracellular loops 1 and 2 (Fig. 1; Table 1).

CB_2 receptor antibodies used in this study

Four different antibodies were used to examine CB_2R expression using WB and IHC assays. Two strains of N-terminal and C-terminal CB_2-KO mice were used as controls. Therefore, we chose different

antibodies that targeted the receptor-deleted region or non-deleted regions. For example, we hypothesized that an antibody with an epitope at the N-terminal of CB_2R (such as Abcam rCB_2-Ab) should detect CB_2R signals in WT and Zimmer strain CB_2-KO mice when the C-terminal-deleted Zimmer strain of CB_2-KO mice was used as a control, and CB_2 antibodies with epitopes at the C-terminal (e.g., the Alomone rCB_2-Ab, the NIH5633 mCB_2-Ab, or the Mackie rCB_2-Ab) should only detect a CB_2R signal in WT mice and not in the Zimmer strain of CB_2-KO mice. Similarly, antibodies with epitopes at the N-terminal or C-terminal of CB_2Rs should detect a CB_2R signal only in WT and not in Deltagen CB_2-KO mice when the N-terminal-deleted Deltagen strain of CB_2-KO mice was used as a control. Table 1 shows the amino acid sequences of CB_2Rs in rats, WT mice and two strains of CB_2-KO mice and the epitopes of the antibodies used in this study. There are 3–5 different amino acid residues (marked by the symbol asterisk (*) in Table 1) in the epitopes of the Abcam and Mackie rCB_2 antibodies between rat and mouse CB_2Rs. The epitope of the Alomone rCB_2 antibody is identical in rat and mouse CB_2Rs.

CB_2-immunoreactive bands are detected in WT and CB_2-KO mice

Figure 2 shows the WB results of the four different antibodies against rCB_2 or mCB_2 receptors. We found that all antibodies detected multiple bands in brain tissue (striatum) and one band in the spleen. We identified one band at ~40 kD that was likely CB_2R-specific in the brain and spleen based on the predicted molecular weight of mCB_2Rs. Antibody preabsorption with specific immune peptides blocked the immunoreactive bands (Fig. 2). The Abcam rCB_2 antibody with an epitope at the N-terminal of CB_2Rs (Fig. 1c) detected similar densities of CB_2R bands in WT, CB_1-KO, and Zimmer CB_2-KO mice. The other three antibodies that targeted the C-terminal-deleted region (Fig. 1c) detected relatively lower CB_2R band densities in the C-terminal-deleted Zimmer CB_2-KO mice compared to WT mice.

Figure 3 shows similar findings in the N-terminal-deleted Deltagen strain CB_2-KO mice. The Abcam rCB_2 antibody with an epitope at the N-terminal-deleted region (Fig. 3a) detected a significantly lower density of CB_2 band in the Deltagen CB_2-KO mice, and the other two antibodies (NIH5633 and Alomone) with epitopes at the C-terminal of mCB_2Rs (Fig. 3a) detected similar CB_2R signal densities in WT and Deltagen CB_2-KO mice (Fig. 3b). The downstream CB_2 gene sequence from the gene-deleted region should not encode receptor protein because of the presence of an early stop codon, and this finding appears to directly undermine the signal specificity detected by these two antibodies.

CB_2R immunostaining is detected in midbrain DA neurons

Previous findings demonstrated functional CB_2Rs in midbrain DA neurons [9, 41, 42]. Therefore, we used double-label fluorescence IHC assays to examine CB_2 immunostaining in midbrain DA neurons in all four strains of WT, CB_1-KO, and CB_2-KO mice. Figure 4 shows the CB_2 immunostaining of the Abcam rCB_2-Ab (with an epitope at the extracellular N-terminal, Fig. 4b, c) and reveals significant CB_2R immunostaining in TH-positive DA neurons in VTA in WT, CB_1-KO, and Zimmer CB_2-KO mice.

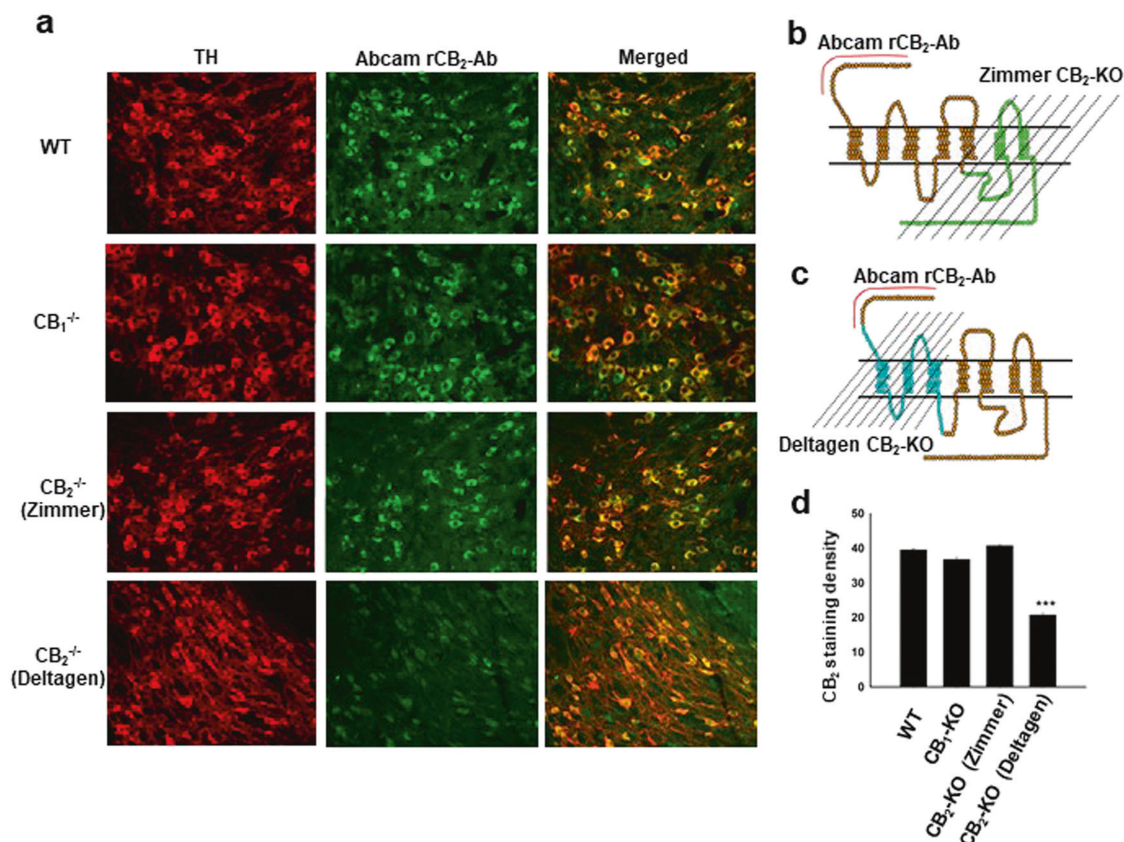


Fig. 4 mCB_2 immunostaining in the midbrain DA neurons using the Abcam rCB_2 antibody. **a** Representative IHC staining results illustrating that the Abcam rCB_2-Ab detected relatively lower CB_2 immunostaining in the Deltagen strain of CB_2-KO mice than in the other strains of mice. **b**, **c** Diagrams showing the receptor-deleted regions and the binding site of the Abcam rCB_2 antibody in both strains of partial CB_2-KO mice. **d** The mean densities of mCB_2 staining in DA neurons (over 200–400 DA neurons from 2 to 3 mice per genotype) illustrating a significant reduction in the Deltagen CB_2-KO mice compared to the other strains. $^{***}P < 0.001$, compared to WT mice

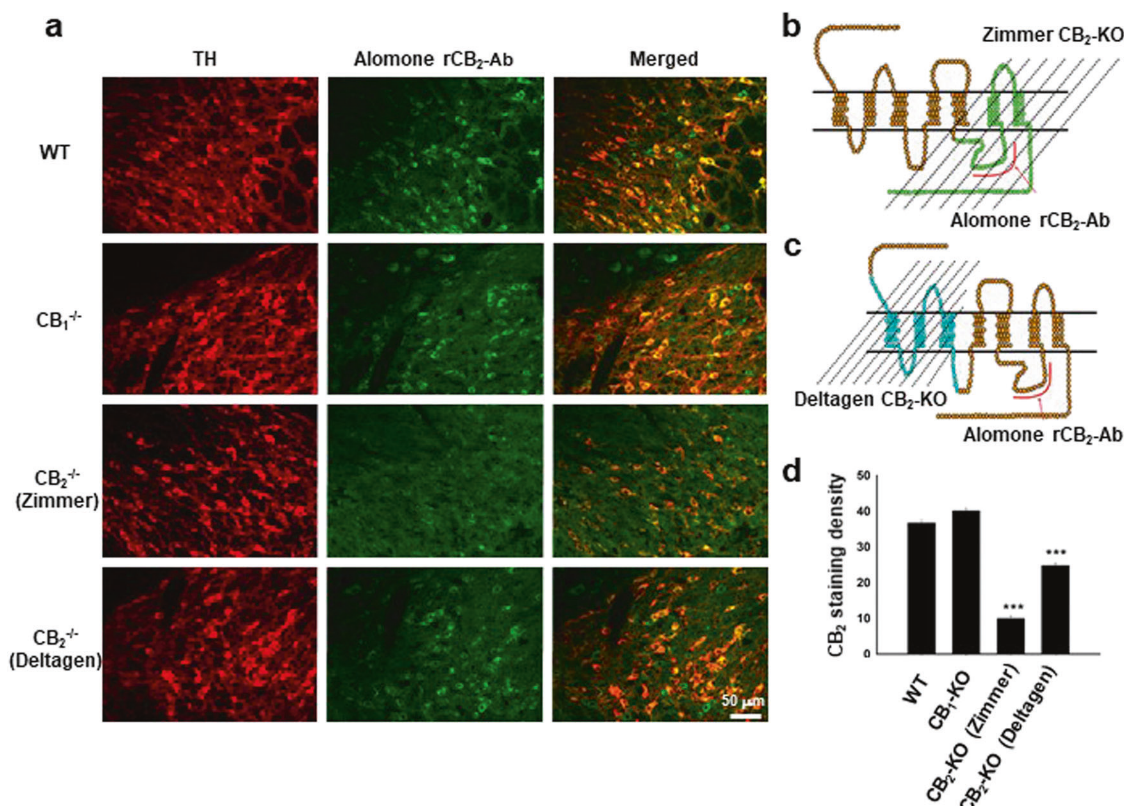


Fig. 5 mCB_2 immunostaining in midbrain DA neurons using the Alomone rCB_2 antibody. **a** Representative IHC staining results illustrating that the Alomone rCB_2 antibody detected significantly lower CB_2 immunostaining in the Zimmer strain of CB_2-KO mice. **b**, **c** Diagrams showing the receptor-deleted regions and the binding site of the Alomone rCB_2 antibody in both strains of CB_2-KO mice. **d** The mean densities of mCB_2 staining in DA neurons (over 200–400 DA neurons from 2 to 3 mice per genotype) illustrating a significant reduction in mCB_2 immunostaining in the Zimmer and Deltagen CB_2-KO mice compared to the other strains. $^{***}P < 0.001$, compared to WT mice

However, the density was significantly lower in the N-terminal-deleted Deltagen CB_2-KO mice compared to WT mice (Fig. 4a–d). Figures 5 and 6 show that the Alomone and NIH5633 antibodies with epitopes at the C-terminal of CB_2Rs detected significantly lower densities of CB_2R immunostaining in C-terminal-deleted Zimmer strain CB_2-KO mice than in WT or CB_1-KO mice. Both antibodies detected marked CB_2R immunostaining in the Deltagen CB_2-KO mice, but the mean density was significantly lower than that in WT mice. Notably, the Mackie rCB_2 antibody detected little-to-no CB_2R immunostaining in the VTA DA neurons in WT mice (Fig. 7).

CB_2 mRNA is detected in the brains of both strains of CB_2-KO mice

We examined CB_2 mRNA in the brain to determine whether the non-specific binding in the above assays was related to the presence of a CB_2R gene that encoded CB_2R proteins in these partial CB_2-KO mice. We previously reported that the upstream sequence of the CB_2R gene is present and detectable in the Zimmer strain of CB_2-KO mice, but the designed gene-deleted region is not [9, 28]. Therefore, we further examined CB_2 mRNA expression in the Deltagen CB_2-KO mice to investigate whether any complete CB_2R gene remained in this strain. Figure 8a shows the mCB_{2A} transcript, CB_2 gene-deleted regions in the Deltagen CB_2-KO mice, and the target regions of three mCB_2 TaqMan probes on mCB_2 transcripts. The mCB_{2A} probe targeted the upstream undeleted gene sequence, and the mCB_2-Deltagen-ko probe targeted the deleted gene sequence in the Deltagen CB_2-KO mice. The mCB_2-Zimmer-ko probe targeted the downstream gene sequence in Deltagen CB_2-KO mice. The mCB_{2A} probe detected the same levels of mCB_2 mRNA signal in the cortex and spleen in WT and Deltagen CB_2-KO mice (Fig. 8b), and the mCB_2-Deltagen-ko probe failed to detect any CB_2 mRNA signal in Deltagen CB_2-KO mice (Fig. 8c). The mCB_2-Zimmer-ko probe detected similar levels of the downstream CB_2 mRNA signals in WT and Deltagen CB_2-KO mice (Fig. 8d). These findings suggest that the complete CB_2 gene sequence is not present, but the gene sequences downstream from the gene-deleted region remain in Deltagen CB_2-KO mice.

DISCUSSION

The present study demonstrated that multiple CB_2R antibodies detected several CB_2-like immunoreactive bands, including one at ~40 kD, in the mouse brain and spleen, as well as CB_2R immunostaining in midbrain DA neurons in WT mice. However, we detected a relatively lower CB_2R signal in the partial CB_2-KO mice compared to WT controls using an antibody that targeted the receptor-deleted region. In contrast, we detected equivalent levels of CB_2R signal in WT and CB_2-KO mice using antibodies that targeted the predicted upstream or downstream receptor regions from the predicted receptor-deleted regions. qRT-PCR assays using three different probes that targeted the upstream, deleted, or downstream gene sequences in CB_2-KO mice detected CB_2 mRNA signals only at the upstream or downstream gene sequences and not in the designed gene-deleted region in the Deltagen CB_2-KO mice. These findings suggest that antibody-based CB_2R signals are not highly mCB_2R-specific when the currently available partial CB_2-KO mice are used as negative controls, which supports the urgent need for further development of full CB_2-KO mice.

The presence of CB_2Rs in the brain is controversial [34]. Extensive research over the past decade indicates that functional CB_2Rs are expressed in glial cells and neurons in the brain

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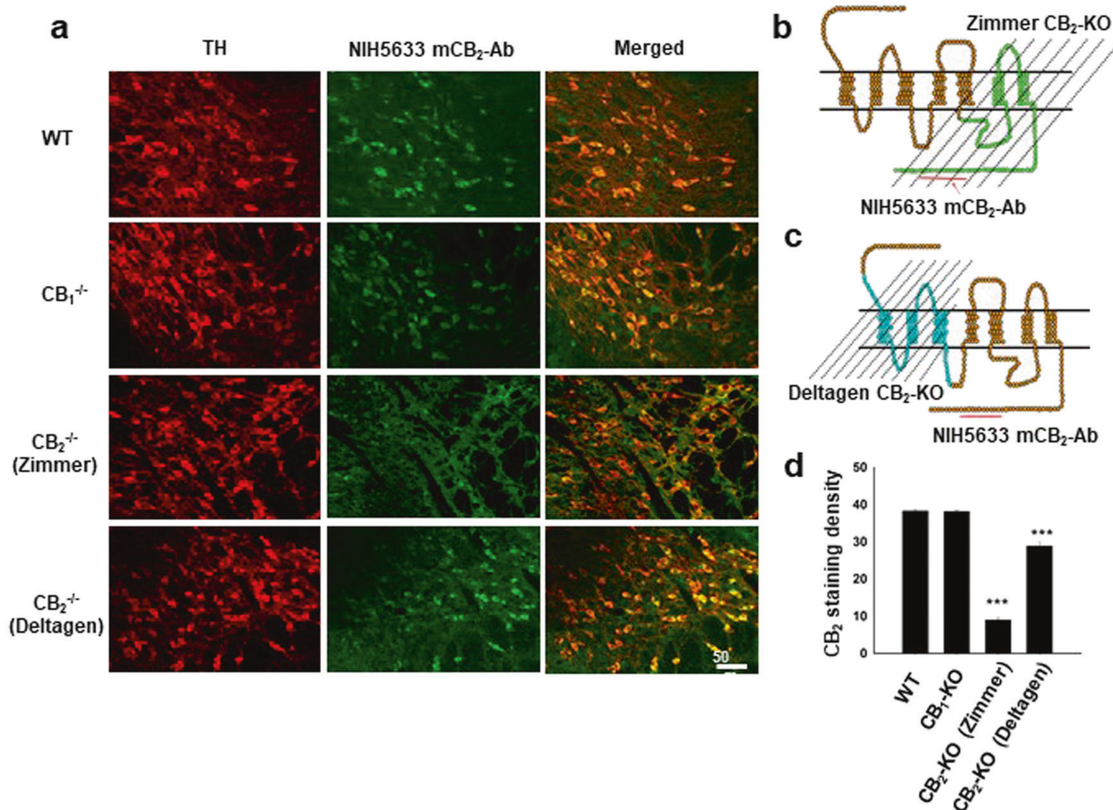

Fig. 6 mCB_2 immunostaining in the midbrain DA neurons using the NIH5633 mCB_2 antibody. **a** Representative IHC staining images illustrating that the NIH5633 mCB_2 antibody detected relatively lower CB_2 immunostaining in the Zimmer strain of CB_2-KO mice than in the other strains of mice. **b**, **c** Diagrams showing the receptor-deleted regions and binding site of the NIH5633 mCB_2 antibody in both strains of CB_2-KO mice. **d** The mean densities of mCB_2 staining in DA neurons (over 200–400 DA neurons from 2 to 3 mice per genotype) illustrating a significant reduction in the Zimmer and Deltagen CB_2-KO mice compared to the other strains. ***$P < 0.001$, compared to WT mice

[1, 43, 44], and evidence from qRT-PCR and ISH assays in our present study and previous reports strongly support this expression [9, 26, 28]. Electrophysiological evidence of brain CB_2R modulation of neuronal activity in the cortex [31–33], hippocampus [22, 25, 30], striatum [29], and midbrain [9, 26] further support the presence of functional CB_2Rs in the brain. We recently reported that systemic or local administration of the selective CB_2R agonist JWH133 or GW405833 into the nucleus accumbens significantly inhibited DA release in this region and attenuated intravenous cocaine self-administration, cocaine-enhanced locomotion, and cocaine-enhanced extracellular DA in the nucleus accumbens. [7] The impact of CB_2R agonism on cocaine's effects was blocked by AM630, a selective CB_2R antagonist, and was absent in Zimmer strain partial CB_2-KO mice. The CB_2R agonist JWH133 also inhibited cocaine-induced conditioned place preference and hyperactivity in rats [45] and cognitive and impulsive-like behavior in mice [20]. The CB_2R antagonist AM630 produced enhanced anxiety [19] and anti-depressive effects [46]. Overexpression of CB_2Rs in the brain decreased anxiety [47] and depression-like behaviors [46], and deletion of CB_2 receptors increased food intake and body weight [48, 49] and caused schizophrenia-like effects [50]. Activation of CB_2 receptors in the brain inhibited emesis in ferrets [5] and produced neuroprotective effects [23, 51–53]. Activation of CB_2 receptors in the spinal cord or thalamus inhibited spontaneous and evoked neuronal responses to noxious stimuli [54–57]. Taken together, these data indicate that functional CB_2Rs modulate a variety of neuronal activities and brain functions and strongly implicate brain CB_2 receptors in behaviors that are reliant on the mesolimbic DA system. However, this conclusion is not fully supported by our present findings of the WB and IHC assays, which indicate that CB_2R antibody signals may not be mCB_2R-specific.

WB and IHC assays are commonly used for the identification of specific protein expression in the brain and the periphery. However, poor antibody specificity is often invoked to invalidate a conclusion that is fully supported by other studies. An ideal antibody should meet most or all of the following criteria [58–60]: (1) immunoblot bands in WB assays should match the correct molecular weight of a target protein; (2) different antibodies against different epitopes of the same proteins should yield the same results in WB and IHC assays; (3) an ideal antibody should produce consistent results in WB and IHC assays; (4) an immunizing peptide should block immunolabeling; (5) immunolabeling should be consistent with the results generated by other independent techniques, such as autoradiography, RT-PCR, ISH, electrophysiology, and in vivo behavioral assays; (6) immunolabeling should be observed in target protein-positive tissues (positive control); and (7) immunolabeling should be abolished in tissues from target gene-deleted animals (i.e., KO animals and negative controls). Our findings in the present study and other previous reports [7–9, 22, 26, 28] clearly meet most of the above criteria, except for the findings in the partial CB_2-KO mice. The results in WB and IHC assays suggest that none of the tested CB_2R antibodies are highly mCB_2-specific. The four antibodies used exhibited only a certain degree of mCB_2 specificity when the appropriate partial CB_2-KO strain was used as a control.

The reasons for non-specific antibody binding in CB_2-KO mice are not clear. There are at least three possibilities. First, the detected signals in this study are not mCB_2R-specific or are totally non-specific. However, this explanation is not supported by the

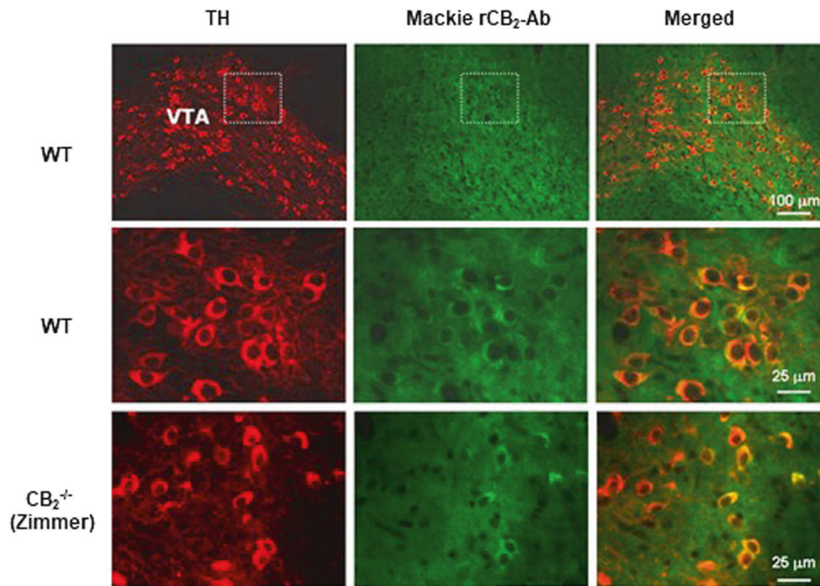

Fig. 7 Representative mCB_2R immunostaining using the Mackie rCB_2 antibody illustrating non-significant CB_2R immunostaining in VTA DA neurons in WT mice

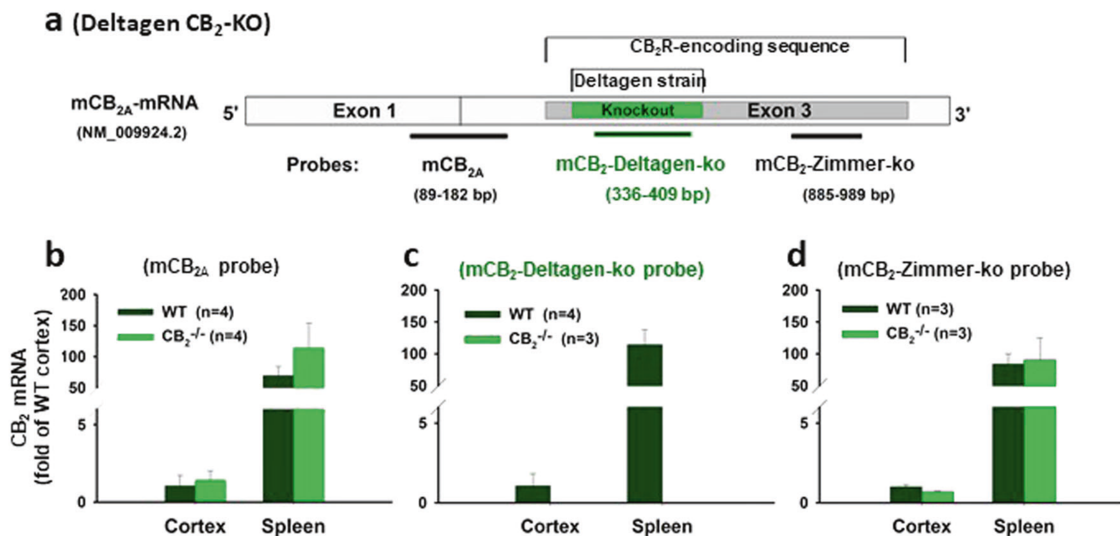

Fig. 8 mCB_2 mRNA expression in the Deltagen CB_2-KO mice. **a** mCB_{2A} transcripts, the CB_2 gene-deleted regions in Deltagen CB_2-KO mice, and the gene sequences detected by three different Taqman probes. **b** The mCB_{2A} probe that targets the undeleted gene regions detected similar levels of mCB_2-mRNA expression in the prefrontal cortex and spleen in WT and CB_2-KO mice. **c** The mCB_2-Deltagen-ko probe detected mCB_2 mRNA only in WT, not in Deltagen CB_2-KO, mice. **d** The mCB_2-Zimmer-ko probe that targets the gene-deleted sequence on exon 3 in Zimmer CB_2-KO mice detected mCB_2 mRNA in WT and Deltagen CB_2-KO mice. NM_009924.2 is the cDNA code in the GenBank of National Center for Biotechnology Information (NCBI)

finding that certain antibodies consistently detected significantly lower (50–70% reduction) CB_2-like signals in WB and IHC assays in CB_2-KO mice compared to WT mice. A second possible reason for the apparent non-specific antibody binding may be related to the presence of mutant or truncated CB_2R fragments in partial CB_2-KO mice. As noted above, the gold standard for a negative control would be full CB_2-KO mice, but these animals are not currently available. We used two CB_2-KO mouse strains with partial CB_2R gene deletion at the N-terminal or the C-terminal in this study as an alternative. We found that the designed gene-deleted sequence was absent, but the upstream and downstream CB_2 gene sequences remained in the Deltagen strain CB_2-KO mice and the Zimmer strain CB_2-KO mice, as reported previously [9]. These findings suggest that the undeleted upstream or downstream gene sequences may encode mutant or truncated CB_2R proteins or fragments in these partial CB_2-KO mice, which may underlie the non-specific binding observed in the present study.

Few studies examined mutant CB_2R expression in either strain of CB_2-KO mice. It is generally believed that upstream CB_2R fragments may be present in the Zimmer CB_2-KO mice (Fig. 1), but these fragments may be unstable and degraded intracellularly. Our finding of the detection of multiple bands in brain tissues of the C-terminal-deleted Zimmer CB_2-KO strain does not support this hypothesis and suggests the possible presence of CB_2R fragments or their coagulations. Notably, multiple antibodies that targeted the downstream CB_2R regions detected CB_2-like signals in the N-terminal-deleted Deltagen CB_2-KO mouse strain. However, the downstream CB_2R fragments should not be expressed

because of the presence of an early stop codon in the gene-replaced region (by neomycin). More studies are required to further address this issue. We recommend caution when using partial CB_2-KO mice as negative controls for WB and IHC assays. It is likely that an antibody may detect CB_2-like band(s) or immunostaining in partial CB_2-KO mice, depending on the antibody epitope and the strain of the partial CB_2-KO mice used.

A third possibility for the non-specific antibody binding may be related to the use of anti-rat CB_2R polyclonal antibodies for the detection of mouse brain mCB_2R expression. Table 1 shows that CB_2Rs exhibit significant species differences in amino acid sequences. Therefore, we urge the use of species-specific CB_2 antibodies to investigate CB_2R expression in different species. For example, mouse CB_2R antibodies with epitopes at the CB_2R N-terminal should be used for investigation of CB_2R signal specificity when N-terminal-deleted Deltagen CB_2-KO mice are used as controls. Unfortunately, mCB_2R-specific antibodies that targets the gene-deleted regions in both partial CB_2-KO strains are not available. Therefore, we used an rCB_2 antibody (Abcam) with epitope at the N-terminal (Fig. 1c) in the present study. This antibody has five different amino acid residues in its epitope between rCB_2R and mCB_2Rs (Table 1), and we presume that these differences may partially contribute to the relatively poor specificity observed in the present study. The use of the Abcam rCB_2 and Mackie rCB_2 antibodies in investigations of CB_2R expression in the mouse is also problematic because the epitopes are significantly different between rCB_2Rs and mCB_2Rs (Fig. 1c; Table 1). We note that the Deltagen CB_2-KO strain is not a full N-terminal KO because the N-terminal 1–25 amino acid residues are likely expressed and only amino acids 26–137 are likely deleted. The presence of the N-terminal fragment (residues 1–25) in the Deltagen CB_2-KO strain may explain the "non-specific" binding observed in that strain. We note that the Alomone rCB_2 and NIH5633 mCB_2 antibodies should not detect CB_2R signals in the C-terminal-deleted Zimmer strain CB_2-KO mice because the epitope of the Alomone antibody is identical in rat and mouse CB_2Rs. The non-specific binding detected by both of these antibodies may be related to the use of polyclonal, rather than monoclonal, CB_2 antibodies.

In conclusion, none of the tested four antibodies were highly mCB_2R-specific. However, the presence of non-specific binding is not surprising because poor specificity is a common problem in all antibody-based assays [58–60], not only in CB_2R research. Unexpected findings (e.g., non-specific binding by polyclonal CB_2 antibodies) in WB and IHC assays should not be used to invalidate a conclusion that is otherwise well supported by many other studies. It is likely that some antibodies detect CB_2R-like signals in partial CB_2-KO mice because mutant or truncated CB_2R proteins may be expressed in these mice. Ideally, the use of anti-mouse monoclonal CB_2R antibodies in combination with full CB_2-KO mice as controls would determine CB_2R antibody signal specificity. The findings in the present study do not invalidate the expression of CB_2Rs in the brain but provide additional evidence to support our previous finding of the expression of functional CB_2Rs in multiple neuronal phenotypes. Our findings do not suggest that partial CB_2-KO mice are useless. In contrast, these mice are valid controls in receptor gene assays and various functional and behavioral assays.

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AUTHOR CONTRIBUTIONS

ELG, AB, and Z-xX designed the experiments. HyZ and HS performed the experiments. CJJ, HyZ, and HS analyzed the data and prepared the figures. QrL and HyZ designed the mCB_2 antibodies (NIH5633) and gene probes (RT-PCR). HS, CJJ and ZxX wrote the manuscript with help from all co-authors.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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