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Novel floxed cannabinoid receptor 2 mouse line combines knockout capability with dual fluorescent reporters

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Background: The cannabinoid receptor 2 (CB_2) is involved in regulating immune responses, yet its specific function in microglia remains poorly defined. This study aimed to generate and validate a microglia-specific, inducible CB_2 knockout mouse model incorporating reporter genes to enable precise detection of CB_2 expression and CB_2 knockout.
Methods: A novel floxed CB_2 mouse line was generated, incorporating GFP and tdTomato reporter genes driven by the *Cnr2* promoter to indicate CB_2 expression and CB_2 knockout, respectively. This line was crossed with Cx3cr1 or Tmem119 tamoxifen-inducible Cre lines to achieve macrophage- or microglia-specific CB_2 knockout, respectively. Behavioural testing, *in vitro* assays, sequencing and *in vivo* immunofluorescence were used to assess the efficiency and specificity of CB_2 knockout as well as potential off-target effects.
Results: The floxed allele did not alter breeding or motor behaviour in mice, nor CB_2 function. CB_2 expression, indicated by GFP, followed expected patterns across tissues and conditions. Sequencing revealed both DNA and RNA of the floxed allele was as anticipated. Tamoxifen-induced Cre activity successfully initiated tdTomato expression exclusively in microglia of tamoxifen treated, Cre positive mice, validating the specificity and inducibility of CB_2 knockout. Microglial tdTomato expression confirmed successful CB_2 knockout in 9.3% of TmemCB_2 and 91.7% of Cx3CB_2 microglia. Peripheral tdTomato expression persisted beyond 3 weeks post-tamoxifen in Cx3CB_2 mice but was minimal in TmemCB_2 mice.
Conclusion: This novel microglia-specific, inducible CB_2 knockout model is the first to combine a floxed CB_2 allele with reporter genes, an essential advancement given the lack of reliable CB_2 antibodies. The findings demonstrate the model's specificity and effectiveness, while highlighting important considerations regarding Cre-mediated effects and recombination specificity. Furthermore, the floxed mouse can be crossed with any Cre line to study CB_2 expression and function in various tissues. This model provides a powerful platform for advancing understanding of CB_2 roles in microglia and supports future exploration of CB_2-targeted therapeutic strategies.

KEYWORDS

cannabinoid, microglia, knockout, neuroinflammation, mouse

1 Introduction

The endocannabinoid system (ECS), a tightly regulated signalling network present in all animal species, is a key regulator of homeostasis, including immune responses (Di Marzo, 2018; Van et al., 2021; Silver, 2019; Lowe et al., 2021; Antignano et al., 2023; Rakotoarivelo et al., 2024). The ECS includes cannabinoid receptors CB_1 and CB_2, along with their endogenous ligands, endocannabinoids. While CB_1 is mostly expressed by neurons, CB_2 is primarily expressed by immune cells. CB_2 expression is upregulated during inflammation and is understood to play a crucial role in immune modulation (Brusco et al., 2008; Ferranti and Foster, 2022; N. Joshi and Onaivi, 2019). Numerous studies have demonstrated the therapeutic effects of CB_2 agonists in a large range of disease models, including Alzheimer's disease (Wu et al., 2013; Jayant et al., 2016; Fakhfouri et al., 2012; C. Li et al., 2019; Aso et al., 2013; Moreno et al., 2012; Sobue et al., 2024), Parkinson's disease (Rentsch et al., 2020; Gómez-Gálvez et al., 2016; Chung et al., 2016; Liu et al., 2022; Espadas et al., 2020; Viveros-Paredes et al., 2017; H. Yu et al., 2021; Joers et al., 2024), Huntington's disease (Sagredo et al., 2009; Palpagama et al., 2019), traumatic brain injury (Braun et al., 2018), cerebrovascular and cardiovascular disorders (Yu et al., 2021; Zarruk et al., 2012; Tang et al., 2016; More et al., 2024), metabolic disorders (Rorato et al., 2022; Youssef, El-Fayoumi, and Mahmoud, 2019; Rohbeck, Eckel, and Romacho, 2021; Verty et al., 2015; Rossi et al., 2016; Hosoki, Asahi, and Nozaki, 2024), pain (Xu et al., 2023; Monory and Lutz, 2005; van den Hoogen et al., 2021; Nan et al., 2023), cancer (Alenabi and Malekinejad, 2021; Gambacorta et al., 2023) and more (Smoum et al., 2022; Whiting et al., 2022; Gasperi et al., 2023; Lowe et al., 2021; Grabon et al., 2023a; Onaivi, 2006; Kong et al., 2014; Espejo-Porras et al., 2019). These studies predominantly attribute the therapeutic effects to CB_2's anti-inflammatory properties; however, the cell-specific mechanisms mediating these effects remain unclear.

In the central nervous system (CNS), CB_2 is minimally expressed under basal conditions, but its expression is upregulated in microglia, the resident immune cells of the brain, during neuroinflammation (Duffy et al., 2021). Microglia are essential for maintaining CNS homeostasis and neuronal health. In their resting state microglia continuously monitor the environment for threats. Their phenotypic plasticity allows them to rapidly adapt to environmental cues, and upon activation by inflammatory stimuli, microglia adopt a pro-inflammatory phenotype, a defining feature of neuroinflammation (Araki, Ikegaya, and Koyama, 2021; Prinz, Jung, and Priller, 2019; Umpierre and Wu, 2021; Gao et al., 2023; Paolicelli et al., 2022). Chronic neuroinflammation is implicated in disease pathogenesis, as demonstrated *in vivo* (Wang P et al., 2022; Kang et al., 2024; Penney et al., 2024; Liang et al., 2023; Shi et al., 2019; Dong et al., 2021; Munro et al., 2024; X. Chen et al., 2023; Bido et al., 2021; Gratuze et al., 2023; Arvanitaki et al., 2024; Jing et al., 2021; Ding et al., 2021; Cui et al., 2021; Rocha et al., 2023; W. Kong et al., 2023; Pan et al., 2023; Ryan et al., 2023; Kitchener, Dundee, and Brown, 2023), *in vitro* (Zhou et al., 2023; Salvadores et al., 2022; C. Zhang et al., 2023), and genetic studies (Takatori et al., 2019; Andersen et al., 2021; Corley et al., 2021; ConsortiumInternational Multiple Sclerosis Genetics, 2019; Rodero et al., 2008). Mechanistically, pro-inflammatory microglia contribute to neuronal damage through various pathways, including direct neuronal interactions (Lindhout et al., 2021; Fricker, Oliva-Martin, and Brown, 2012; Butler et al., 2021), the release of neurotoxic cytokines (Lindhout et al., 2021; Rodriguez-Gomez et al., 2020; Oyarce et al., 2022; X. Liu et al., 2021), propagation of pathogenic proteins (Zheng and Zhang, 2021; Wang C et al., 2022; Odfalk, Bieniek, and Hopp, 2022; Xia et al., 2021), and recruitment of peripheral immune cells (Liddelow et al., 2017; X. Chen et al., 2023; Green et al., 2024; Joshi et al., 2019). These pathological activities are compounded by the loss of microglial homeostatic functions (Della Valle et al., 2024; Borst, Dumas, and Prinz, 2021; Zrzavy et al., 2017; Sobue et al., 2021; Kwon and Koh, 2020; Angelova and Brown, 2019). Conversely, microglia in anti-inflammatory states can exert neuroprotective effects, and there is growing evidence that therapies aimed at shifting microglia from a pro-inflammatory to an anti-inflammatory phenotype is beneficial in treating neurodegenerative diseases (Q. Li et al., 2021; Wang W et al., 2023; Willis et al., 2020; Shibuya et al., 2022; Mader et al., 2024; Yoo et al., 2023; Chadarevian et al., 2024; Munro et al., 2024; Daria et al., 2017; Lee et al., 2020; Tao et al., 2021; Jang et al., 2022; Pan et al., 2023; Piano et al., 2023; Birkle and Brown, 2023; Z. Yang et al., 2019; Guo, Wang, and Yin, 2022; Rizzi et al., 2018; Gao et al., 2023). CB_2 is upregulated in pro-inflammatory microglia, and CB_2 stimulation has been shown to mitigate their activation, making CB_2 an attractive therapeutic target (Wang M et al., 2023; Chung et al., 2016; Ojha et al., 2016; Chen et al., 2025).

Although microglia are the principal CB_2-expressing cells in the CNS, studies have reported CB_2 expression in certain neuronal populations, other glial cells, and by immune cells that infiltrate the CNS during inflammation (Ziring et al., 2006; Robinson et al., 2015; Jia et al., 2020). However, the extent and functional significance of CB_2 expression in these cell types remains debated, in part due to challenges in antibody specificity and detection methods (Atwood and Mackie, 2010; Grabon et al., 2023a; Eraso-Pichot et al., 2023). This raises a critical question: Are the therapeutic effects of CB_2 agonists primarily mediated through microglia, or do other CNS and peripheral cell types contribute significantly?

To address this knowledge gap, we have generated a novel floxed CB_2 mouse line (CB_2flx). This model allows for conditional knockout (KO) of the entire coding region of *Cnr2*, the gene encoding CB_2, in specific cell populations by crossing with appropriate Cre driver lines. This line is novel in that it incorporates dual fluorescent reporter genes for visualising CB_2-expressing cells and cells in which CB_2 has been deleted. The incorporation of reporter genes overcomes the limitations posed by a lack of reliable CB_2 antibodies (Grabon et al., 2023a; Grabon et al., 2023b; Zhang et al., 2019), providing a robust tool for investigating CB_2 expression and function. This line was then crossed with inducible microglia-specific Cre lines, allowing for precise, cell type-specific deletion of CB_2 in microglia. Given the complexity of the genetic modifications, rigorous validation is essential to ensure the specificity, efficiency, and functional neutrality of the system prior to its application in disease models.

Abbreviations: BL6, C57BL/6JAusb; CB_1, Cannabinoid receptor type 1; CB_2, Cannabinoid receptor type 2; CB_2flx, C57BL/6-*Cnr2*$^{tm1(GFP.tdTomato)BViss}$/J mouse line; CNS, Central nervous system; Cx3CB_2, B6.129P2(Cg)-*Cx3cr1*$^{tm2.1(Cre/ERT2)Litt}$/WganJ x CB_2flx mouse line; ECS, Endocannabinoid system; GFP, Green fluorescent protein; KO, Knockout; LPS, Lipopolysaccharide; SN, Substantia nigra; Tam, Tamoxifen; tdT, tdTomato; TmemCB_2, C57BL/6-*Tmem119*$^{em1(Cre/ERT2)Gfng}$/J x CB_2flx mouse line; YFP, Yellow fluorescent protein.

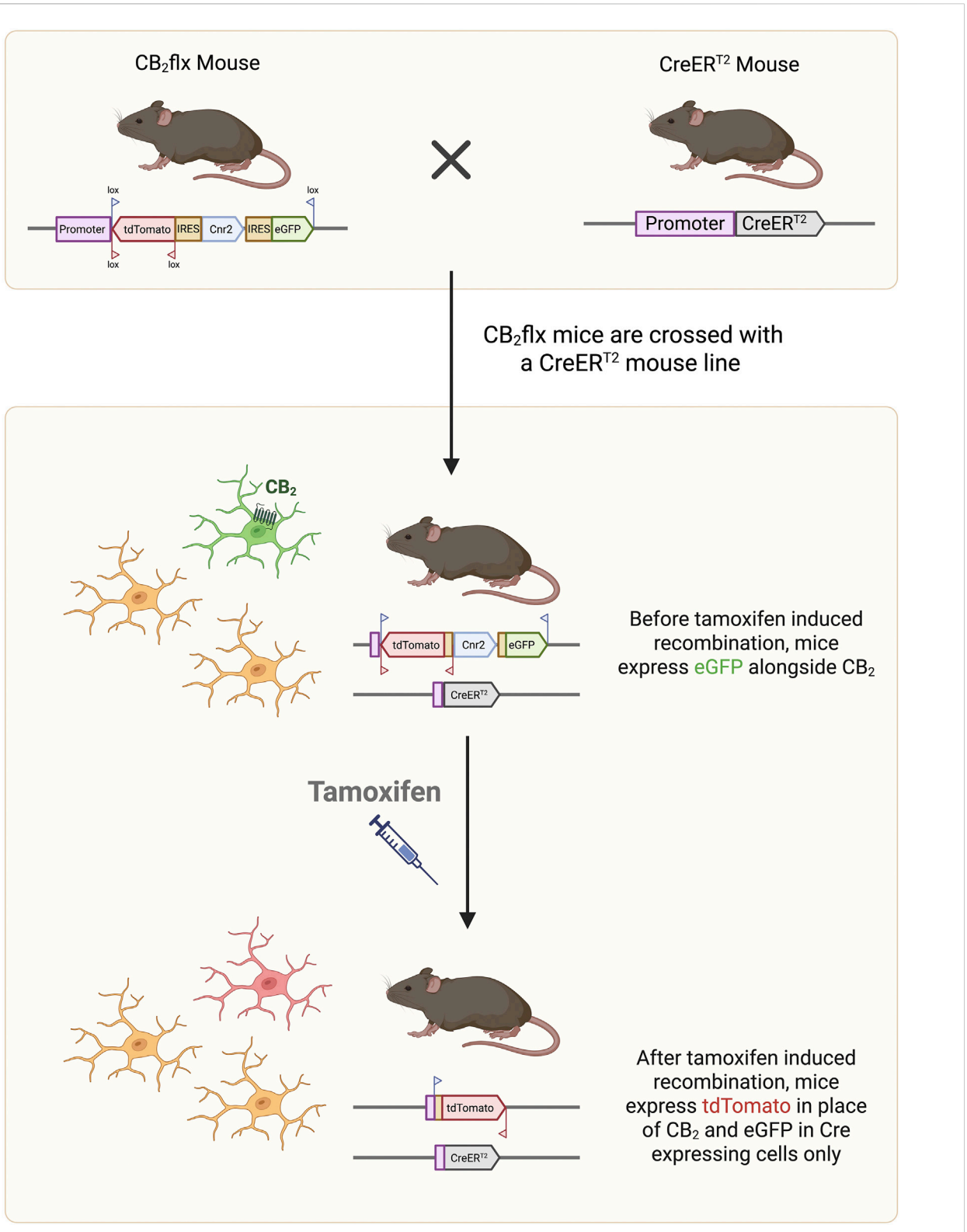

FIGURE 1
Conditional CB_2 knockout mechanism. The CB_2flx mouse line contains genetic modifications in which the entire *Cnr2* coding region (blue box) is surrounded by lox sites. The reporter genes *GFP* (sense orientation) and *tdT* (antisense orientation) are inserted into the *Cnr2* locus under control of the *Cnr2* promoter, such that cells expressing CB_2 also express GFP. The CB_2flx line is crossed with a Cre/ERT2 line to create an inducible CB_2-knockout line. Following tamoxifen injection, Cre mediated recombination excises the floxed *Cnr2* coding region and GFP, while flipping tdT into the sense orientation. Consequently, cells in which CB_2 has been knocked out now express tdT as a marker of recombination. GFP, green fluorescent protein. tdT, tdTomato.

2 Methods

2.1 Animals

2.1.1 Cre-Lox FLEx switch mechanism

A spatially and temporally specific CB_2-KO mouse line was generated using the Cre-Lox recombination system. CB_2 is encoded by the *Cnr2* gene, which contains a single protein coding exon (exon 2). The entire coding region of *Cnr2* was flanked by loxP sites using homologous recombination in embryonic stem cells, enabling complete KO of CB_2 protein expression upon Cre-mediated recombination. To facilitate monitoring of *Cnr2* expression and recombination, *GFP* and *tdTomato* (*tdT*) reporter genes were inserted under the control of the *Cnr2* promoter. The *tdT* cassette was placed in an inverse orientation and flanked by lox2272 sites as part of a flip-excision switch (Supplementary Figure S1A). In the absence of Cre recombinase, CB_2 and GFP are expressed under the control of the *Cnr2* promoter. Following Cre-mediated recombination, the floxed *Cnr2* coding region and *GFP* are excised, while *tdT* is simultaneously flipped into the sense orientation and will now be expressed under the *Cnr2* promoter, providing a permanent marker of recombination (Figure 1; Supplementary Figure S1B). This novel line was named C57BL/6-*Cnr2*$^{tm1(GFP,tdTomato)BViss}$/J line ($CB_2$flx).

Genotyping of all genetically modified mice was performed for the *Cnr2* floxed allele and relevant *Cre* alleles using real-time PCR with SYTO9-based melt curve analysis on a LightCycler 480 system. Primer sequences and PCR conditions are detailed in Supplementary Table S1.

2.1.2 Housing and husbandry

Mice were housed under specific-pathogen-free conditions at 21 °C ± 1 °C with a 12-h light-dark cycle, in plastic cages with *ad libitum* access to water and standard chow. A maximum of five same-sex mice were housed per cage. Mice were acclimated to the facility for at least 1 week prior to experiments. Experimental groups comprised 8–12-week-old male and female mice, and mice of the same sex and genotype were randomly assigned to experimental groups.

All genetically modified mice used in experiments were homozygous for the floxed *Cnr2* allele. Experimental TmemCB_2 and Cx3CB_2 mice were bred so that littermates were either heterozygous (Cre/+) or wildtype (+/+) for the Cre/ERT2 allele.

All animal research and care procedures were approved by the Garvan Institute/St. Vincent's Animal Ethics Committee (ARA numbers 23/13, 20/10, 18/37), per guidelines issued by the National Health and Medical Research Council of Australia and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Tamoxifen administration

Tamoxifen (Tam) (20 mg/mL) was dissolved in sunflower oil. Mice received i.p. injections of Tam or vehicle control according to three regimens: 150 mg/kg every other day for four doses, 100 mg/kg daily for 8 days, or 100 mg/kg twice daily for ten doses. Tissue was collected 3 weeks after the final injection.

2.3 Motor behaviour

Locomotor activity was assessed in a 30 × 30 cm plexiglass open field arena within a sound-attenuated chamber under dim illumination (MedAssociates). One day following a 10 min habituation session, mouse movements were tracked for 10 min. Total distance travelled, centre zone (19 × 19 cm) entries, and time spent in the centre zone were recorded. Blinding was maintained during testing.

Mice were trained on an accelerating rotarod (0–40 RPM) whereby they were placed on the rotarod and returned to it each time they fell off, for 5 min. The following day, latency to fall was measured over three 5-min trials, separated by 30-min intervals. The average of the highest two trials was used for analysis. Experimenters were blinded to treatment groups.

2.4 Stereotaxic lipopolysaccharide injection

To induce inflammation-induced neurodegeneration, stereotaxic surgery was performed to ipsilaterally inject the striatum with the endotoxin lipopolysaccharide (LPS). Intrastriatal LPS is well established to produce a robust neuroinflammatory response in the substantia nigra (SN), while its large volume allows for more accurate targeting and reduced mechanical damage (Deng et al., 2020; Jang et al., 2022; Skrzypczak-Wiercioch and Salat, 2022). Before surgery, mice received 4 × 150 mg/kg doses of Tam or vehicle control, once every other day, followed by a 4-week washout period. Mice were then anesthetised via an anaesthetic cocktail (10 mL/kg, i.p.), containing ketamine (Mavlab, Australia; 10 mg/mL) and xylazine (Troy Laboratories, Australia; 2 mg/mL). LPS was administered as previously described (Gómez-Gálvez et al., 2016). Briefly, LPS (*Salmonella enterica*, Minnesota) was dissolved in 0.9% sterile saline to a concentration of 5 mg/mL. Two injections of 1 µL of LPS were injected into the right striatum at AP +1.18, ML -1.5, DV -3.5 and AP -0.34, ML -2.5, DV -3.2, relative to bregma and the dural surface. Injections were given at a rate of 0.5 µL/min using a 2 µL Neuros syringe (Hamilton, Germany).

Mice were acrificed for histological analysis 15 days after surgery.

2.5 Histology

2.5.1 Tissue collection and processing

Animals were anaesthetised before cardiac perfusion with 4% paraformaldehyde. The brain was immediately dissected out and submerged in paraformaldehyde at 4 °C for 24 h, before being transferred to cryoprotectant solution (30% sucrose in PBS).

2.5.2 Immunofluorescence

Free-floating 40 µm sections were blocked in 3% BSA +0.25% Triton for 1 h before primary antibody (Supplementary Table S2) was applied for 72 h, at 4 °C. Tissue was then incubated with the corresponding Alexa Fluor-conjugated secondary antibody for 24 h at 4 °C, protected from light. Finally, the sections were incubated in DAPI (Thermo Fisher; 1:1000 in PBS) for 10 min.

2.5.3 FIJI image analysis

For colocalization of GFP, tdT and Iba1, four representative images of each brain were taken in consistent areas of the striatum, SN, and hippocampus with a Zeiss Axio Imager.Z2 microscope. For each image, four z-stacks were taken using the ×40 objective. Percentages of GFP, tdT and Iba1 colocalization were quantified using the FIJI (ImageJ) Colocalization Object Counter plugin (Lunde and Glover, 2020). Both the max projection and stack were analysed. A cell would only be counted if the nuclear marker DAPI was present.

2.6 *In vitro* cAMP assay

Primary glia cultures were prepared from P0–P3 C57BL/6JAusb (BL6), CB_2flx and $Cx3CB_2$ pups, as previously described (Schildge et al., 2013). Briefly, cortices were dissected in dissection medium (HBSS, 1% P/S) dissociated with trypsin-EDTA (0.005%), and a single cell suspension in glia medium (DMEM/F12, 10% FBS, 1% P/S) was added to PDL coated flasks. Media was changed after 24–36 h and then every 2–3 days thereafter, until the cells reached approximately 90% confluence (~9 days). Microglia and astrocytes were separated by shaking cultures at 200 RPM for 6 h. Microglia and astrocyte cell pellets were resuspended in stimulation buffer (HBSS + 5 mM HEPES +0.5 mM IBMX +0.075% BSA; pH 7.4) for use in cAMP assays. Forskolin-stimulated cAMP levels were measured using the LANCE Ultra cAMP kit (PerkinElmer), following manufacturer's instructions.

For assay optimisation, forskolin concentration-response curves were established for both microglia and astrocytes at various cell densities to determine the optimum cell density and forskolin concentrations for subsequent CB_2 agonist cAMP assays. It was concluded that the ideal conditions for microglia were 2000 cells/well with a forskolin concentration of 71.4 μM (Supplementary Figure S2A). For astrocytes, 500 cells/well with a forskolin concentration of 2.6 μM (Supplementary Figure S2B).

For the agonist assays, Hu308 was diluted in DMSO to generate a concentration response curve of forskolin-stimulated cAMP levels for each cell type. For all assays, samples were run in triplicate and a cAMP standard curve was run on every plate. TR-FRET signal was measured with the PHERAstar FSX microplate reader (BMG LabTech).

2.7 Statistics

All statistics were performed using Prism 10 software (GraphPad). Unless otherwise stated, $p < 0.05$ was considered significant and data are reported as mean ± standard error of the mean. Before undergoing parametric tests, D'Agostino-Pearson normality test was carried out and if a dataset failed this test, the Q-Q plot was inspected to determine if there were major violations of a Gaussian distribution. Further, Spearman's rank test was used to test for homoscedasticity, and if this test failed, the residual plot was inspected for major violations. Data that passed these tests underwent one-, two- or three-way ANOVAs. For *post hoc* corrections, Tukey's was used when comparing every mean with every other mean, Dunnett's for comparing every mean to a control mean, Dunn's correction for non-parametric comparisons, and Šídák's correction for all other *post hoc* comparisons.

Details of all mouse lines, reagents, equipment and software used are listed in Supplementary Table S3.

3 Results

3.1 Validation of *Cnr2* gene targeting

To validate the genetic modifications in the floxed *Cnr2* locus, DNA sequencing was performed by OzGene. As expected, the sequencing results confirmed alignment with the anticipated sequence across the targeted region, except for a 510 bp stretch within the 5′homology arm, for which incomplete sequencing data were obtained due to poor trace quality. Despite this limitation, the low-quality sequenced portion of the region exhibited 100% identity to the predicted sequence (Supplementary Figure S3). Furthermore, sequencing of *Cnr2* mRNA, performed by The Garvan Institute's Genetics Core Facility, indicating precise alignment with the anticipated sequence and therefore no unintended disruptions in *Cnr2* transcription. These in-depth sequencing analyses confirm the successful incorporation of the intended genetic modifications in the floxed *Cnr2* locus, with no evidence of off-target alterations affecting *Cnr2* transcription. Full sequencing data for both DNA and mRNA is available on GenBank.

3.2 Genetic modifications do not impair reproductive fitness

The reproductive fitness of the genetically modified mouse lines was assessed compared to BL6 controls by a prospective analysis of breeding outcomes. There was no significant difference between groups for litter size ($F(3, 196) = 1.31$, $p = 0.27$; Figure 2A), mortality rate ($KW(4, 200) = 3.79$, $p = 0.28$; Figure 2B), or sex ratio ($F(3, 192) = 0.094$, $p = 0.96$; Figure 2C). Furthermore, no overt developmental or behavioural abnormalities were observed during routine breeding and handling. These findings demonstrate that the genetic modifications in the CB_2flx, $Cx3CB_2$, and $TmemCB_2$ mouse lines do not adversely affect reproductive capacity or offspring viability.

3.3 Motor behaviour is unaffected in CB_2flx mice

To assess the effects of the floxed *Cnr2* allele on motor behaviour, CB_2flx mice were subjected to open field and rotarod test. In the open field test, no significant differences were observed between CB_2flx and BL6 mice in total distance travelled ($t(11) = 0.670$, $p = 0.516$; Figure 3A), number of ambulatory episodes ($t(11) = 0.421$, $p = 0.682$; Figure 3B), or time spent in the central zone ($t(11) = 1.138$, $p = 0.279$; Figure 3C). Similarly, performance on the rotarod test revealed no differences in latency to fall ($t(11) = 0.346$, $p = 0.736$; Figure 3D). Collectively, these findings demonstrate that the presence of the floxed *Cnr2* allele does not affect locomotor activity or motor coordination.

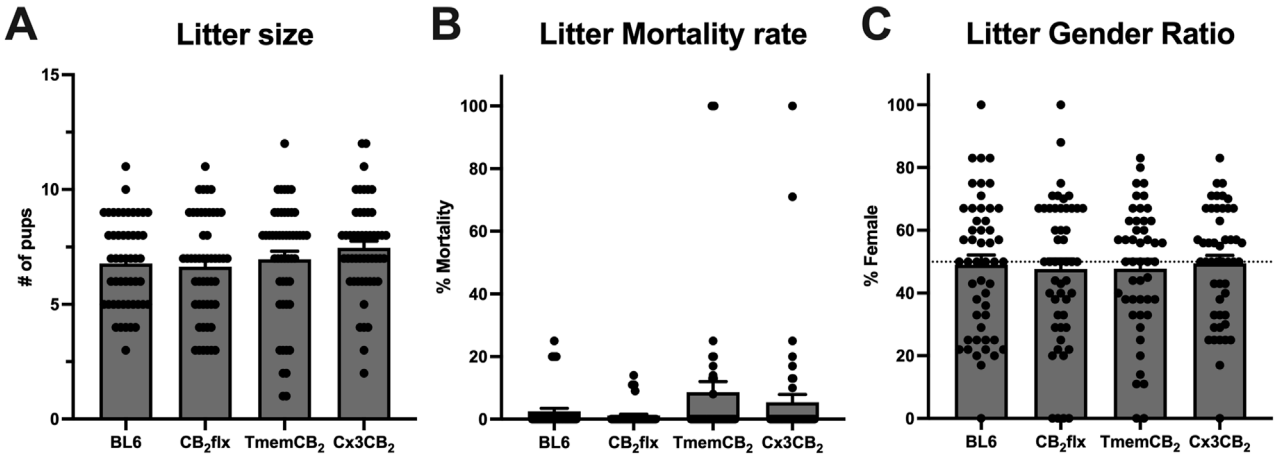

FIGURE 2
Litter characteristics are not impaired in genetically modified strains. **(A)** Number of pups born per litter. One-way ANOVA, Dunnett's *post hoc* test. **(B)** Proportion of litter mortality between birth and weaning. One-way ANOVA, Dunnett's *post hoc* test. **(C)** Proportion of pups that were female at weaning. One-way ANOVA, Dunn's *post hoc* test. Data were collected from 50 litters per strain over a similar timeframe. Data are represented as mean ± SEM.

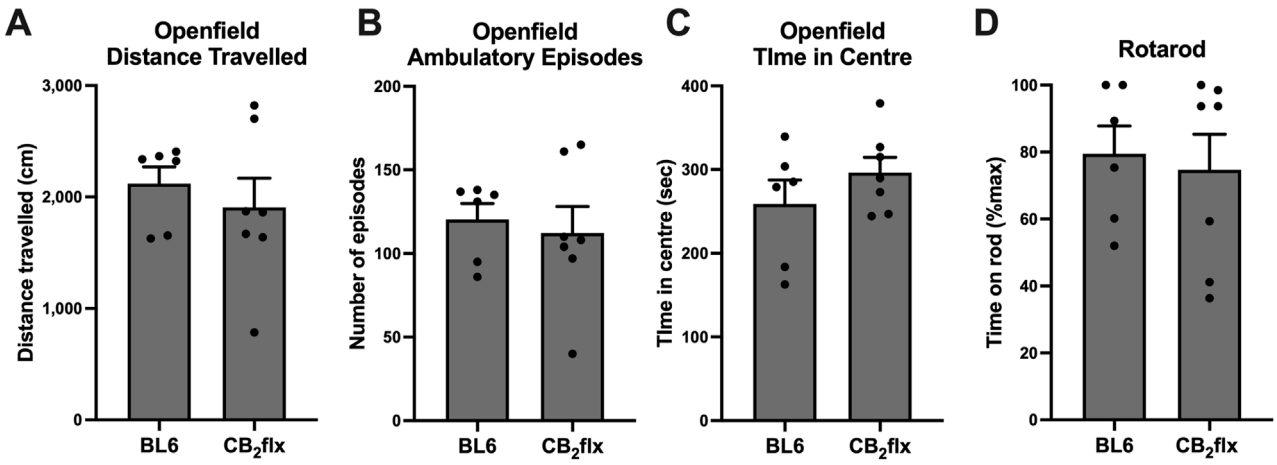

FIGURE 3
Motor behaviour is not impaired in the CB_2flx strain. **(A–C)** Open field and **(D)** rotarod motor behaviour tests in CB_2flx mice. N = 6-7/group. Unpaired t-test. Data are represented as mean ± SEM.

3.4 CB_2 function is preserved in glial cells

As a $G_{\alpha i}$-coupled receptor, CB_2 activation inhibits cAMP production (Kaminski, 1998). Therefore, a cAMP assay was performed to assess receptor function in primary glial cultures from CB_2flx and $Cx3CB_2$ mice. First, optimisation experiments were conducted to determine appropriate cell densities and forskolin concentrations for both microglia and astrocytes (Supplementary Figure S2).

Upon stimulation with the CB_2 agonist Hu308, forskolin-induced cAMP levels were reduced in a dose-dependent manner across all groups. No significant differences were observed in Hu308-induced cAMP inhibition between CB_2flx, $Cx3CB_2$, and BL6 controls for microglia (F (2,28) = 0.027, p = 0.973; Figure 4A) or astrocytes (F (2,32) = 0.432, p = 0.653; Figure 4B). These results indicate that CB_2 function is preserved in glial cells derived from genetically modified strains.

3.5 Reporter gene expression in the CB_2flx mouse line

Given the challenges in using immunohistochemistry to detect CB_2, a fluorescent reporter system was incorporated into the CB_2flx line to enable visualisation of CB_2 expression. In the CB_2flx line, GFP expression is driven by the *Cnr2* promoter, allowing detection of CB_2-expressing cells through histological analysis. In the absence of Cre-mediated recombination, tdT should not be expressed. To

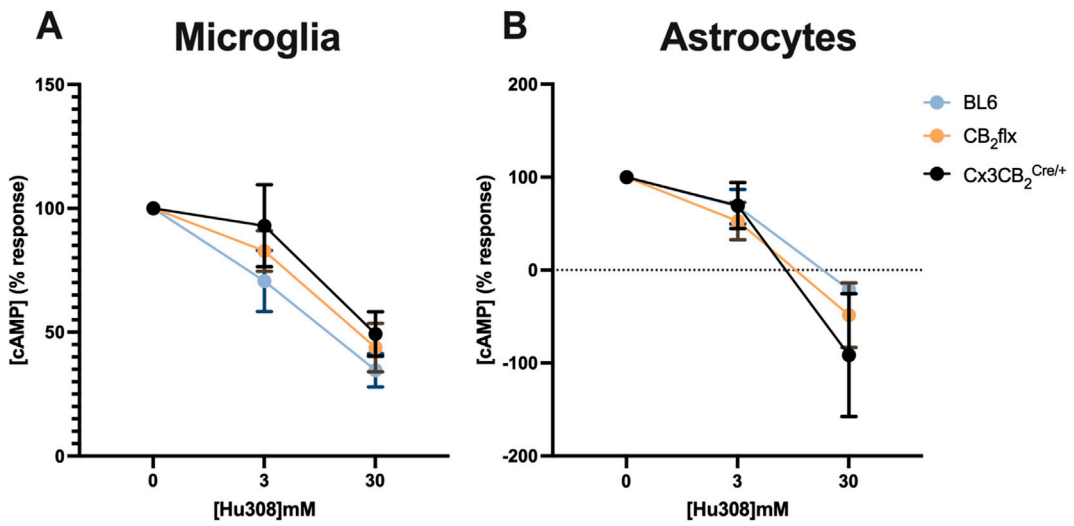

FIGURE 4
CB_2 function is conserved in primary glia cultures. The CB_2 agonist Hu308 inhibited cAMP production in primary **(A)** microglia and **(B)** astrocytes in a dose dependent manner in all strains. Data was normalised whereby 100% represents 0 mM Hu308 and 0% represents no forskolin control. N = 5-8/group. Two-way ANOVA, Dunnett's *post hoc* test. Data are represented as mean ± SEM. Each point represents the mean of three technical replicates.

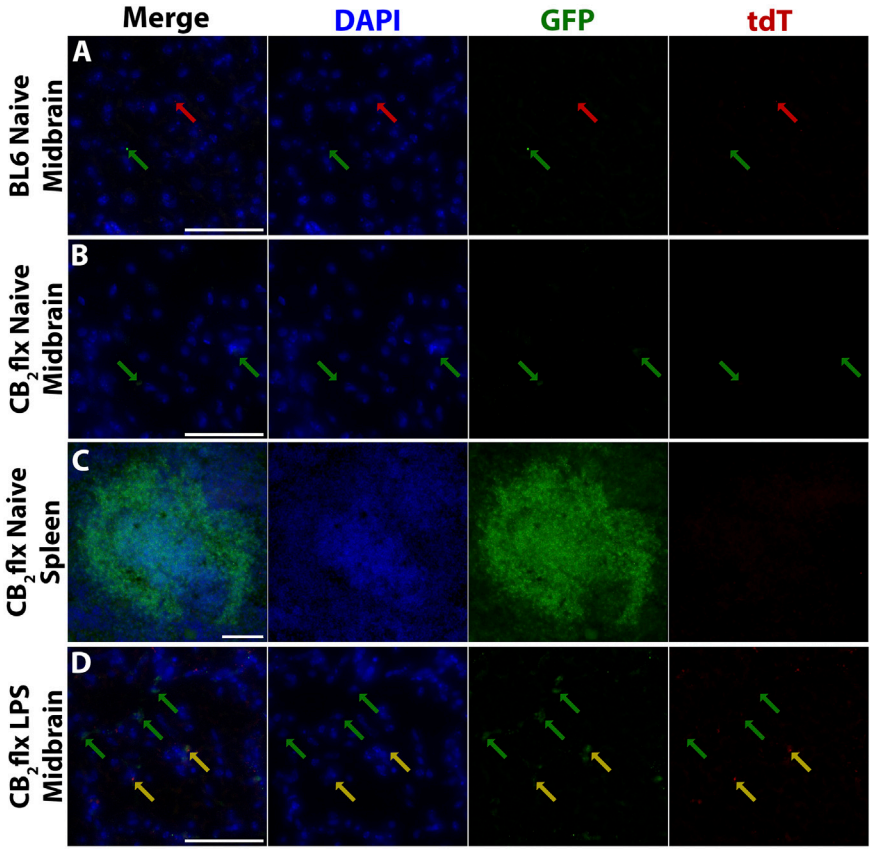

FIGURE 5
Reporter gene expression reflects expected CB_2 expression in CB_2flx mice. Representative images of green fluorescent protein (GFP) and tdTomato (tdT) reporter gene expression in **(A)** the naive BL6 CNS, **(B)** naive CB_2flx CNS, **(C)** naive CB_2flx spleen, and **(D)** CB_2flx CNS treated with intrastriatal LPS. Green arrows = GFP expression. Red arrows = tdT expression. Yellow arrows = GFP/tdT colocalization. Scale bar = 100 μm.

confirm this, GFP and tdT expression were examined using immunofluorescence in the periphery, as well as in the brain under naive and inflammatory conditions.

BL6 controls displayed some small points of autofluorescence for both GFP and tdT, which served as an essential benchmark to distinguish true signal from artefacts (Figure 5A). In the naive CB_2flx brain (Figure 5B), minimal GFP expression was observed, consistent with the low basal expression of CB_2 in the CNS, while the spleen exhibited strong GFP expression, particularly in white pulp regions, which are enriched in CB_2 expressing immune cells (Figure 5C) (Galiègue et al., 1995; Simard et al., 2022; Lewis, Williams, and Eisenbarth, 2019).

To assess inflammation-induced CB_2 expression, neuroinflammation was elicited via intrastriatal injection of LPS. GFP expression increased markedly in the inflamed CNS, aligning with the expected upregulation of CB_2 during neuroinflammation. Occasional tdT expression was observed, but this was restricted to regions with intense GFP signals, suggesting fluorescence channel bleed-through rather than true tdT expression (Figure 5D).

Overall, GFP expression in CB_2flx mice mirrored the expected CB_2 expression profile: low in the naive CNS, elevated in the CNS during inflammation, and pronounced in peripheral tissues. These findings validate the utility of this mouse model for studying CB_2 expression and function.

3.6 Tam induces microglial specific tdT expression in Cx3CB$_2$ mice

Given that CNS CB_2 is primarily expressed by microglia (Duffy et al., 2021), the CB_2flx line was crossed with a macrophage-specific Cre line (JAX, strain#: 021160), resulting in the establishment of the Cx3CB$_2$ line. In these mice, Cre expression is limited to Cx3cr1-expressing cells, which include microglia and peripheral macrophages (Subbarayan et al., 2022; Mizutani et al., 2012). Upon Tam administration, Cre-mediated CB_2-KO is expected in all Cx3cr1-expressing cells.

To determine the most efficient Tam protocol for inducing Cre-mediated recombination in Cx3CB$_2$ mice and to assess the effects of Cre and Tam on CB_2 expression, we analysed reporter gene expression in BL6, CB_2flx, and Cx3CB$_2$ mice. In Cx3CB$_2$ mice heterozygous (Cre/+) for the Cre allele, GFP serves as a marker of CB_2 expression, while yellow fluorescent protein (YFP) is expressed in Cx3cr1-Cre cells as part of the Cre driver line. A technical limitation of this experimental design is that the overlapping emission spectra of GFP and YFP could not be distinguished using our immunofluorescence approach. Consequently, in Cx3CB$_2^{Cre/+}$ microglia, which express both YFP (from the Cre allele) and potentially GFP (from CB_2 expression), the fluorescent signal primarily reflects YFP from Cre expression, and prevents direct assessment of CB_2-driven GFP in these cells. In contrast, cells from other genotypes (CB_2flx, Cx3CB$_2^{+/+}$) and non-microglial cell types do not express YFP, allowing GFP signal in these groups to be unambiguously attributed to CB_2 expression. Despite this limitation in directly visualising CB_2 expression in Cre-positive microglia, the tdT reporter system provides definitive confirmation of successful Cre-mediated CB_2 deletion in these cells.

In Cx3CB$_2^{Cre/+}$ mice, nearly all Iba1+ microglia (>92%) expressed GFP/YFP, with no significant differences between treatment groups (F (6, 19) = 923.3, $p < 0.0001$; Figure 6A). Furthermore, over 96% of GFP/YFP signal colocalized with Iba1+ microglia, again with no significant differences between treatment groups (F (3, 9) = 0.413, $p = 0.747$; Figure 6B). These results confirm that Cre expression is restricted to microglia in Cx3CB$_2^{Cre/+}$ mice, and that Tam treatment does not influence Cre expression levels.

Microglia from all other genotypes exhibited minimal GFP/YFP signal, confirming the absence of Cre and supporting prior findings of low basal CB_2 expression in microglia. Additionally, no significant differences were observed between Tam-treated Cx3CB$_2^{+/+}$ and BL6 controls, demonstrating that Tam administration does not induce CB_2 expression in microglia (Figure 6A).

tdT expression serves as a reporter for successful CB_2-KO in Cx3CB$_2$ mice, therefore tdT should be restricted to microglia in Tam-treated and Cre/+ mice. As expected, no significant tdT expression was detected in any control group, confirming the absence of CB_2-KO. In contrast, nearly all Iba1+ microglia in Tam-treated Cx3CB$_2^{Cre/+}$ mice expressed tdT (Figure 6C). Within these groups, over 92% of tdT expression colocalized with microglia, with no significant differences between Tam administration protocols, confirming high recombination efficiency across all protocols (F (2, 7) = 4.07, p = 0.067; Figure 6D). High-magnification (Figures 6E–I) and low-magnification (Supplementary Figure S4) representative images further emphasise the stark differences in reporter gene expression between groups. Furthermore, no GFP/YFP or tdT signal was observed in neurons or astrocytes, confirming that Cre expression, CB_2 expression, and CB_2-KO were microglia/macrophage-specific (Supplementary Figure S5).

Given that Cx3cr1-expressing macrophages are also present in peripheral tissues, we next examined CB_2 expression outside the CNS. Peripheral macrophages have been reported to undergo renewal every 3 weeks, suggesting that tdT should be absent in the spleen at this timepoint, rendering CB_2-KO microglia-specific thereafter (Wang et al., 2020; Dick et al., 2019; Goldmann et al., 2013; Peng et al., 2016; Bedolla et al., 2023). As expected, Tam-treated Cx3CB$_2^{+/+}$ spleens exhibited GFP but lacked tdT, similar to CB_2flx controls (Supplementary Figure S6A). Conversely, tdT was detected in Cx3CB$_2^{Cre/+}$ spleens 1-week post-Tam, as anticipated (Supplementary Figure S6B). However, tdT expression persisted at 3 weeks, contrary to prior reports, suggesting that CB_2-KO is not specific to microglia at this timepoint (Supplementary Figure S6C). This finding challenges the conventional assumption that a 3-week waiting period is sufficient to ensure microglia specificity in this Cre line.

These results confirm the microglia-specific expression of Cre in the CNS of Cx3CB$_2^{Cre/+}$ mice and its Tam-dependent activity. All Tam protocols efficiently induced CB_2-KO in macrophages, with no significant differences between protocols. Based on these results, four doses of 150 mg/kg administered every other day was selected for continued use, as it exhibited the highest mean microglial tdT expression and specificity of 91.7%. Notably, CB_2-KO persisted in peripheral macrophages beyond 3 weeks post-Tam, highlighting the need for extended evaluation periods if exclusive microglial specificity is required in this model.

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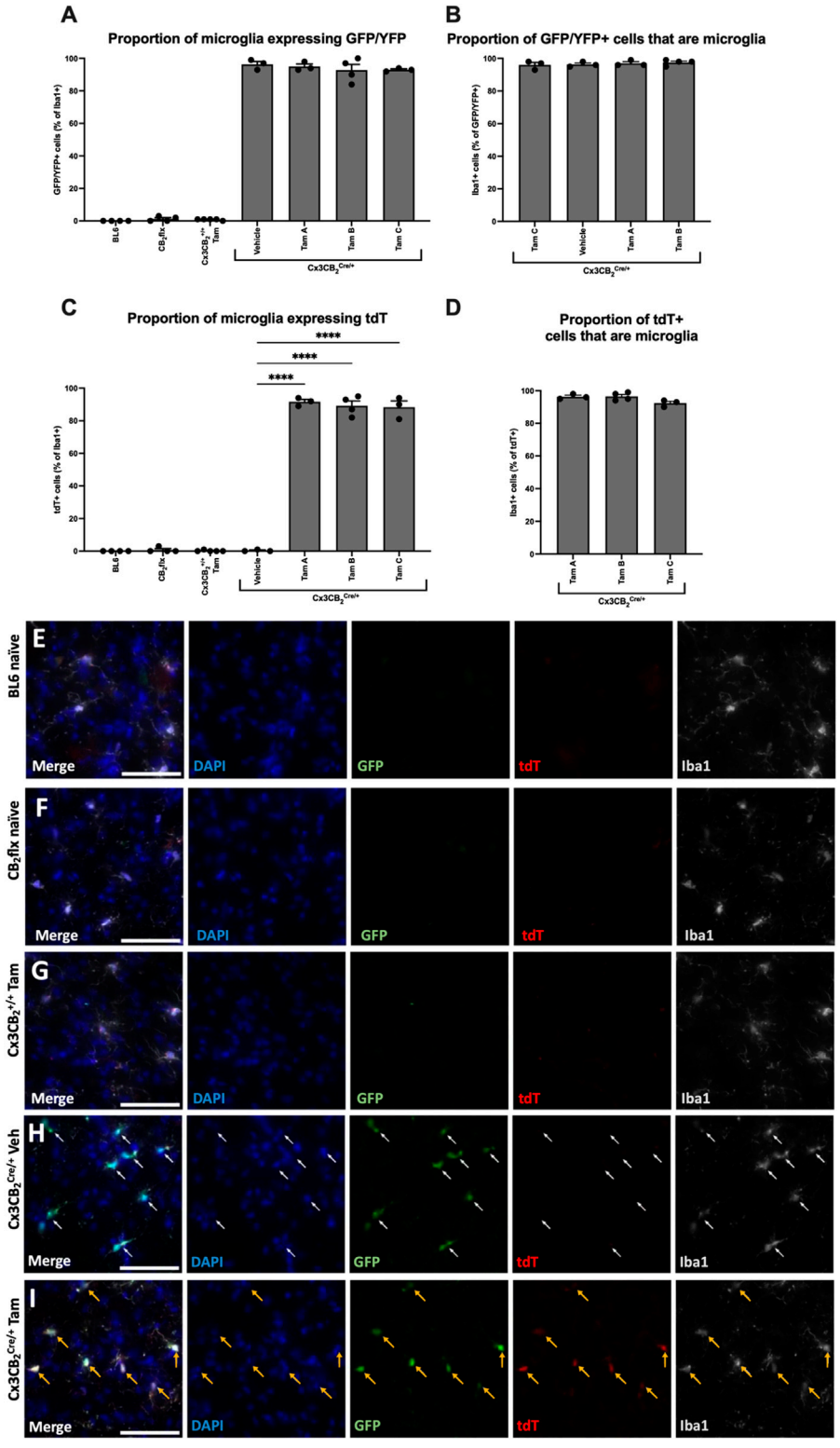

FIGURE 6
In $Cx3CB_2$ mice, tdT expression is microglia specific and is Tam/Cre dependent. **(A–D)** Quantification of GFP and tdT colocalization with Iba1. Significance bars between $Cx3CB_2^{Cre/+}$ groups only are shown. N = 3-4/group. An average of 133.4 (±4.3) Iba1+ cells were counted per n. Data are represented as mean ± SEM. One-way ANOVA with Tukey's *post hoc* test, ****$p \leq 0.0001$. Representative images of substantia nigra immunostaining in **(E)** BL6, **(F)** CB_2flx, **(G)** Tam treated $Cx3CB_2^{+/+}$, **(H)** Vehicle-treated $Cx3CB_2^{Cre/+}$ and **(I)** Tam-treated $Cx3CB_2^{Cre/+}$ animals. White arrows = GFP expression. Yellow arrows = GFP/tdT colocalization. Scale bar = 50 µm.

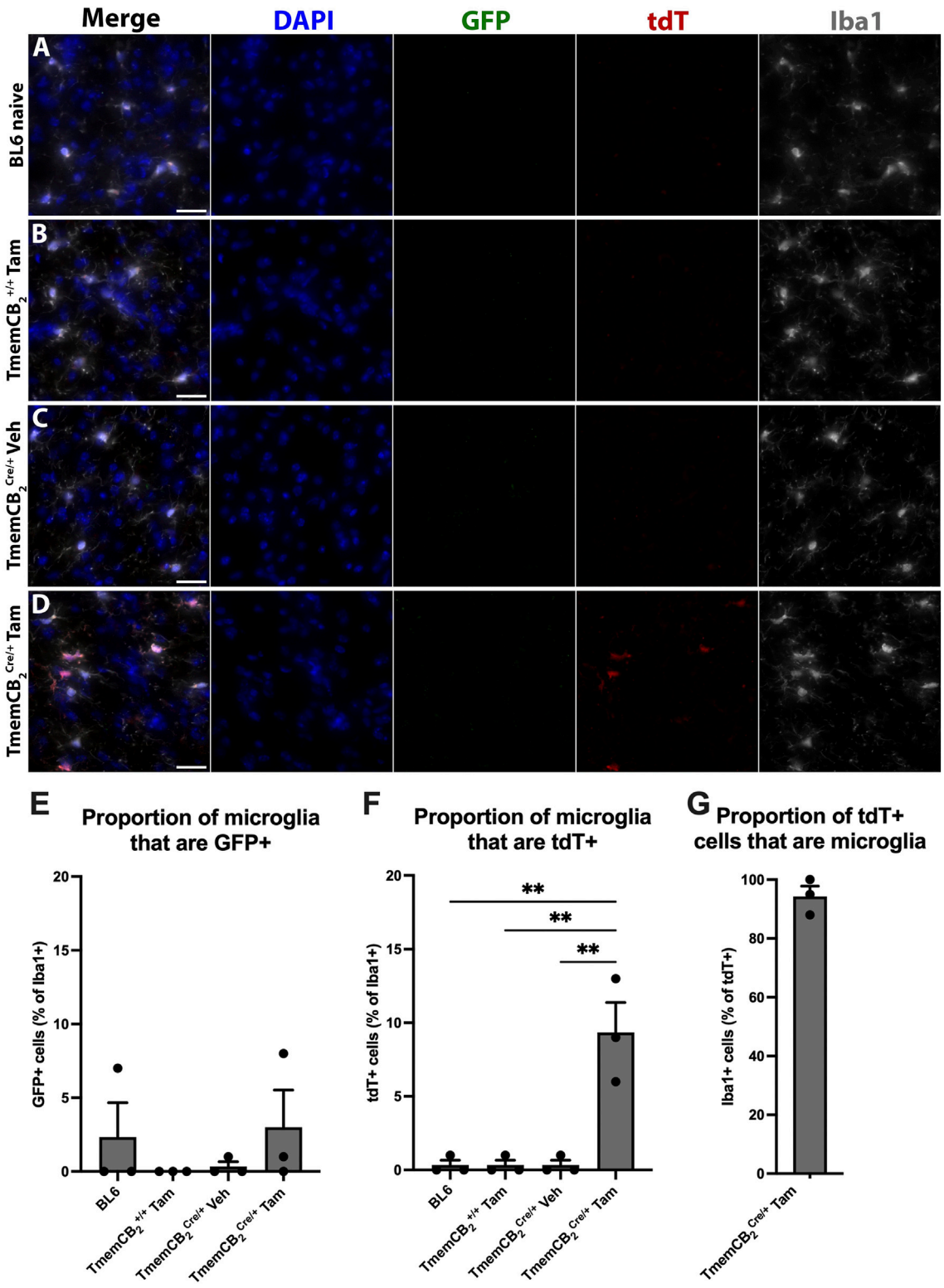

FIGURE 7
In $TmemCB_2$ mice, tdT expression is microglia specific and is Tam/Cre dependent. **(A–D)** Representative images of substantia nigra GFP/tdT/Iba1 immunostaining. **(A)** BL6, **(B)** Tam treated $TmemCB_2^{+/+}$, **(C)** Vehicle treated $TmemCB_2^{Cre/+}$ and **(D)** Tam treated $TmemCB_2^{Cre/+}$. Scale bar = 50 μm. **(E–G)** Quantification of GFP and tdT with Iba1+ microglia. As only one group expressed tdT, G contains one panel only. N = 3/group. An average of 115.8 (±6.1) Iba1+ cells were counted per n. Data are represented as mean ± SEM. One-way ANOVA, Tukey's *post hoc* test **p ≤ 0.01.

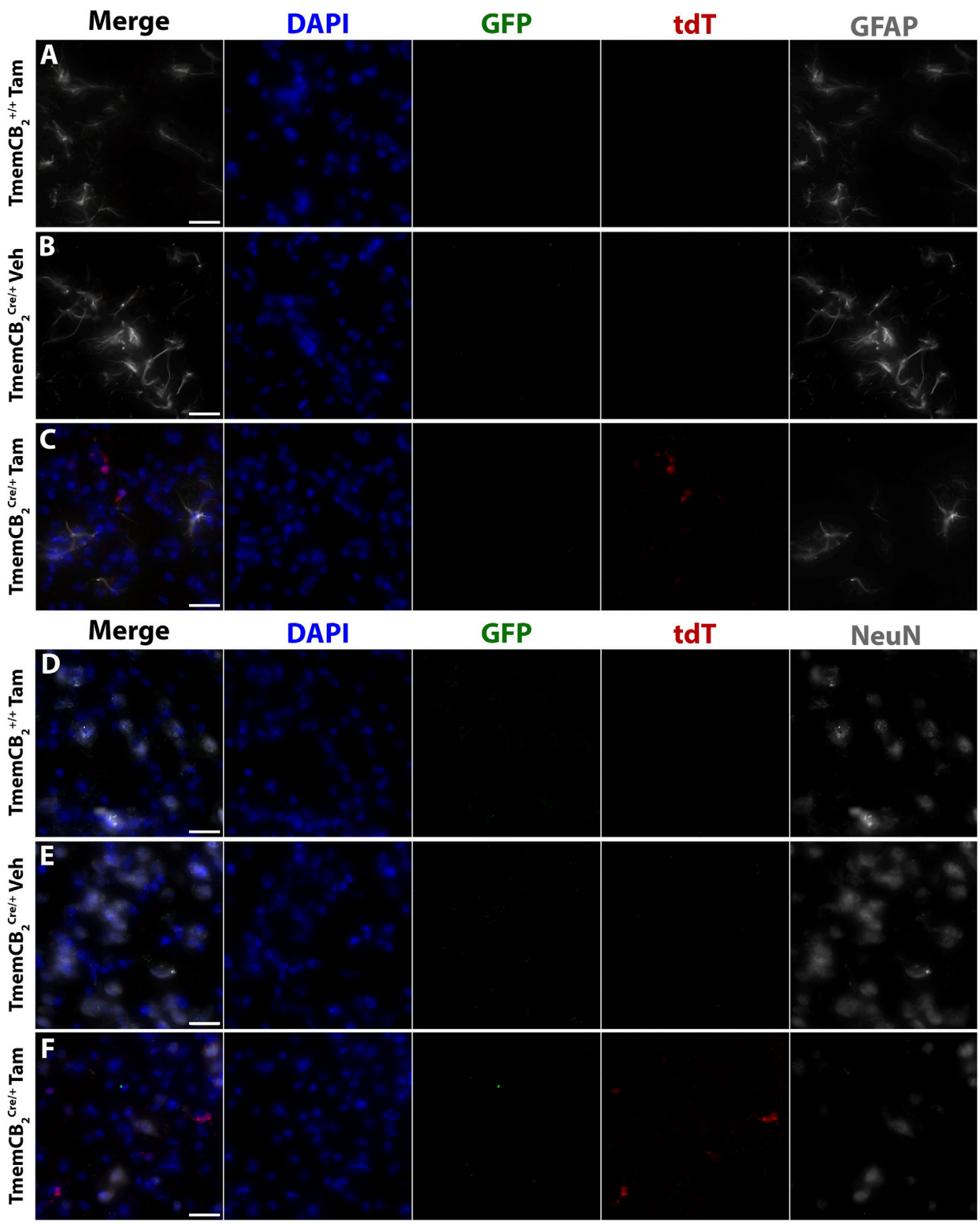

FIGURE 8
In $TmemCB_2$ mice, reporter gene expression does not colocalize with astrocytes or neurons. Representative images of immunostaining in the substantia nigra. GFP and tdT with either **(A–C)** GFAP or **(D–F)** NeuN. **(A,D)** Tam treated $TmemCB_2^{+/+}$, **(B,E)** Vehicle treated $TmemCB_2^{Cre/+}$ **(C,F)** Tam treated $TmemCB_2^{Cre/+}$. Scale bar = 20 μm.

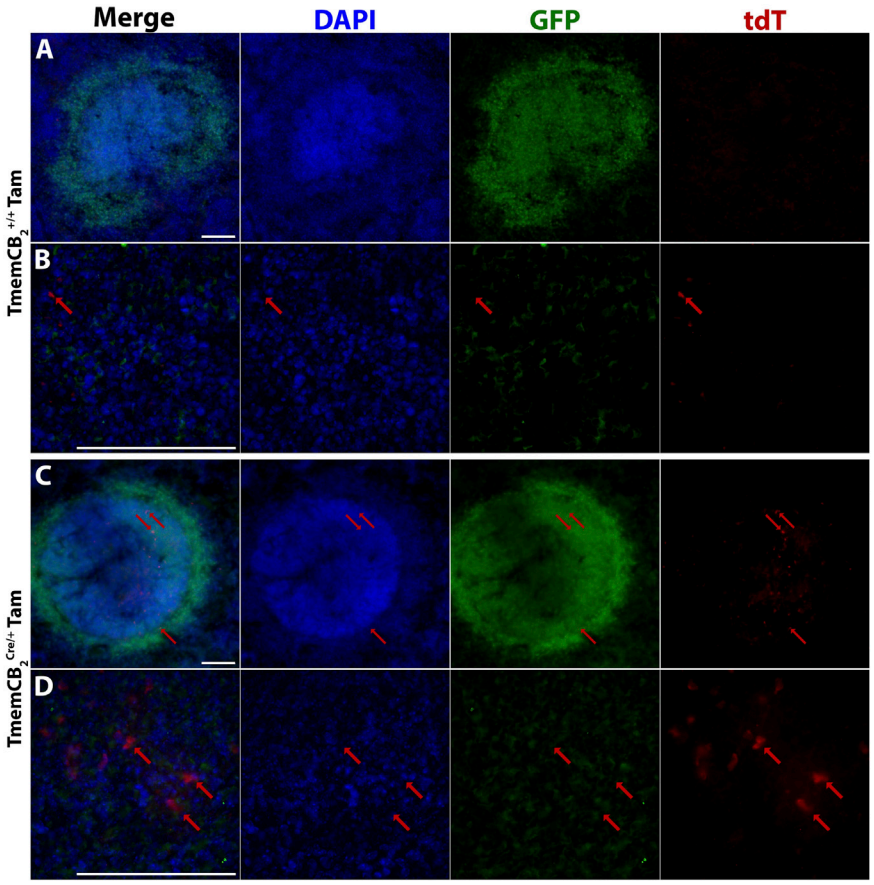

FIGURE 9
Reporter gene expression is present in the TmemCB$_2$ spleen. Representative immunofluorescent images of Tam treated **(A,B)** TmemCB$_2^{+/+}$ and **(C,D)** TmemCB$_2^{Cre/+}$ spleens. Red arrows = tdT expression. Scale bar = 100 μm.

3.7 Tamoxifen induced reporter gene expression in the TmemCB$_2$ line

To improve microglia specificity of the CB$_2$-KO, we generated the TmemCB$_2$ line by crossing the CB$_2$flx line with a Tmem119$^{Cre/ERT2}$ line (JAX, strain# 031820), in which Cre expression is driven by the highly microglia-specific Tmem119 promoter (Kaiser and Feng, 2019). We assessed CB$_2$-KO efficiency and specificity in the TmemCB$_2$ line using the optimised Tam protocol established in Cx3CB$_2$ mice (four doses of 150 mg/kg administered every other day). Tam was administered to TmemCB$_2$ mice either homozygous for the wild-type *Tmem119* allele (+/+) or heterozygous for the Tmem119$^{Cre/ERT2}$ allele (Cre/+), alongside untreated BL6 controls. Only microglia in the Tam-treated TmemCB$_2^{Cre/+}$ group were anticipated to express tdT.

Reporter gene expression and colocalization with Iba1 were analysed to determine KO efficiency (representative images in Figures 7A–D). GFP expression in Iba1+ microglia did not differ between TmemCB$_2$ groups and BL6 controls, suggesting a lack of basal CB$_2$ expression in microglia under naive conditions (F (3, 8) = 0.732, p = 0.561; Figure 7E). Similarly, tdT expression in TmemCB$_2^{+/+}$ and vehicle-treated controls did not differ from BL6 mice, confirming an absence of CB$_2$-KO cells. However, in Tam-treated TmemCB$_2^{Cre/+}$ mice, 9.3% of microglia expressed tdT, indicating a significant increase in CB$_2$-KO microglia (F (3, 8) = 18.23, p = 0.0006; Figure 7F). This confirms that tdT expression in microglia is Tam-dependent. Furthermore, in this group, 94.3% of tdT signal colocalized with Iba1+ microglia, demonstrating that CB$_2$-KO is microglia-specific in the CNS (Figure 7G). Low magnification representative images further demonstrate the differences in reporter gene expression between groups (Supplementary Figure S7).

Neither GFP nor tdT colocalized with astrocytes (Figures 8A–C) or neurons (Figures 8D–F) in any group, further confirming that CB$_2$ is not detectably expressed in these CNS types under basal conditions and that CB$_2$-KO is microglia-specific.

Finally, we examined reporter gene expression in the periphery of Tam- or vehicle-treated TmemCB$_2$ mice. As expected, minimal tdT expression was observed in the spleen of Tam-treated TmemCB$_2^{+/+}$ mice (Figures 9A,B). However, rare tdT expression was detected in the spleen of Tam-treated TmemCB$_2^{Cre/+}$ mice (Figures 9C,D), though at significantly lower levels than in Cx3CB$_2$ mice. This suggests that the TmemCB$_2$ line exhibits greater specificity for CNS microglia.

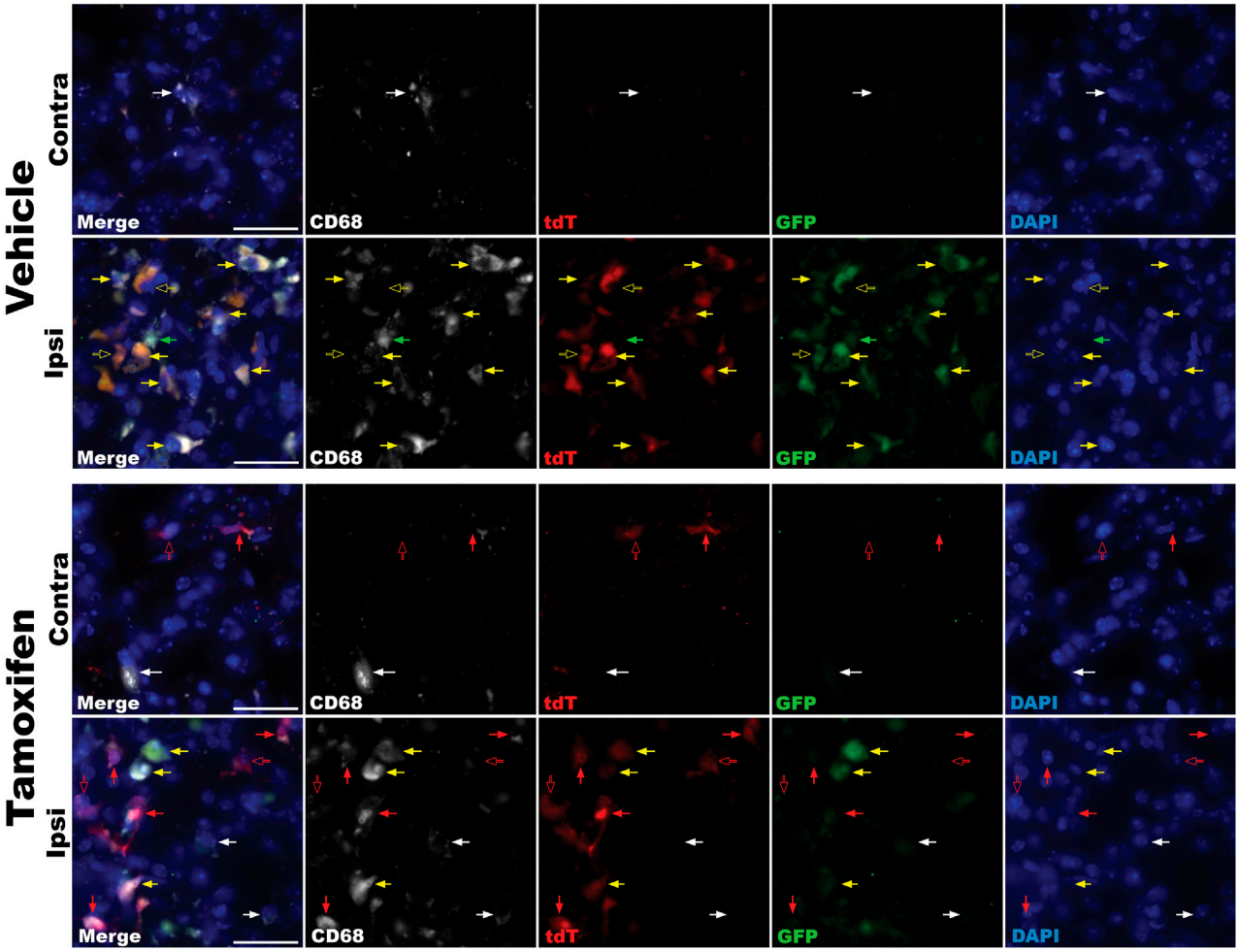

FIGURE 10
Immunofluorescence analysis of reporter gene expression in the striatum of LPS-lesioned TmemCB$_2^{Cre/+}$ mice. Representative images from vehicle- and tamoxifen-treated mice are shown for both ipsilateral and contralateral striata. Red arrows = tdT expression. Green arrows = GFP expression. Yellow arrows = GFP and tdT coexpression. Solid arrows = reporter gene colocalizing with CD68. Hollow arrows = reporter gene not colocalizing with CD68. White arrows indicate CD68 expression only. Scale bar = 25uM.

3.8 Reporter gene expression is upregulated in LPS lesioned mice

To assess whether reporter gene expression accurately reflects CB_2 upregulation in an inflammatory environment, we examined Tam- and vehicle-treated TmemCB$_2^{Cre/+}$ mice following LPS-induced neuroinflammation. GFP and tdT expression were analysed in the SN after an ipsilateral intrastriatal injection of LPS to evaluate CB_2 expression and KO persistence. CD68 is expressed by phagocytic macrophages and is considered a marker for activated microglia within the CNS (Walker and Lue, 2015). Since CB_2 is upregulated in activated microglia, the colocalization of CD68 with GFP and tdT was assessed to determine the extent of CB_2 expression in pro-inflammatory microglia in the striatum of this model.

In both treatment groups, GFP expression was highly upregulated in the ipsilateral striatum, indicating a robust increase in CB_2 expression in response to LPS. GFP almost always colocalized with CD68+ cells, which mark activated microglia, although rare GFP+ CD68− cells were observed (Figure 10).

In vehicle-treated mice, tdT expression was faint and restricted to the lesion site. This tdT signal exclusively colocalized with areas of intense GFP immunoreactivity, suggesting that it arose from channel bleed-through rather than true tdT expression (Figure 10).

In Tam-treated mice, tdT expression was widespread, observed throughout the brain and most prominently at the lesion site. Bright tdT signals were found in the ipsilateral and contralateral striatum, not colocalizing with GFP, indicating true signal and KO of CB_2 in these cells. In the ipsilateral striatum, tdT predominantly, but not exclusively, colocalized with CD68 (Figure 10).

These findings demonstrate that neuroinflammation induces CB_2 upregulation, as indicated by increased ipsilateral reporter gene expression in both groups. Importantly, Tam-treated mice exhibited persistent and specific CB_2-KO throughout the brain under inflammatory conditions.

4 Discussion

This study successfully developed and validated a novel microglia-specific, inducible CB_2-KO mouse model that incorporates a dual reporter system to enable precise, simultaneous visualisation of CB_2 expression and gene deletion. While floxed *Cnr2* mouse lines (Q.-R. Liu et al., 2017; Stempel et al., 2016) and CB_2-reporter lines (López et al., 2018; Schmöle et al., 2015) have been generated previously, this is the first model to combine an inducible CB_2-KO with reporter genes. This unique configuration allows both tracking of endogenous CB_2 promoter activity and permanent labelling of cells that have undergone CB_2-KO, a feature not present in any previously described CB_2-KO models. The CB_2flx line can be crossed with any Cre driver line, providing a versatile tool to investigate CB_2 function and regulation in both the CNS and the periphery. This flexibility represents a significant advancement for CB_2 research.

Given the well-documented lack of reliable CB_2-specific antibodies (Atwood and Mackie, 2010; Grabon et al., 2023b; Eraso-Pichot et al., 2023), traditional biochemical and histological methods for detecting CB_2 expression remain limited. By placing the reporter genes under the endogenous *Cnr2* promoter, our approach overcomes this critical methodological barrier: GFP fluorescence enables identification of cells actively expressing CB_2, while tdT provides a permanent genetic marker of recombination. This allows the monitoring of *Cnr2* promoter activity even after CB_2 protein has been knocked out, providing unique insights into transcriptional regulation that are not possible with antibody-based methods. Moreover, this design enables assessment of how CB_2 deletion in 1 cell type may influence CB_2 expression in other cell populations, supporting more nuanced investigation of intercellular signalling dynamics. While alternative methods such as RNAscope *in situ* hybridisation can detect low-abundance CB_2 mRNA with high sensitivity, including in neuronal populations where CB_2 expression is minimal (Eraso-Pichot et al., 2023), our model offers complementary advantages for long-term *in vivo* tracking and functional manipulation of CB_2-expressing cells without requiring tissue processing or probe optimisation for each experimental condition. However, we do note the limitations of reporter gene lines in detecting low abundance CB_2 expression.

CB_2 is known to play critical roles in reproduction, development, and motor control (Atwood and Mackie, 2010), so it was essential to confirm that genetic modifications made to the *Cnr2* gene did not disrupt these functions before microglial KO was induced. Our findings demonstrate these physiological processes remain intact. Additionally, *in vitro* studies revealed no differences in the responses of genetically modified glia to a CB_2 agonist, indicating that CB_2 signalling remained intact prior to conditional KO.

Under basal conditions, GFP expression, marking CB_2 activity, was detected in peripheral tissues but was minimal in the CNS, consistent with previous GFP-CB_2 models (López et al., 2018; Ruiz De Martín Esteban et al., 2022). Following LPS-induced neuroinflammation, GFP expression increased in microglia, confirming its association with microglial activation and mirroring results from previous research, including those using CB_2-GFP mice in disease models (López et al., 2018). These findings validate the responsiveness and fidelity of the *Cnr2* promoter in driving reporter expression under neuroinflammatory conditions. Furthermore, Tam-induced Cre activity in the TmemCB_2 and Cx3CB_2 lines successfully triggered expression of tdT, indicating successful CB_2-KO. Importantly, tdT expression was exclusively observed in microglia of Cre/+ mice following Tam administration, validating the inducibility and specificity of the Cre-lox system.

Under inflammatory conditions, reporter gene expression was upregulated in microglia, confirming CB_2 upregulation, consistent with previous reports (Grabon et al., 2023b). While CB_2 primarily co-localised with pro-inflammatory microglia, distinct populations were observed, suggesting that CB_2 expression is heterogeneous among microglial subtypes. The diversity of microglia expression profiles and how this translates to differences in phenotype and function is only beginning to be understood (Paolicelli et al., 2022), so it is not surprising that not all activated microglia ubiquitously express CB_2 and CD68. Another explanation is that CD68+CB_2+ cells at the LPS lesion site could be non-microglial cells expressing CB_2, but given that no GFP expression was observed in neurons or astrocytes, this is unlikely. Future studies incorporating additional markers of reactive microglia could provide further insight into microglial heterogeneity and CB_2 expression. Additionally, investigation into reporter gene colocalization with markers for other CNS cell types under various physiological and pathological conditions may reveal context-specific upregulation of CB_2 in these populations. Although there is little evidence for CB_2 expression in other CNS cell types, such as oligodendrocytes, future studies may wish to validate reporter gene expression in other cell types (Bernal-Chico et al., 2023). One limitation of this model in assessing CB_2-KO efficiency is that tdT, the marker of successful CB_2-KO, is only expressed if the *Cnr2* promoter is active. Since basal CB_2 expression is very low in the CNS, most cells with CB_2-KO would not be expected to express tdT. Nonetheless, our findings confirm that at least 9.3% of microglia in the TmemCB_2 line and 91.7% in the Cx3CB_2 line exhibited CB_2-KO. Additionally, it is important to note that almost all microglia in the Cx3CB_2 line and almost all reporter gene expressing microglia in the TmemCB_2 line expressed tdT, indicating high KO efficiency. To address challenges posed by low basal CB_2 expression, DNA-based methods such as *in situ* hybridisation will be essential for definitive confirmation of CB_2-KO efficiency.

Expression levels of tdT in Cre+ animals receiving Tam were significantly higher than GFP expression in control groups, despite both reporters being driven by the *Cnr2* promoter. This discrepancy suggests that *Cnr2* promoter activity is increased following Cre-mediated recombination, complicating the accurate assessment of CB_2-KO efficiency through reporter genes. This is unlikely to be an effect of Tam, as Tam treated +/+ groups did not have increased *Cnr2* promoter activity, as indicated by GFP expression (Figures 6A, 7E).

A potential explanation for both the increased tdT expression compared to GFP expression in controls and the higher tdT expression in the Cx3CB_2 line compared to the TmemCB_2 line may be Cre toxicity. Cre recombinase, after Tam-induced translocation to the nucleus, has been shown to cause DNA damage or other toxic effects that alter cellular function (Sahasrabuddhe and Ghosh, 2022), potentially resulting in increased *Cnr2* promoter activity. Notably, this effect appears to

be more pronounced in the $Cx3CB_2$ line, where tdT expression was nearly tenfold higher than in the $TmemCB_2$ line. This aligns with previous reports suggesting that Cre toxicity is more significant in Cx3cr1-Cre mice compared to Tmem119-Cre lines, possibly due to differences in Cre expression levels (Sahasrabuddhe and Ghosh, 2022). These findings highlight the importance of considering Cre-mediated effects when interpreting data from Cre-lox models, particularly in systems where promoter activity is under investigation; however, further research is required to validate this hypothesis.

Analysis of tdT expression in peripheral tissues also highlighted limitations in the specificity of the Cre drivers used. In the $Cx3CB_2$ line, tdT expression persisted in the spleen 3 weeks post-Tam, despite the expectation that Cre-mediated recombination would be restricted to CNS microglia due to rapid turnover of peripheral Cx3cr1+ cells after this time. This persistence suggests that a longer washout period may be necessary to achieve microglia-specific recombination in this line. In contrast, tdT expression in $TmemCB_2$ spleens was minimal, supporting the evidence that Tmem119 provides greater specificity to microglia. However, our findings also contribute to emerging evidence that Tmem119 is expressed at low levels outside the CNS. Additionally, given that Tmem119 is downregulated in activated microglia, further investigation is needed to determine its specificity and sensitivity as a microglial marker under both homeostatic and inflammatory conditions (Vankriekelsvenne et al., 2022; Bedolla et al., 2024).

Although most studies using $Cx3cr1^{Cre/ERT2}$ lines to target microglia suggest that a 3-week washout period will result in microglia specificity (Bedolla et al., 2023; Costa et al., 2021; Peng et al., 2016; Mo et al., 2019; Hohsfield et al., 2021; Y. Yang et al., 2023), evidence confirming the clearance of peripheral cells with Cre-mediated recombination at this time point is lacking. The assumption that a 3-week washout period is sufficient for peripheral clearance of KO cells is based on studies using neonate (Parkhurst et al., 2013) or adolescent (Goldmann et al., 2013) mice with low-dose Tam administration protocols. As the majority of studies employing $Cx3cr1^{Cre/ERT2}$ lines use adult mice with longer and higher-dose Tam regimens, the persistence of Cre-mediated modification in peripheral macrophages under these conditions remains unclear, and more recent evidence supports our finding that a washout period is not sufficient (Bedolla et al., 2024). Therefore, if microglia-specific CB_2-KO is required in the $Cx3CB_2$ line, a time course study of peripheral tdT expression is needed to determine the minimum washout period required to achieve specificity, as we have confirmed here that 3 weeks is insufficient.

In summary, this study presents the development and validation of a unique CB_2 transgenic model that integrates inducible KO with dual fluorescent reporters, offering a versatile and powerful tool to investigate CB_2 function across tissues, disease states, and developmental stages. The flexibility of the CB_2flx line, which can be crossed with any Cre line, allows for the study of CB_2 function and expression across various cell types and tissues. This versatility makes the CB_2flx line an invaluable tool for exploring CB_2 in a range of contexts, from neuroinflammation to other disease models in which CB_2 has been suggested to play a role. By directly addressing existing methodological limitations in CB_2 research, this model enables experiments that were previously not possible, laying the groundwork for future studies aimed at understanding CB_2's contribution to disease processes and assessing the therapeutic potential of CB_2 modulation.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: Genbank database, accession number PRJNA1356642.

Ethics statement

The animal study was approved by The Garvan Institute and St Vincent's Animal Ethics Committee. The study was conducted in accordance with local legislation and institutional requirements.

Author contributions

KL: Investigation, Data curation, Conceptualization, Formal Analysis, Project administration, Writing – review and editing, Writing – original draft, Methodology. PR: Conceptualization, Writing – review and editing, Supervision. SS: Conceptualization, Writing – review and editing, Supervision. BV: Writing – review and editing, Funding acquisition, Conceptualization, Supervision.

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Conflict of interest

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

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SUPPLEMENTARY FIGURE S1
Floxed Cnr2 construct. Modified Cnr2 gene **(A)** before and **(B)** after Cre mediated recombination. Blue boxes indicate exons. SA = Splice acceptor. pA = poly **(A)**. IRES = internal ribosome entry site. eGFP = enhanced green flouresent protein. UTR = untranslated region.

SUPPLEMENTARY FIGURE S2
Optimisation of cAMP assay for primary glia cultures. Forskolin concentration-response curves were generated for different cell densities to determine optimum cell density and forskolin concentration.

SUPPLEMENTARY FIGURE S3
Sequencing of floxed *Cnr2* gene.

SUPPLEMENTARY FIGURE S4
Representative, low magnification images of $Cx3CB_2$ substantia nigra **(A)** Tam treated $Cx3CB_2^{+/+}$, **(B)** Vehicle treated $Cx3CB_2^{Cre/+}$, **(C)** Tam treated $Cx3CB_2^{Cre/+}$. Scale bar = 100 µm.

SUPPLEMENTARY FIGURE S5
In $Cx3CB_2$ mice, reporter gene expression does not colocalize with astrocytes or neurons. Representative images of immunostaining in the substantia nigra of GFP and tdT with either **(A–C)** GFAP or **(D–F)** NeuN. **(A,D)** Tam treated $Cx3CB_2^{+/+}$, **(B,E)** Vehicle treated $Cx3CB_2^{Cre/+}$, **(C,F)** Tam treated $Cx3CB_2^{Cre/+}$. Scale bar = 50 µm.

SUPPLEMENTARY FIGURE S6
Reporter gene expression is present in the $Cx3CB_2$ spleen. Representative images of spleen immunofluorescence for **(A)** $Cx3CB_2^{+/+}$ Tam treated, 1 week-post Tam. **(B)** $Cx3CB_2^{Cre/+}$ Tam treated, 1 week-post Tam. **(C)** $Cx3CB_2^{Cre/+}$ Tam treated, 3 weeks-post Tam. Scale bar = 100 µm.

SUPPLEMENTARY FIGURE S7
Low magnification representative images of $TmemCB_2$ substantia nigra **(A)** Tam treated $TmemCB_2^{+/+}$, **(B)** Vehicle treated $TmemCB_2^{Cre/+}$, **(C)** Tam treated $TmemCB_2^{Cre/+}$. Arrows indicate colocalization of reporter gene with Iba1. Scale bar = 100 µm.

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