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RESEARCH ARTICLE

Δ^9-Tetrahydrocannabinol promotes oligodendrocyte development and CNS myelination in vivo

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Abstract

Δ^9-Tetrahydrocannabinol (THC), the main bioactive compound found in the plant *Cannabis sativa*, exerts its effects by activating cannabinoid receptors present in many neural cells. Cannabinoid receptors are also physiologically engaged by endogenous cannabinoid compounds, the so-called endocannabinoids. Specifically, the endocannabinoid 2-arachidonoylglycerol has been highlighted as an important modulator of oligodendrocyte (OL) development at embryonic stages and in animal models of demyelination. However, the potential impact of THC exposure on OL lineage progression during the critical periods of postnatal myelination has never been explored. Here, we show that acute THC administration at early postnatal ages in mice enhanced OL development and CNS myelination in the subcortical white matter by promoting oligodendrocyte precursor cell cycle exit and differentiation. Mechanistically, THC-induced-myelination was mediated by CB_1 and CB_2 cannabinoid receptors, as demonstrated by the blockade of THC actions by selective receptor antagonists. Moreover, the THC-mediated modulation of oligodendroglial differentiation relied on the activation of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, as mTORC1 pharmacological inhibition prevented

Abbreviations: 2-AG, 2-arachidonoylglycerol; CNP, 2′-3′-cyclic nucleotide 3′-phosphodiesterase; BrdU, 5-bromo-2′-deoxyuridine; CC, corpus callosum; CC1, anti-adenomatous polyposis coli; eCB, endocannabinoid; eCBS, endocannabinoid system; GSTπ, glutathione-S-transferase π; MAG, myelin associated glycoprotein; MBP, myelin basic protein; MGL, monoacylglycerol lipase; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; mTORC1, mammalian target of rapamycin complex 1; NG2, neuron-glial antigen 2; Olig2, oligodendrocyte transcription factor 2; PLP, proteolipid protein; SCWM, subcortical white matter; THC, Δ9-tetrahydrocannabinol.

Alba Huerga-Gómez and Tania Aguado contributed equally to this study.

the THC effects. Our study identifies THC as an effective pharmacological strategy to enhance oligodendrogenesis and CNS myelination in vivo.

KEYWORDS

cannabinoid receptors, cannabinoids, CB_1, CB_2, mTORC1, myelination, oligodendrocyte precursor cells

1 | INTRODUCTION

In the mammalian CNS, the oligodendrocyte (OL) developmental program begins with the specification of oligodendrocyte precursor cells (OPCs), which emerge and expand in the late prenatal and postnatal periods (Kessaris et al., 2006; Lu et al., 2002). OPCs differentiate through a premyelinating stage to become the mature myelinating OL cell, which generates axonal myelin to fine-tune neural circuitry (Monje, 2018). Progression through the OL lineage is tightly regulated by a multitude of intrinsic and extrinsic cues, which control myelination both spatially and temporally during development, along with adult lifespan and under pathological conditions (Elbaz & Popko, 2019).

The endocannabinoid system (eCBS) is a lipid-based signaling system constituted by at least two G protein-coupled receptors (CB_1 and CB_2 cannabinoid receptors), their endogenous ligands (endocannabinoids [eCB]), and the enzymes responsible for eCB synthesis and degradation (Pertwee et al., 2010). Pharmacological and genetic approaches have revealed the relevance of the eCBS in modulating normal brain development (Galve-Roperh et al., 2013). eCBs, cannabinoid receptors and eCB-metabolizing enzymes are expressed from early stages of neural development and guide major processes such as neural progenitor proliferation, cell differentiation, cell migration, and synaptogenesis (Galve-Roperh et al., 2013; Harkany et al., 2007; Maccarrone, Guzman, Mackie, Doherty, & Harkany, 2014). In the context of oligodendrogenesis, the eCB 2-arachidonoylglycerol (2-AG) has been highlighted as an important modulator of OL functions in different physiopathological settings (Ilyasov, Milligan, Pharr, & Howlett, 2018). Elevation of 2-AG levels by pharmacological inhibition of the 2-AG-degrading enzyme monoacylglycerol lipase (MAGL) induces cell proliferation in cultured OPC, whereas 2-AG enhances OL maturation when OPCs are maintained under differentiating conditions (Arevalo-Martin et al., 2007; Molina-Holgado et al., 2002). MAGL pharmacological inhibition in vivo induces premature OL development at embryonic stages (Alpar et al., 2014) and enhances OL regeneration in the Theiler's murine encephalomyelitis virus progressive mouse model of MS (Feliu et al., 2017). Increasing 2-AG levels also attenuates myelin degeneration and inflammation in the cuprizone animal model of primary demyelination and prevents mitochondrial dysfunction upon excitotoxic insults in OL in vitro (Bernal-Chico et al., 2015).

Δ^9-Tetrahydrocannabinol (THC), the main cannabinoid compound present in the plant *Cannabis sativa*, exerts most of its effects by activating CB_1 and CB_2 cannabinoid receptors (Pertwee et al., 2010). Besides the available evidence of eCBs modulating OL development and survival, the capacity of THC to target OPC and modulate OL development during CNS myelination has never been explored. Here, by using transgenic reporter mouse lines, we studied the effect of administering THC on OL development during the critical period of postnatal CNS myelination. We show that acute THC administration induces OL development in the postnatal white matter by promoting OPC cell cycle exit and differentiation. THC administration also favored OL maturation and CNS myelination. In summary, our findings identify THC as a novel pharmacological candidate to enhance OL development and CNS myelination in vivo.

2 | MATERIALS AND METHODS

2.1 | Animal procedures

All the experimental procedures used were ethically reviewed and performed under the guidelines and with the approval of the Animal Welfare Committee of Universidad Complutense de Madrid and Comunidad de Madrid, and under the directives of the European Commission (Directive 2010/63/EU). All animals, of either sex, used in this work were bred into the C57BL/6J background. Mouse lines used were from Jackson Laboratories: *CNP*-mGFP (Cnp-EGFP* 1Qrlu/J, Cat# JAX:026105) (Deng et al., 2014), neuron-glial antigen 2 (*NG2*)-dsRed (Cspg4-DsRed.T1 1Akik/J Cat# JAX:008241) (Zhu, Bergles, & Nishiyama, 2008), *NG2*-CreER^{tm} (NG2-Cre, Cspg4-cre/Esr1* BAkik/J, Cat# JAX:008538) (Zhu et al., 2008), and Rosa-Ai6(B6.Cg-Gt(ROSA)26Sor$^{tm6(CAG-ZsGreen1)Hze/J}$, Cat# JAX:007906) (Madisen et al., 2010). By crossing the *NG2*-Cre with Ai6 mouse lines we generated the *NG2*-Cre:Rosa-Ai6 mouse line (referred to here as *NG2*-Cre:Ai6). THC (3 mg/kg), SR141716 (SR_1, 2 mg/kg), SR144528 (SR_2, 2 mg/kg) or vehicle (100 μL Tween/NaCl (1:18, vol/vol) and 1% (vol/vol) dimethylsulfoxide) were administered by intraperitoneal injection to the animals. The Rapamicine analog temsirolimus (Tem) was dissolved in 0.9% NaCl and administered by intraperitoneal injection at 2.5 mg/kg. Vehicle, SR_1, SR_2, or Tem were administered 30 min before THC, once a day for four consecutive days. 5-Bromo-2′-deoxyuridine (BrdU) administration in vivo was performed as described previously (Palazuelos, Klingener, & Aguirre, 2014). Briefly, when THC was used to conduct cell cycle exit experiments (Ki67/BrdU ratios), BrdU was first administered at 100 mg/kg; 3 hr later, animals received THC or vehicle

twice a day for two consecutive days, and brain tissue was analyzed 48 hr later.

2.2 | FAC-sorting

FAC-sorting purification of *NG2*-Cre:Ai6 cells was performed as previously described (Palazuelos et al., 2014). Six-day-old *NG2*-Cre:Ai6 pups received Tamoxifen (37.5 mg/kg) for two consecutive days, and subcortical white matter tissue was microdissected 24 hr after the last Tamoxifen injection and processed for single-cell dissociation. Cell suspensions were analyzed for light forward and side scatter using a FACS Aria cell sorter, (BD Bioscience). Purified *NG2*-Cre:Ai6^{+} cells were frozen until processed for RNA extraction.

2.3 | Organotypic cerebellar slice culture

Cerebellar organotypic slice cultures were prepared by using brain tissue isolated from P8 C57BL/6J or *CNP*-mGFP animals, as described (Rutkowska, Sailer, & Dev, 2017). Briefly, mouse pups were decapitated, and their brains were dissected into ice-cold Hank's Balanced Salt Solution (HBSS). Cerebellar 300-μm sagittal slices were cut with a tissue chopper and placed on Millicell culture inserts with 0.45 μm pore size (Millipore) in medium containing 50% Minimal Essential Medium with Earle's Salts, 25% HBSS, 25% horse serum, 25 mM HEPES buffer, UltraGlutamine, penicillin–streptomycin, amphotericin B, and 5 mg/ml glucose. Media were changed every 2 days.

2.4 | Sudan black staining

Floating sections were mounted on to TESPA-coated glass slides. After air-drying, sections were dehydrated in 70% ethanol and stained with 0.5% Sudan black in 70% ethanol for 30 min. Excess staining was removed by washing the slides in 70% ethanol and finally rinsed with water. Samples were examined under light microscopy in a Zeiss Axioplan2 microscope.

2.5 | Immunofluorescence

For the characterization of OL lineage cells and myelination, brain tissue was processed as previously described (Palazuelos, Klingener, Raines, Crawford, & Aguirre, 2015). In brief, 30-μm-thick coronal free-floating brain sections obtained from paraformaldehyde-perfused mice were washed in PBS, blocked with 10% goat serum, and then incubated with the indicated primary antibodies (overnight at 4°C). The following day, sections were washed and fluorescent secondary antibodies were used. Antigen retrieval for Olig2 and Ki67 immunostainings was performed with citric acid (10 mM, pH 6, 60°C for 25 min), and for BrdU immunostaining with hydrochloric acid (2 M, 37°C for 30 min). Immunofluorescence analysis of organotypic slices was performed by firstly washing the slices in PBS and then fixing and permeabilizing them by incubation in 4% paraformaldehyde for 10 min on ice. Slices were then washed twice for 10 min in PBS and incubated for 4 hr in PBS supplemented with 0.5% Triton-X100 and 10% goat serum. Primary antibodies were applied for 48 hr at 4°C and subsequently incubated with the appropriate Alexa Fluor-conjugated secondary antibodies for 4 hr at room temperature. Nuclei were stained with DAPI. The primary antibodies used were the following: anti-CC1 (1:400, Millipore, Cat#OP80, RRID:AB_2057371), anti-Olig2 (1:250, Millipore Cat#AB9610, RRID:AB_570666), anti-GSTπ (1:200, MBL International Cat#312, RRID:AB_591792), anti-MAG (1:500, Abcam, Cat#ab89780, RRID:AB_2042411), anti-MBP (1:200, BioLegend, Cat#836504, RRID:AB_2616694), anti-myelin oligodendrocyte glycoprotein (MOG; 1:500, Abcam Cat#ab32760, RRID:AB_2145529), anti-BrdU (1:200, Abcam Cat#ab6326, RRID:AB_305426), anti-Ki67 (1:400, Thermo Fisher Scientific Cat#RM-9106-S0, RRID:AB_2341197), anti-pAkt (Thr308) (1:500, Cell Signaling Technology, Cat#9275, RRID:AB_329828), anti-pS6 (Ser240/244) (1:800, Cell Signaling Technology, Cat#5364, RRID:AB_10694233), and FluoroMyelin (1:300, Thermo Fisher Scientific Cat#F34652, RRID:AB_2572213). The appropriate mouse, rat, and rabbit highly cross-adsorbed Alexa Fluor 488, Alexa Fluor 547, and Alexa Fluor 647 secondary antibodies (1:1,000, Invitrogen) were used.

2.6 | Confocal microscopy

A confocal laser-scanning microscope TCS-SP8 (Leica DMI6000 B instrument) was used for image acquisition of green fluorescent protein (GFP), dsRed protein, Alexa 488, 547, and 647. Optical sections (z = 0.5 μm) of confocal epifluorescence images were sequentially acquired using a ×20 or ×63 objectives, with Leica Application Suite X (LAS X) software. Merged images were processed in Photoshop Cs6 software with minimal changes of contrast. At least four different brains for each strain and each experimental condition were analyzed. Cell counting was performed blindly, and tissue sections were matched across samples. For subcortical white matter analysis, a minimum of six correlative slices from a 1-in-10 series located between +1 and −1 mm from bregma was analyzed. All cell quantification data were obtained by using ImageJ (NIH), and results are presented as the mean cell number per mm^2 or as the percentage of positive cells within Olig2^{+} or in BrdU^{+} populations. CC1^{+} and NG2^{+} or CC1^{+} in Olig2 or BrdU cell quantifications were performed in the *corpus callosum* (CC) of the specified regions. When counting the percentage of Olig2^{+} cells that expressed either *NG2*-dsRed or CC1, only *NG2*-dsRed^{+}CC1^{neg} and *NG2*-dsRed^{neg}CC1^{+} cells were included. Double-positive *NG2*-dsRed^{+}/CC1^{+} cells were excluded from quantifications. GSTπ^{+}, *CNP*-mGFP^{+}, and MAG^{+} cell quantifications were performed in the CC and *cingulum* areas. All digital images were acquired using the same exposure parameters. To quantify FluoroMyelin, MBP, MOG, and proteolipid protein (PLP) immunofluorescence levels pixel intensity was measured. For

quantification of organotypic cultures, apical ends of each lobe and slices with integral cytoarchitecture were chosen. Three to five slices per condition and three to five images per slice were analyzed per experiment.

2.7 | Western blot

SCWM tissue from wild-type and transgenic mice was microdissected from 300-μm-thick coronal sections, processed for whole-protein extraction using RIPA lysis buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0, in PBS) containing PMSF, protease inhibitors, and sodium orthovanadate (Sigma). Protein samples (2–15 μg total protein) were separated on 12% acrylamide (Bio-Rad) gels and transferred to polyvinylidene difluoride membranes (Millipore). The primary antibodies used for detection of the indicated proteins were the following: anti-CNPase (1:1,000, BioLegend, Cat#836404, RRID:AB_2566639), anti-MBP (1:1,000, BioLegend, Cat#836504, RRID:AB_2616694), anti-MAG (1:2,000, Abcam, Cat#ab89780, RRID:AB_2042411), anti-MOG (1:2,000, Abcam Cat#ab32760, RRID:AB_2145529), anti-PLP (1:2,000, Abcam, Cat#ab28486, RRID:AB_776593), anti-pAkt (Thr308) (1:500, Cell Signaling Technology, Cat#9275, RRID:AB_329828), anti-pP70S6 kinase (Thr389) (1:500, Cell Signaling Technology, Cat#9206, RRID:AB_2285392), anti-p4E-binding protein 1 (BP1) (Ser65) (1:500, Cell Signaling Technology, Cat#9451, RRID:AB_330947), anti-pS6 (Ser240/244) (1:1,000, Cell Signaling Technology, Cat#5364, RRID:AB_10694233), and anti-α-tubulin (1:5,000, Sigma-Aldrich, Cat#T9026, RRID:AB_477593). Primary antibodies were used in combination with secondary horseradish peroxidase-conjugate antibodies to detect the protein in question using an enhanced chemiluminescence substrate mixture (ECL Plus; GE Healthcare; Santa Cruz Biotechnology; 1:5,000). Protein levels were quantified using Adobe Photoshop software. Protein levels were normalized to the internal control α-tubulin, referred to the Veh-treated group, and expressed as arbitrary units (a.u.).

2.8 | Real-time PCR

RNA was extracted from *NG2*-Cre:Ai6$^+$ FAC-sorted cells using Arcturus PicoPure isolation kit (Thermo Fisher Scientific). RNA from each sample was reverse-transcribed using the SuperScript First-Strand cDNA Synthesis kit (Invitrogen). Mouse gene-specific primers were obtained from Integrated DNA Technologies. Semiquantitative PCR was performed with DreamTaq Mastermix (Thermo Fisher Scientific) using the following conditions: 93°C for 2 min, and 35 cycles (1 min at 95°C, 1 min at 58°C, 1 min at 72°C). After a final extension step at 72°C for 5 min, PCR products were separated on 1.5% agarose gels. GAPDH was used as a positive control. The sequences of the primers used are the following:

CB_1-F: 5′-TCTCTGGAAGGCTCACAG-3′ and CB_1-R: 5′-TGTCTGTGGACACAGACATG-3′ (508 bp PCR product);

CB_2-F: 5′-CTCATGGGGTGGACTTGTTG-3′ and CB_2-R: 5′-ACCTTGGGCCTTCTTCTTTC-3′ (500 bp PCR product);

GAPDH-F: 5′-GGGAAGCTCACTGGCATGGCCTTCC-3′ and GAPDH-R: 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (318 bp PCR product).

2.9 | Statistics

Data shown represent the mean ± *SEM* and the *n* number of animals and experiments indicated in every case. Statistical analysis was performed by unpaired Student's *t* test using a confidence interval of 95% for pairwise comparisons or by one-way ANOVA, followed by uncorrected Fisher's LSD or Bonferroni's post hoc test, for comparisons of more than two groups (GraphPad Prism 7).

3 | RESULTS

3.1 | THC induces OPC cell cycle exit and differentiation

To evaluate the potential capacity of THC to modulate OPC cell fate in vivo, we studied the effect of an acute administration of THC in the process of oligodendrogenesis during the critical periods of postnatal CNS myelination. For this purpose, we used the *NG2*-dsRed reporter mouse line (Zhu et al., 2008), in which the fluorescent red protein is expressed under the control of the *NG2* gene promoter, an OPC marker thus allowing a strong fluorescence intensity in cells bodies for high-resolution characterization of the differentiation state of the oligodendroglial population. THC (3 mg/kg) or vehicle was administered daily at postnatal Day 6 (P6) to *NG2*-dsRed mouse pups for two or four consecutive days, and brain tissue sections were analyzed at P8 and P15 (Figure 1a). OL differentiation was analyzed by immunofluorescence in the CC by using OL-lineage (oligodendrocyte transcription factor 2, Olig2), OPC (*NG2*-dsRed), and immature/mature OL (CC1, anti-adenomatous polyposis coli) markers. We found increased CC1$^+$ OL cell densities in the CC of THC-treated-pups as fast as 2 days after the first THC administration as compared to the vehicle-treated group (Figure 1b,c). These differences were maintained at later stages of development. Moreover, THC-treated mice showed an increased percentage of Olig2$^+$ cells that co-expressed the OL marker CC1 and a reduced percentage of *NG2*-dsRed$^+$ OPCs in the CC at P8 and P15 (Figure 1d–f), indicating that THC administration promoted OL differentiation. To complement these data, we performed organotypic cerebellar cultures from P8 *NG2*-dsRed mice (Figure 1g–i). Cerebellar slices were incubated with THC (1 μM) for 2 days and the number of Olig2$^+$ cells that express OPC or OL markers was quantified. Immunofluorescence analysis showed that THC increased the proportion of Olig2$^+$ cells that express the OL differentiation marker CC1, with a concomitant reduction of *NG2*-dsRed$^+$ OPCs, as compared to controls (Figure 1h,i), thus indicating that THC also induced OPC differentiation in the cerebellar white matter ex vivo.

To further study oligodendrogenesis upon THC treatment, we performed OPC cell cycle exit experiments by administering BrdU before THC, in order to label proliferating OPCs and track cell cycle exit and differentiation dynamics in the developing CC. Immunofluorescence analysis at 2 days after THC administration showed a reduced percentage of proliferating Ki67$^+$ or NG2$^+$ cells concomitant

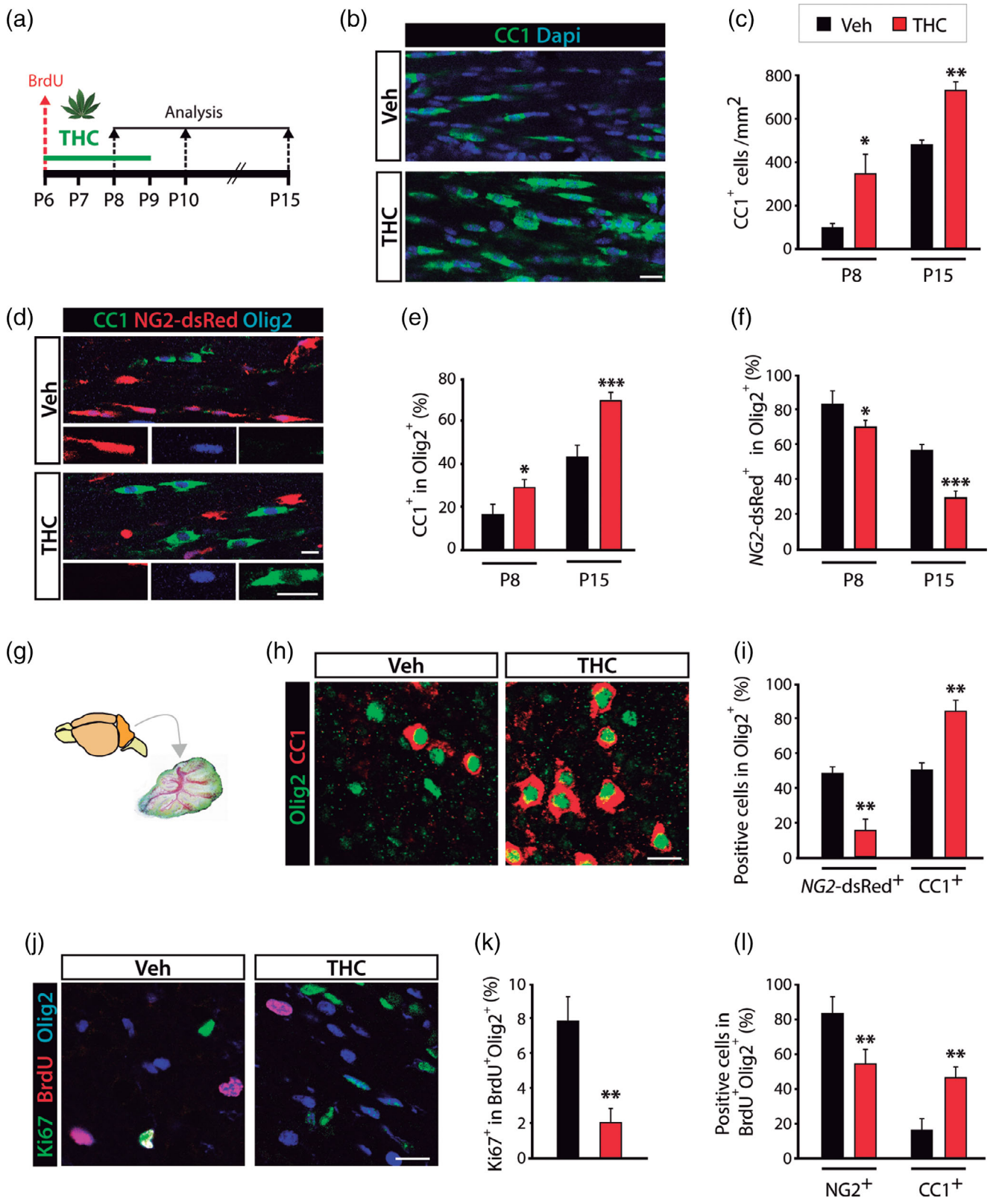

FIGURE 1 Legend on next page.

with an increased percentage of $CC1^+$ cells within the $BrdU^+Olig2^+$ population, in the CC at P8, as compared to their controls (Figure 1j–l). Together, these data support that THC induces OPC cell cycle exit and OL differentiation in the postnatal CNS.

3.2 | THC enhances OL maturation

The complete process of oligodendrogenesis compromises the transition through multiple developmental stages before the generation of mature fully differentiated myelinating cells. Thus, we evaluated whether THC administration not only induces OL differentiation but also enhances the generation of mature myelinating OLs. To address this question, we used the *CNP*-mGFP reporter mouse line (Deng et al., 2014), in which the mGFP expression is driven by the 2′-3′-cyclic nucleotide 3′-phosphodiesterase (*CNP*) promoter, that is expressed in the myelinating cell lineage, thus allowing the identification of mature myelinating OLs processes, but also the inner layers of myelin sheaths for quantifying CNS myelination. Thus, 6-day-old *CNP*-mGFP mouse pups were injected with THC or vehicle for four consecutive days, and brains were collected at different time points (P10 and P15). We performed immunofluorescence analysis and quantified the state of OL maturation in the SCWM, including the CC (Figure 2a–c), and *cingulum* (Figure 2d). We found an increased density of mature myelinating OLs, as reflected by the higher number of *CNP*-$mGFP^+CC1^+$ double-positive cells (Figure 2c), in concert with a higher percentage of $CC1^+$ OLs that expressed the mature marker GSTπ (glutathione-S-transferase π) and higher density of myelin-associated glycoprotein (MAG^+) cells in the SCWM of THC-treated mice (Figure 2d–g). In sum, these data show that THC-induced OL differentiation is accompanied by an enhanced OL maturation during postnatal development.

To complement these observations, we analyzed the impact of THC on organotypic cerebellar slices obtained from P8 *CNP*-mGFP pups (Figure 2h). Immunofluorescence analysis revealed an increased maturation of the OL population in the white matter of THC-treated cerebellar slices as compared to controls, measured as the percentage of *CNP*-$mGFP^+$ or $GST\pi^+$ cells within $CC1^+$ cells, and as MAG^+ cell density (Figure 2i–l). Overall, these findings show that THC induces OPC differentiation and enhances OL maturation.

3.3 | THC promotes CNS myelination

Then, we assessed whether THC-induced OL differentiation is fully accomplished and translates into enhanced myelination. Thus, THC or vehicle was administered to P6 *CNP*-mGFP pups for four consecutive days, and SCWM myelination was analyzed during the critical periods of postnatal myelination. Quantification of FluoroMyelin (Figure 3a), Sudan black staining (Figure 3b), GFP signals from the *CNP*-mGFP transgenic line, as well as the expression of the myelin proteins MBP (myelin basic protein) and MOG (Figure 3c) showed increased myelin and myelin-associated proteins levels in the THC-treated mouse groups when compared to controls. In addition, we quantified myelin-associated protein levels in SCWM extracts from THC-treated and control mice at P10 and P15 by western blot analyses. We found increased MBP, PLP, CNP, MOG, and MAG protein levels in SCWM extracts from THC-treated mice as compared to their controls (Figure 3d,e).

We extended these data by performing cerebellar organotypic slice culture experiments (Figure 3f). Cerebellar slices from 8-day-old *CNP*-mGFP pups were exposed to THC (1 μM) or vehicle for four consecutive days. Immunofluorescence analysis revealed increased immunoreactivity to MBP, MOG, and *CNP*-mGFP in the cerebellar white matter of THC-treated slices as compared to controls (Figure 3f). Altogether, these observations indicate that acute administration of THC at early postnatal stages enhances OL maturation and CNS myelination.

3.4 | THC modulates CNS myelination through CB_1 and CB_2 cannabinoid receptor activation

In order to address the mechanism by which THC modulates OL development during postnatal myelination, we first sought to analyze CB_1 and CB_2 receptor expression in OLs in vivo. For this purpose, *NG2*-Cre mice were bred with Rosa-Ai6 mice, an inducible reporter strain, thus allowing Cre-dependent ZsGreen expression in OPC-derived cells. RT-PCR analysis of SCWM FAC-sorted cells at P8 evidenced CB_1 and CB_2 receptor transcript expression in OLs lineage cells (Figure 4a). In order to address the possible contribution of both receptors to THC-induced CNS myelination we administered cannabinoid CB_1 receptor (SR141716A, SR_1) and CB_2 receptor (SR144528,

FIGURE 1 THC induces OPC cycle exit and differentiation. (a) Timeline of THC (3 mg/kg) or Veh administration at postnatal ages, from P6 to P9, and oligodendrogenesis was analyzed in the CC at P8, P10, and P15. (b–f) Immunofluorescence analysis of OL (CC1), OPC (*NG2*-dsRed), and OL-lineage (Olig2) markers in CC sections from *NG2*-dsRed mice at P8 or P15. Representative confocal images at P15 (b,d) and quantification of $CC1^+$ cell densities (c) and the percentage of $CC1^+$ OLs (e) or *NG2*-$dsRed^+$ OPCs (f) within the $Olig2^+$ population. (g–i) Cerebellar organotypic slice cultures from P8 NG2-dsRed mice were incubated with THC (1 μM) for 2 days. (h,i) Immunofluorescence analysis and quantification of the percentage of $Olig2^+$ cells that expressed OPC (*NG2*-dsRed) or OL (CC1) markers. (j) Cell cycle exit experiments were performed by administering BrdU before THC at P6 and quantifying the percentage of $BrdU^+$ cells that were actively dividing at P8 ($Ki67^+$). (k,l) Immunofluorescence analysis and quantification of $Ki67^+$ (k), $NG2^+$ or $CC1^+$ (l) cells within the $BrdU^+Olig2^+$ population at P8. Results are expressed in cell number per mm^2 or percentage of cells. Data are shown as mean values ± *SEM*. $n = 3$ (P8) or $n = 6$ (P15) animals for (c,e,f), $n = 3$ experiments for (i) and $n = 3$ animals for (k,l). Scale bars: (b,d,h,j) = 10 μm. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$. BrdU, 5-bromo-2′-deoxyuridine; CC, corpus callosum; OL, oligodendrocyte; OPC, oligodendrocyte precursor cell; THC, Δ^9-tetrahydrocannabinol; Veh, vehicle

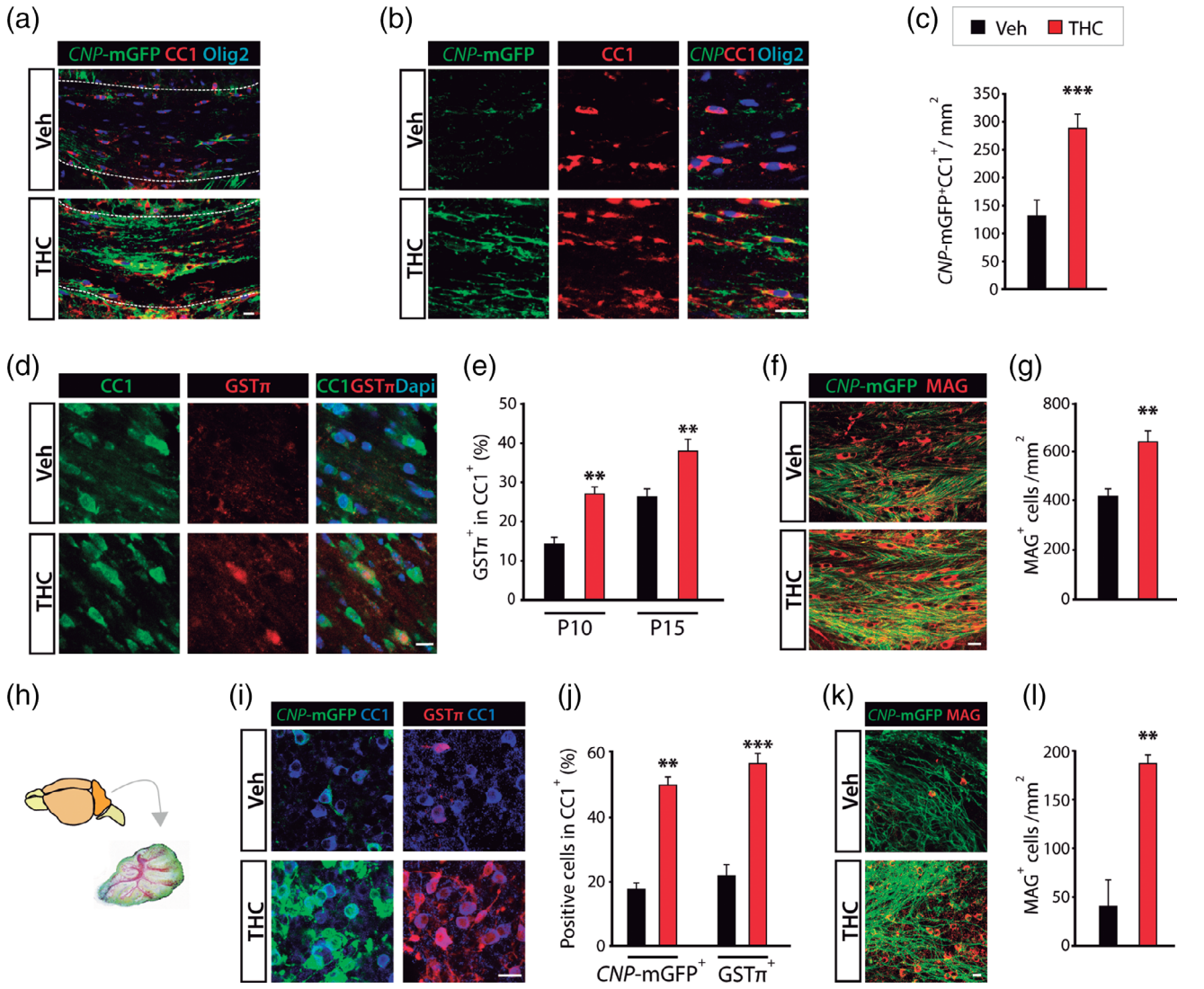

FIGURE 2 THC accelerates oligodendrocyte maturation. THC administration at early postnatal ages induces OL maturation in the SCWM. THC (3 mg/kg) or Veh was administered from P6 to P9 into *CNP*-mGFP mice and oligodendrocyte maturation was analyzed in the SCWM at P10 and P15. (a–g) Immunofluorescence analysis of brain sections from *CNP*-mGFP mice stained for OL (CC1), mature OL (GSTπ, or MAG), and OL-lineage (Olig2) markers in the SCWM at P10 and P15. Representative confocal images at P10 (a) or P15 (b,d,f) and quantification of mature OLs $CC1^+$*CNP*-$mGFP^+$ (c) and MAG^+ (g) cell densities, and the percentage of $GST\pi^+$ cells within the $CC1^+$ population (e). Results are expressed in cell number per mm^2 or percentage of cells. (h–l) Organotypic slice cultures from P8 *CNP*-mGFP mice were stimulated with THC (1 μm) or Veh for 4 days and processed for immunofluorescence analysis. (i,k) Representative confocal images and (j,l) quantification of *CNP*-$mGFP^+$ and $GST\pi^+$ cells in $CC1^+$ cells or MAG^+ cell densities in the cerebellar white matter. Data are shown as mean values ± *SEM*. n = 5 animals for (c), n = 3 animals for (e,g), and n = 3 experiments for (j,l). $^{**}p < .01$, $^{***}p < .001$. Scale bars: (a,b,d,f,i) = 10 μm, (k) = 15 μm. CC, corpus callosum; CNP, 2′-3′-cyclic nucleotide 3′-phosphodiesterase; OL, oligodendrocyte; MBP, myelin basic protein; MAG, myelin-associated glycoprotein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; SCWM, subcortical white matter; THC, Δ^9-tetrahydrocannabinol; Veh, vehicle

SR_2) selective antagonists, at 2 mg/kg, alone or in combination with THC, at 30 min before THC, once a day from P6 to P9 to *CNP*-mGFP pups, and analyzed OL differentiation and SCWM myelination at P15. Immunofluorescence analysis of OL markers in SCWM sections revealed that either CB_1 or CB_2 receptor antagonist prevented the acceleration of OL differentiation induced by THC (Figure 4b–e). Western blot analysis of SCWM extracts at P15 showed that both receptor antagonists prevented the THC-induced increase in myelin protein levels in the SCWM, such as MAG, MOG, or CNP (Figure 4f,g). These findings indicate that THC induces OPC differentiation and SCWM myelination by activating CB_1 and CB_2 cannabinoid receptors.

3.5 | THC modulates CNS myelination by activating the Akt/mTORC1 axis

In recent years, several studies have reported the role of the Akt/mTORC1 (protein kinase B, PKB/mammalian target of rapamycin

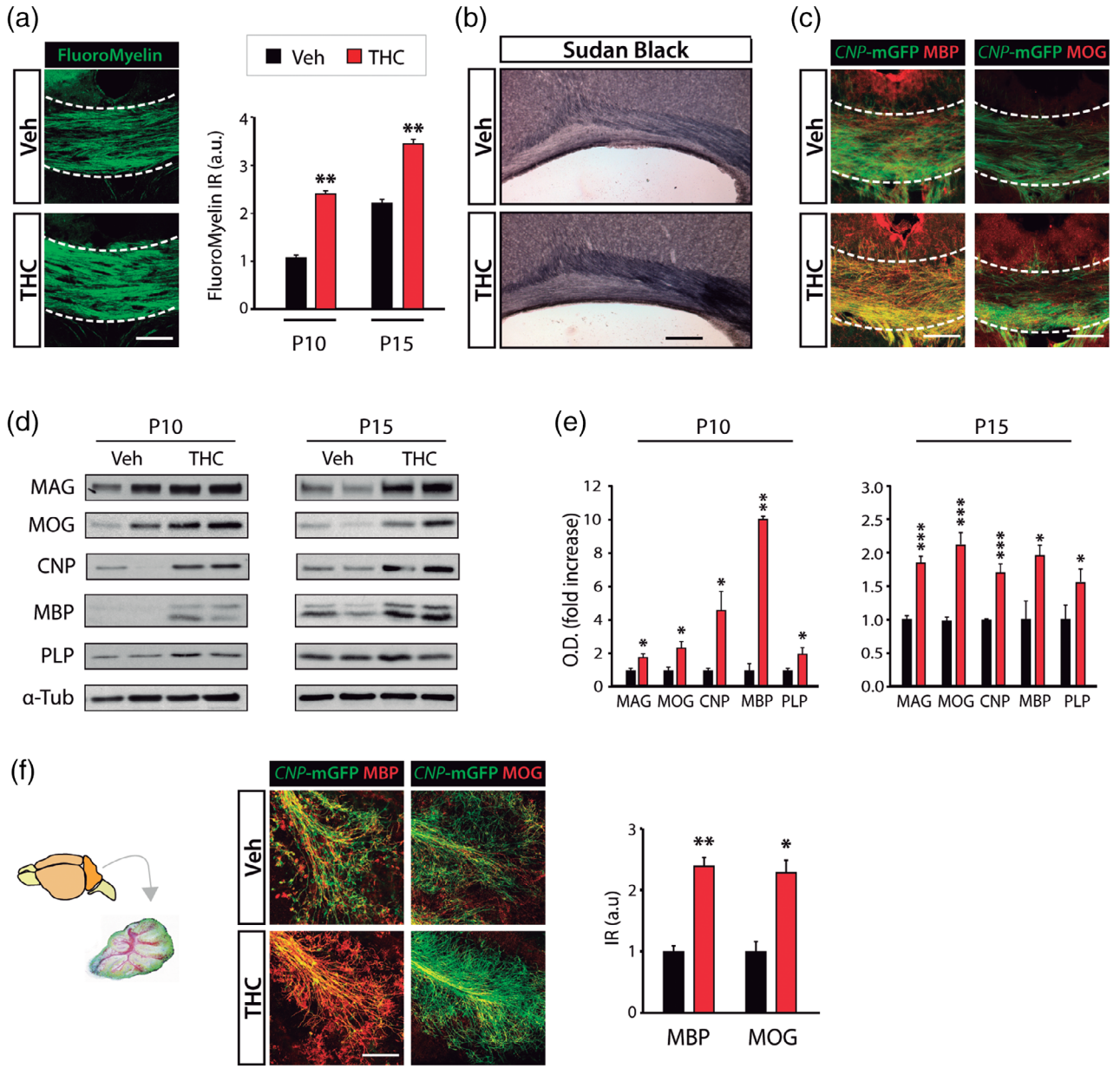

FIGURE 3 THC induces CNS myelination. THC (3 mg/kg) was administered from P6 to P9 to *CNP*-mGFP mouse. (a,c) Immunofluorescence analysis of myelin in the SCWM. (a) FluoroMyelin staining at P15 and reactivity quantification both at P10 and P15 in the SCWM of THC or Veh-treated mice. (b) Sudan black staining in the CC of THC or Veh-treated mice at P15. (c) Veh or THC-treated *CNP*-mGFP mice at P15 were immunostained for MBP and MOG. (d) Representative western blots scan images and (e) quantification of myelin protein levels in SWCM extracts from Veh or THC-treated mice at P10 and P15. Protein levels were normalized to the internal control α-tubulin, referred to the Veh-treated group, and expressed as arbitrary units (a.u.) (f) Organotypic slice cultures from P8 *CNP*-mGFP mice were stimulated with THC (1 μM) or Veh for four consecutive days and immunofluorescence analysis was performed. Representative confocal images and quantification of MBP and MOG immunoreactivity (I.R.) expressed in arbitrary units (a.u.) in the white matter of THC or Veh-treated cerebellar slice cultures. Data are shown as mean values ± *SEM*. n = 3 animals for (a), n = 3–6 animals for (e) and n = 3 experiments for (f). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. Scale bars: (a, c) = 150 μm, (b) = 220 μm, and (f) = 120 μm. CC, corpus callosum; CNP, 2′-3′-cyclic nucleotide 3′-phosphodiesterase; MBP, myelin basic protein; MAG, myelin-associated glycoprotein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; SCWM, subcortical white matter; THC, Δ^9-tetrahydrocannabinol; Veh, vehicle

complex 1) signaling pathway as an essential modulator of OL differentiation and CNS myelination (Gaesser & Fyffe-Maricich, 2016; Wood et al., 2013). Indeed, the cannabinoid CB_1 and CB_2 G protein-coupled receptors regulate this signaling pathway in several neural cell types, including neurons, neuroblasts, glioma cells, neural progenitor cells, and OPCs (Blazquez et al., 2015; Diaz-Alonso et al., 2015; Gomez et al., 2015; Palazuelos, Ortega, Diaz-Alonso, Guzman, & Galve-Roperh, 2012; Puighermanal et al., 2009; Salazar et al., 2009). Therefore, we explored the involvement of the Akt/mTORC1 signaling pathway in THC-induced OL development and CNS myelination.

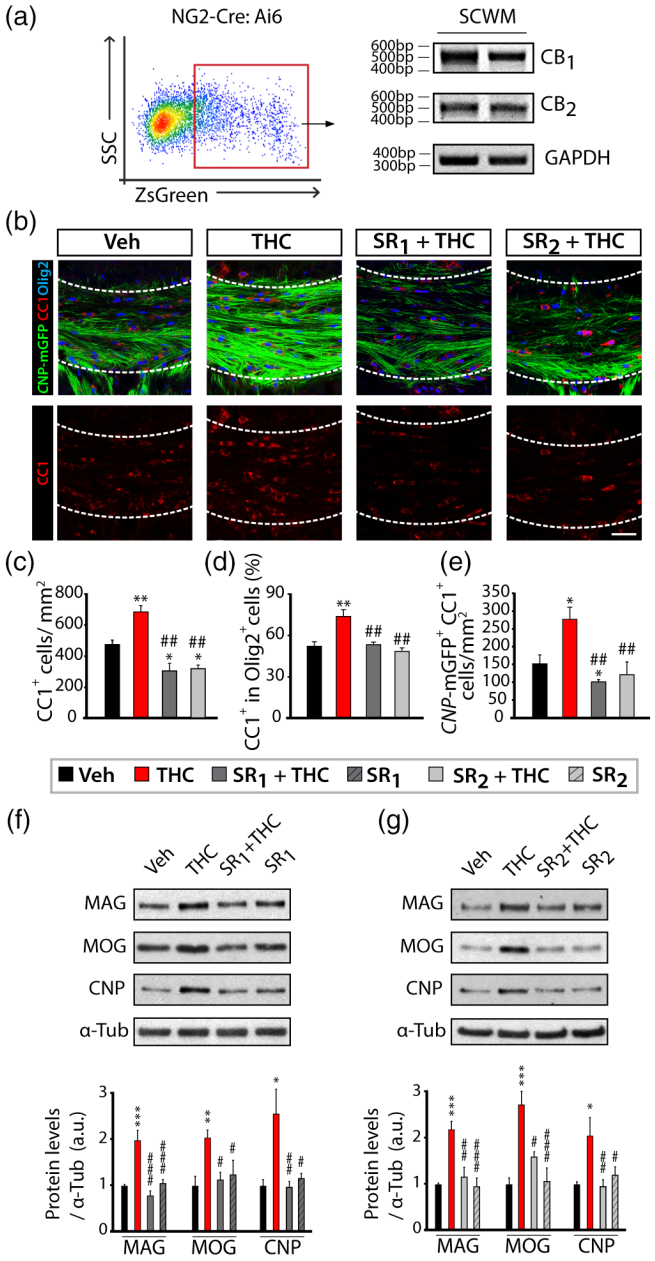

FIGURE 4 THC modulates CNS myelination through CB_1 and CB_2 cannabinoid receptors activation. (a) FAC-sorting plot of the ZsGreen population of SCWM dissected from *NG2*-Cre:Ai6 mice at Postnatal Day 8 (P8, Tamoxifen at P6 and P7). RT-PCR analysis showed the presence of CB_1 and CB_2 receptor mRNA in FAC-sorted OL lineage cells. (b–g) SR_1 (SR-141716A) or SR_2 (SR-144528), selective CB_1 or CB_2 cannabinoid receptors antagonists, respectively, were administered at 2 mg/kg 30 min before THC to P6 *CNP*-mGFP mouse pups for four consecutive days (P6–P9) and tissue was analyzed at P15 in the SCWM. (b) Immunofluorescence analysis and quantification of $CC1^+$ cell densities (c), the percentage of $CC1^+$ in $Olig2^+$ cells (d), and $CC1^+$*CNP*-$mGFP^+$ cell densities (e) at P15 in the SCWM. (f,g) Western blot analysis and quantification of myelin protein levels in SCWM extracts at P15. Protein levels were normalized to the internal control α-tubulin, referred to the Veh-treated group, and expressed as arbitrary units (a.u.). Data are shown as mean values ± *SEM*. n = 3–4 animals for (b,c,d), and n = 3–6 animals for (f,g). $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus Veh-treated mice; $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ versus THC-treated mice. Scale bars: (a) = 20 μm. SCWM, subcortical white matter; THC, Δ^9-tetrahydrocannabinol; Veh, vehicle

THC or vehicle was administered to P6 pups for four consecutive days, and SCWMs were analyzed at P15. Western blot analysis of SCWM extracts revealed increased phosphorylation levels of several representative Akt/mTORC1 axis readout proteins, such as Akt, p70 ribosomal protein S6 kinase (p70S6K), ribosomal protein S6 and eukaryotic translation initiation factor 4E-BP1 in THC-treated mice as compared to vehicle-treated animals (Figure 5a,b). Consistent with these data, immunofluorescence characterization showed that THC administration increased $pAkt^+$ (Figure 5c) and $pS6^+$ (Figure 5d,f) $CC1^+$ OLs in the SCWM, as compared to the control group. Hence, THC administration activates the Akt/mTORC1 signaling pathway in cells of the OL lineage in vivo.

In order to evaluate the involvement of mTORC1 signaling in THC-induced OL differentiation, we administered the mTORC1 inhibitor temsirolimus (Tem, 2.5 mg/kg), alone or in combination with THC (at 30 min before THC), once a day for four consecutive days, and analyzed postnatal oligodendrogenesis and SCWM myelination. Immunofluorescence analysis in the SCWM showed that mTORC1 pharmacological blockade prevented the effect of THC on OL development. Thus, temsirolimus administration abrogated the THC-induced increase in $CC1^+$ cell densities and $pS6^+$ cell numbers in the $CC1^+$ cell population (Figure 5d–g). Finally, Temsirolimus also efficiently prevented the THC-induced increase in *CNP*-mGFP reactivity in tissue sections (Figure 5d,h) and myelin protein levels in the SCWM (Figure 5i,j). In summary, these findings indicate that THC modulates OL development and SCWM myelination, at least in part, by activating the Akt/mTORC1 signaling pathway in cells of the OL lineage.

4 | DISCUSSION

In the present study, we show that THC induces OPC differentiation and CNS myelinization in vivo, which, in line with previous in vitro evidence, allows extrapolating the effect of cannabinoid compounds during cell-culture oligodendrogenesis to the developing mouse brain. THC typically binds and activates both CB_1 and CB_2 cannabinoid receptors, and OPCs express both receptors (Arevalo-Martin et al., 2007; Gomez et al., 2015; Mato, Alberdi, Ledent, Watanabe, & Matute, 2009; Molina-Holgado et al., 2002). Our finding on the presence of both receptors in *NG2*-Cre:Rosa-$Ai6^+$ OL lineage cells in the postnatal CNS are consistent with previous RNA sequencing studies of purified CNS cell types (Zhang et al., 2014) or single-cell RNA sequencing on cells of the OL lineage from the mouse juvenile and adult CNS (Marques et al., 2018). Specifically, *Cnr1* transcripts were reported in 11 out of 12 identified OL populations, ranging from OPCs to myelin-forming oligodendrocytes, suggesting that CB_1 receptors regulate cell function at multiple stages of OL lineage progression. Moreover, our results are in line with previous findings in which synthetic CB_1/CB_2 receptor-mixed agonists and eCBs were found to modulate OPC functions, and blocking pharmacologically either of the two receptors prevented those effects (Feliu et al., 2017; Gomez et al., 2011). This may raise the question of whether there is a functional and/or physical interaction between both receptors, and what

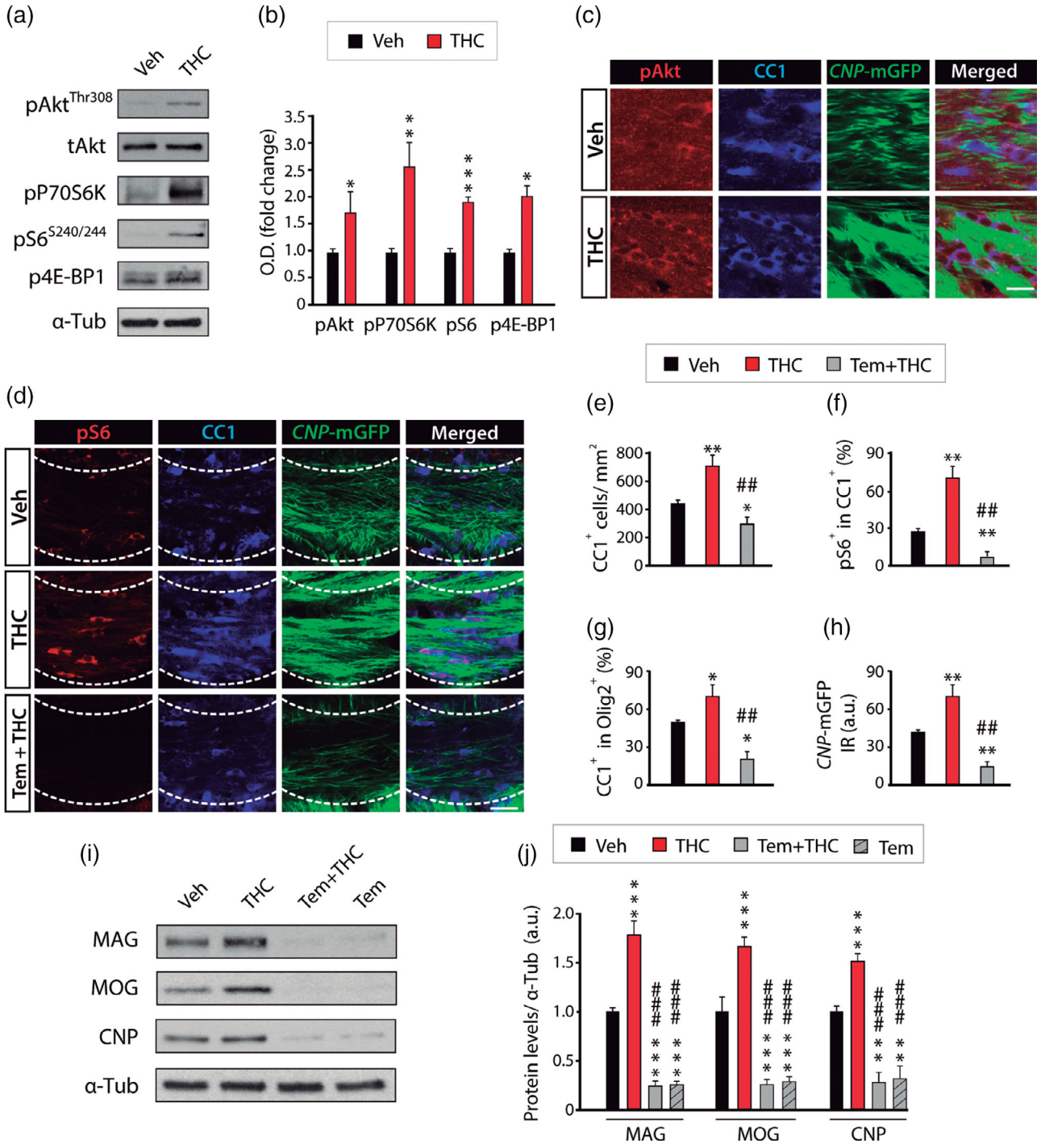

FIGURE 5 THC modulates CNS myelination by activating Akt/mTORC1 signaling pathway. The mTORC1 inhibitor temsiolimus (Tem) was administered at 5 mg/kg 30 min before THC to P6 pups for four consecutive days, and tissue was analyzed at P15 in the SCWM. (a,b) Western blot analysis and quantification of the activation of several Akt/mTORC1 axis proteins (pAkt, pP70S6K, pS6, and p4E-BP1) of SCWM extracts from Veh- or THC-treated mice at P15. (c) *CNP*-mGFP mice were immunostained for pAKT and CC1. (d–h) Immunofluorescence analysis with anti-CC1 and pS6 antibodies and quantification of $CC1^+$ cell densities (e), the percentage of $pS6^+$ in $CC1^+$ cells (f), percentage of $CC1^+$ in $Olig2^+$cells (g), and *CNP*-mGFP immunoreactivity (IR) (h) in the SCWM of *CNP*-mGFP-treated mice at P15. (i,j) Western blot analysis and quantification of myelin protein levels (MAG, MOG, CNP) in SCWM extracts from treated mice at P15. Protein levels were normalized to the internal control α-tubulin, referred to the Veh-treated group, and expressed as arbitrary units (a.u.). Data are shown as mean values ± *SEM*. $n = 3$ animals per experimental group. Scale bars: (c) = 5 μm, (d) = 20 μm. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus Veh-treated mice; $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ versus THC-treated mice. mTORC1, mammalian target of rapamycin complex 1; SCWM, subcortical white matter; temsirolimus, Tem; THC, Δ^9-tetrahydrocannabinol; Veh, vehicle

the physiopathological significance of these effects may be. In *post-mortem* human MS samples, CB_1 receptors were present in certain subpopulations of OPC and differentiated OLs in active plaques, while CB_2 receptor expression was not studied in cells of the oligodendroglial lineage (Benito et al., 2007). Therefore, the potential presence and cross talk of CB_1 and CB_2 receptors in human oligodendroglial cells in health and disease needs further analysis. An extensive number of studies have pointed out some similarities in the mode of action of the molecules that modulate oligodendrogenesis during postnatal development and in the adult CNS (Itoh, Maki, Lok, & Arai, 2015; Patel & Klein, 2011). Indeed, chronic cannabis use has been associated with structural differences in the gray matter, but more prominently in white matter tracts (Manza, Yuan, Shokri-Kojori, Tomasi, & Volkow, 2019; Orr, Paschall, & Banich, 2016), with greater functional connectivity in orbitofrontal cortex network and increased structural connectivity of the forceps minor of cannabis users, which in turn has been suggested to reflect better myelination and/or intact axons (Filbey et al., 2014; Song et al., 2002). Therefore, the identification in this study of THC as a potent inductor of OPC differentiation in vivo opens the possibility that this molecule could potentially modulate CNS myelination in the adult human brain.

While many studies have shown the capacity of temsirolimus to cross the blood–brain barrier (BBB) at the preclinical (Zhao et al., 2012) and clinical levels (Galanis et al., 2005), some studies have reported poor BBB permeability of cannabinoid receptor antagonists following administration to adult mice, specifically SR_2 (Bouchard et al., 2012; Soethoudt et al., 2017). However, as in the mammalian brain, and in particular in rodents, it is generally assumed that BBB properties probably require some refinement during the 2–3 weeks of age, later developmental stages than period used in this study (Engelhardt & Liebner, 2014). In any event, we cannot exclude the possibility that at least part of the effect observed in this study with cannabinoid receptor antagonists may be non-centrally mediated.

The process of OL development during different physiological and pathological settings needs the proper coordination of many intracellular signaling pathways to drive the processes of OPC differentiation and CNS myelination (Gaesser & Fyffe-Maricich, 2016). Among them, the Akt/mTORC1 axis has been highlighted as a central route controlling these processes (Figlia, Gerber, & Suter, 2018; Wood et al., 2013). Akt/mTORC1 exerts an essential function in modulating the initial steps of OPC differentiation and myelination (Bercury et al., 2014; Narayanan, Flores, Wang, & Macklin, 2009; Tyler et al., 2009; Wahl, McLane, Bercury, Macklin, & Wood, 2014; Wood et al., 2013). Upstream Akt/mTORC1, the main extracellular factors identified at the moment that positively modulate OL differentiation and/or CNS myelination are insulin-like growth factor, Neuregulin/erb2, and thyroid hormone (Gaesser & Fyffe-Maricich, 2016; Wood et al., 2013). Our study points to cannabinoid receptors as important regulators of OL development in vivo and identifies the Akt/mTORC1 as an intracellular pathway mediating these effects. In the context of cannabinoid signaling, exogenous and endogenous cannabinoid molecules and their receptors have been shown to modulate many processes within the CNS by controlling the Akt/mTORC1 axis. Thus, for example, during development, cannabinoid signaling through Akt/mTORC1 modulates neural progenitor cell proliferation, specification, and pyramidal neuron generation (Diaz-Alonso et al., 2015; Palazuelos et al., 2012). Moreover, deregulation of the eCBS contributes to an overactive mTORC1 signaling in focal cortical dysplasia (Garcia-Rincon et al., 2018) and to mTORC1-mediated loss of striatal neuron protection from excitotoxic injury (Blazquez et al., 2015). In the context of OLs, in vitro evidence has implicated the Akt/mTORC1 axis in mediating the effect of endogenous or synthetic cannabinoids on both OPC proliferation and OL differentiation (Gomez et al., 2011; Gomez et al., 2015). Hence, this study provides new evidence for a pivotal role of the Akt/mTORC1 axis as a mediator of OL differentiation by cannabinoid compounds in vivo.

The globally increasing recreational use of cannabis has stressed the potentially harmful effects of consumption during pregnancy. Many studies have also evidenced the relevance of the eCBS in modulating essential processes during CNS development. Thus, for example, THC exposure during embryonic development has been linked to psychiatric disorders, such as depression and anxiety (Fried & Smith, 2001; Volkow, Baler, Compton, & Weiss, 2014). In mice, cannabinoid exposure during pregnancy leads to an epileptogenic phenotype in the adult, at least in part by modulating basal progenitor expansion, as well neuronal differentiation and migration at specific embryonic stages (Bara et al., 2018; de Salas-Quiroga et al., 2015; Diaz-Alonso et al., 2012; Diaz-Alonso et al., 2015). Moreover, administration at early postnatal ages induces developmental alterations and behavioral defects in adult mice, such as altered social interaction, or spontaneous behavior (Philippot, Nyberg, Gordh, Fredriksson, & Viberg, 2016; Trezza et al., 2008). In the context of OL and myelination, increasing evidence has linked deregulated developmental myelination to impaired cognitive functions and neuropsychiatric alterations (Fields, 2008; Nave & Ehrenreich, 2014). Specifically, alterations in postnatal myelination have been associated with altered motor function (Ishii, Furusho, Dupree, & Bansal, 2014; Schneider et al., 2016), memory (Poggi et al., 2016), social interaction (Poggi et al., 2016; Roy et al., 2007), and anxiety (Carson et al., 2015; Chen et al., 2015; Roy et al., 2007). Thus, the effect of THC administration at postnatal ages observed in OL development and CNS myelination reported in this study suggests that at least part of these developmental and neuropsychiatric alterations observed after THC exposure may be due to an impact on the OL lineage.

Overall, our study identifies THC as a potent inductor of OPC differentiation and CNS myelination in vivo and supports the relevance of cannabinoid receptors in modulating OL functions and myelination during postnatal myelination in mice. It also suggests that THC administration may potentially modulate OL functions in other contexts, such as OL turnover or myelin homeostasis during adulthood, as wells as control OPC functions and enhance OL regeneration and CNS remyelination under demyelinating conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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