Activation of CB2 receptor is required for the therapeutic effect of ABHD6 inhibition in experimental autoimmune encephalomyelitis

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Abstract

Alpha/beta-hydrolase domain 6 (ABHD6) is a novel 2-arachidonoylglycerol (2-AG) hydrolytic enzyme, that can fine-tune the endocannabinoid signaling in the central nervous system. Recently we and others have demonstrated the protective effect of ABHD6 inhibition in the animal models of traumatic brain injury and epileptic seizures. In this study, we investigated the role of targeting ABHD6 in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). Post-symptom treatment with an ABHD6 inhibitor WWL70 **increased the brain levels of 2-AG** and ameliorated the clinical signs of EAE, T cells infiltration, microglia activation and the expression of activated leukocyte cell adhesion molecules. The production of iNOS, COX-2, TNF- α and IL-1 β and the phosphorylation of NF- κ B were also significantly reduced by WWL70 treatment. The neuroprotective effect of WWL70 was demonstrated by increased survival of mature oligodendrocytes, reduced demyelination and axonal loss in WWL70 treated EAE mouse spinal cord. The therapeutic effect of WWL70 on EAE was absent by co-administration of CB2 receptor antagonist, but not CB1 receptor antagonist. Consistently, WWL70 did not afford any protection in CB2 receptor knockout mice after EAE induction. Given the increased expression of ABHD6 in microglia/macrophages, but not in T cells, we speculated that inhibition of ABHD6 might enhance 2-AG signaling particularly in microglia/macrophages to exert anti-inflammatory effects via activation of CB2 receptors. These results suggest that inhibition of ABHD6 might be used as an ideal strategy for the treatment of MS and other neurodegenerative diseases.

Key words: ABHD6; 2-arachidonoylglycerol; cannabinoid receptors; microglia; inflammation; demyelination

1. Introduction

Modulation of the endocannabinoid system has emerged as a novel strategy for the treatment of neuroinflammatory and neurodegenerative diseases, including multiple sclerosis (MS) (Centonze et al., 2007b). The endocannabinoid system consists of cannabinoid type 1 and type 2 receptors (CB1R and CB2R), endogenous ligands, and enzymes for their synthesis and inactivation. N-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are the two most studied endocannabinoids, that are synthesized on demand and can activate CBRs in a region- and time- specific manner (Pertwee, 2014). Dysregulation of the endocannabinoid system observed in both animal models and patients of MS (Centonze et al., 2007a; Witting et al., 2006) suggests that targeting this system can impact the pathogenesis of MS.

Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model of MS, characterized by enhanced inflammatory and immune response, demyelination and axonal injury (Kubajewska and Constantinescu, 2010). The regulatory action of cannabinoids in EAE is mostly controlled by CB2R-mediated anti-inflammation and CB1R-mediated neuroprotection (Cabral et al., 2008; Maresz et al., 2007). Administration of exogenous 2-AG has been shown to be effective in the EAE mouse models (Lourbopoulos et al., 2011), suggesting **that** modulation of the endogenous levels of 2-AG might trigger a self-defense mechanism.

2-AG is produced from the membrane phosphatidylinositol-4,5-bisphosphate by phospholipase C and diacylglycerol lipase (DAGL), and inactivated primarily by monoacylglycerol lipase (MAGL) and to a less extent by alpha/beta-hydrolase domain 6 (ABHD6) (Murataeva et al., 2014; Ueda et al., 2011). Chronic inactivation or deletion of MAGL

causes 2-AG overload, desensitization of CB1R and behavioral tolerance (Chanda et al., 2010; Schlosburg et al., 2010). Although ABHD6 contributes to a small portion of 2-AG hydrolysis, it can complement with MAGL in neurons (Marrs et al., 2010). Combined inhibition of MAGL and ABHD6 has an additive effect to elevate 2-AG levels in neurons, suggesting **that** these enzymes may be located in different cellular compartments and thus control the selectivity and efficacy of 2-AG at the CBRs (Marrs et al., 2010). The expression of ABHD6, but not MAGL in BV2 microglia and several cell lines of macrophages implies that the function of these enzymes might be cell type specific (Alhouayek et al., 2013; Marrs et al., 2010).

Augmentation of the endogenous cannabinoids can fine-tune the cannabinoid signaling to afford neuroprotection without producing psychotropic side effects (Hwang et al., 2010). However, the role of targeting 2-AG hydrolysis in the animal models of MS has not been well-elucidated. Several recent studies have found that sustained use of MAGL inhibitors at low concentrations maintains the therapeutic efficacy to inflammatory and neuropathic pain without causing CB1R desensitization and behavioral tolerance (Guindon et al., 2013; Kinsey et al., 2013; Sciolino et al., 2011), indicating that partial, rather than a complete inhibition of 2-AG hydrolysis might be more therapeutically useful. We have recently shown that inhibition of ABHD6 has anti-inflammatory and neuroprotective effects in the mouse model of traumatic brain injury (Tchantchou and Zhang, 2013). In this study, we aim to investigate the role of ABHD6 inhibition in the treatment of EAE and the underlying mechanisms.

2. Materials and Methods:

2.1. Materials

An ABHD6 inhibitor WWL70, the CB1R antagonist AM 281 and the CB2R antagonist AM 630 were purchased from Cayman Chemicals (Ann Arbor, MI). All other chemicals and reagents were purchased from Sigma (St. Louis, MO), unless stated otherwise.

2.2. Animals

Female 7-week-old wild type (C57BL/6J) and the CB2R knockout (CB2R^{-/-}; B6.129P2-Cnr2tm1Dgen/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animal care and experimental procedures were carried out in accordance with NIH guidelines and approved by the Uniformed Services University Animal Care and Use Committee.

2.3. Induction of EAE

Following a sterile preparation of the skin, female 8-week-old wild type and CB2R^{-/-} mice were injected subcutaneously with 200 µg MOG 35–55 peptide emulsified in Complete Freund's Adjuvant (CFA, DIFCO) containing 500 µg Mycobacterium tuberculosis (DIFCO). Immediately and 24 h after immunization, mice were administered 200 ng pertussis toxin (List Biological Labs, Campbell, CA) intraperitoneally (i.p.). At 1 week after induction, EAE mice received a booster of 200 µg of MOG in incomplete Freund's Adjuvant without Mycobacterium tuberculosis. Animals were monitored blindly and daily by two independent observers and neurological signs were assessed as follows: 0, normal mouse; 1, piloerection, tail weakness; 2, tail paralysis; 3, tail paralysis plus hind limb weakness/paralysis; 4, tail, hind limb and fore limb

paralysis; 5, moribund/death, using increment of 0.5 points for intermediate clinical findings. The onset of EAE was defined as the first day when an animal showed a clinical score ≥ 0.5 .

2.4. Drug treatment

The selective ABHD6 inhibitor WWL70 at **5 mg/kg** and 10 mg/kg (i.p.) was given starting at the disease onset (usually occurs around day 10 after EAE induction) and then once a day until **day 21** or 28. To determine the cannabinoid receptor dependency, WWL70 (10 mg/kg) was co-administered with the CB1R antagonist AM281 (3 mg/kg, i.p.) and the CB2R antagonist AM630 (3 mg/kg, i.p.), respectively. All drugs were dissolved in DMSO-cremophor-saline (1:1:18), which was used as a vehicle control. For CB2R^{-/-} mice, WWL70 at 10 mg/kg was also given starting at the disease onset and then once daily until the end of experiment.

2.5. qRT-PCR

On day 28 after EAE induction, mice were euthanized and the spinal cords were dissected out. Total RNA was extracted from fresh spinal cord tissues by using Trizol reagent (Sigma, St Louis, MO). SYBR green based qRT-PCR was applied to assess the gene expression. Following the manufacture's instruction, RNA (1 μ g) was reversely transcribed to cDNA and then subjected to PCR. The reaction mixture contained 1xTagman fast universal PCR master mix (Applied Biosystems, Grand Island, NY), 50 ng cDNA and 100 nM of each primer. qRT-PCR was run in a CFX96TM Real Time System (Bio-Rad, Hercules, CA). The cycling condition was 95°C for 15 s, 75°C for 1 min, 55°C for 30 s for various cycles, followed by a melting point determination or dissociation curves. The expression level of each gene was indicated by the number of cycles needed for the cDNA amplification to reach a threshold. The amount of DNA

is calculated from the number of cycles normalized by GAPDH. The relative levels of gene expression were determined by the $2^{-\Delta\Delta C_t}$ method. The following primers were used: CB1 (Accession Number: BC079564): forward, 5'-tcttagacggccttgccagat-3' and reverse, 5'-agggactaccctgaaggaa-3'; CB2 (NM_001305278): forward, 5'-tgctgtcatatgctgggc-3' and reverse, 5'-atcctggctcctagtggtt-3'; ABHD6 (NM_025341): forward, 5'- cattcaatcctggcatttggtg-3' and reverse, 5'-atggtgtgcgtagcgaact-3'. IL-1 β (BC011437): forward, 5'-gcaactgttctgaactcaact-3' and reverse, 5'-atcttttggggtccgtcaact-3'; TNF- α (NM_013693): forward, 5'-ccctcacactagatcatcttct-3' and reverse, 5'-gctacgacgtgggctacag-3'; iNOS (AY090567): forward, 5'- acctgttcagctacgcctt-3' and reverse, 5'- cattcccaaatgtgcttgct-3'; and GAPDH (GU214026): forward, 5'- aggtcggtgtgaacggatttg-3' and reverse, 5'- ttagaccatgtagttgaggtca-3'.

2.6. Mass spectrometry 2-AG analysis

2-AG analysis was performed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as described (Zhang et al., 2010). Briefly, at 21 days after vehicle or drug injection, animals were sacrificed by cervical dislocation and the cerebral cortices were dissected, flash frozen in liquid nitrogen and stored at -80°C. Approximately 2 mg of brain tissues were homogenized with 150 μ l of 0.02% trifluoroacetic acid (TFA) and 1 nmole of internal standard (IS) 2-AG-d₈, and then added 1 ml acetonitrile. After centrifuge at 5,000 g for 5 min, the supernatants were transferred to a new glass tube, and evaporated at a temperature of 40°C under a gentle stream of nitrogen. The residues were resuspended with 0.1 ml of acetonitrile and stored at -80°C until mass analysis.

HPLC system (Agilent Technologies, Santa Clara, CA) was used with a reverse phase column RP-18 (100 $\times$ 3.0 mm i.d) (Agilent Technologies) maintained at 40°C. The mobile phase was

consisted of solvent A: 0.2% acetic acid in water–methanol (H₂O:MeOH = 95 : 5, v/v) and solvent B: 0.2% acetic acid in water–methanol (H₂O:MeOH = 5:95, v/v) with a gradient system. The flow rate was 0.5 ml/min. The HPLC flow was split before the MS and ~0.2ml/min effluent was directed into the electrospray ionization (ESI) source of the mass spectrometer. The injection volume was 20 µl. On-line LC-MS/MS analyses were performed using a tandem quadrupole mass spectrometer operated in positive ESI mode with the ion source temperature of 125°C. The positive ESI conditions for 2-AG and 2-AG-d₈ were optimized to a desolvation temperature of 350°C, a spray voltage of 3.5 kV and a cone voltage of 20 V. The MRM transitions of m/z 379 →287 for 2-AG and 387 →295 for 2-AG-d₈ were simultaneously monitored. The concentrations of 2-AG were determined by calculating their corresponding peak area ratio to that of the IS using a linear fit weighting to the calibration curve.

2.7. 2-AG hydrolysis assay

Dissected whole spinal cord was homogenized in 150 µl 1x PBS and the homogenate was centrifuged at 1,400 g for 5 min to remove debris, followed by centrifugation at 100,000 g for 45 min at 4°C. The pellet was suspended in 200 µl 1x PBS with a brief sonication, and the membrane fraction was used for the measurement of 2-AG hydrolysis assay. Twenty µg of the membrane protein was incubated with 10 µM WWL70 at 37°C for 5 min prior to mixing with 10 µM 2-AG and 33 nCi ³H-2-AG (American Radiolabeled Chemicals, St Louis MO) in 100 µl 1x PBS containing 0.1% fatty acid-free BSA. After 2 min incubation, the ice cold methanol/chloroform (1:1 ratio) was added to stop reaction. The mixture was briefly vortexed, centrifuged at 3,000 g for 5 min to separate aqueous phase from organic phase. The aqueous

phase was mixed with scintillation cocktail and the radioactivity was measured using the scintillation counter LS6500 (Beckman Coulter, Brea, CA).

2.8. Activity Based Protein Profiling (ABPP)

The ABPP assay was conducted according to the previous report (Hsu et al., 2013). Briefly, 10 μ g of the membrane fraction prepared as above was pre-incubated with WWL70 (10 μ M) for 10 min at room temperature, then mixed with a novel serine hydrolase probe HT-01 (1 μ M) at 37°C for 40 min. The HT-01 probe was kindly provided by Drs. Hsu and Cravatt, and was shown to recognize ABHD6 specifically (Hsu et al., 2013). The reaction mixture was denatured and loaded on 4-15% SDS-PAGE gel. The gel was scanned with a fluorescence monitor (FUJI FAS5100) at 473 nm excitation with FITC mode and was then subject to western blotting. The expression of calnexin detected by using an anti-calnexin antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control for membrane proteins (Spencer et al., 2004).

2.9. Immunostaining

Animals were euthanized using a combination of ketamine and xylazine solution (90 mg ketamine/10 mg xylazine per ml, i.p.), then intracardially perfused with ice cold 1x PBS followed by 4% paraformaldehyde in the same buffer. Spinal cords were dissected out and kept in 4% paraformaldehyde at 4°C overnight. Tissues were then transferred into 1x PBS containing 30% sucrose at 4°C overnight and stored at -80°C after being embedded in Tissue Tek OCT. Transverse sections of lumbar spinal cords were cut at 14 μ m using a cryostat (Leica model CM1900, Bannockburn, IL) and mounted onto Superfrost Plus slides for immunoassay.

Primary antibodies including anti-mouse F4/80 (1:300; eBioscience, San Diego, CA), anti-rabbit iNOS (1:50; Abcam, Cambridge, MA), anti-mouse CD4 (1:100; BD Pharmingen, San Jose, CA), anti-rabbit phosphorylated NF- κ B (1:100; Full moon Biosystem, Sunnyvale, CA); anti-mouse APC monoclonal (CC1) (1:100; Calbiochem, Billarica, MA), anti-rabbit MBP (1:200; Covance, Dedham, MA), anti-rabbit NF-200 (1:250; Calbiochem, Billarica, MA) and anti-rabbit ABHD6 (1:100; Abcam, Cambridge, MA) were used for immunofluorescence assay. Briefly, the slides were washed with 1x PBS twice, and then incubated in 1x PBS containing 1% donkey serum and 0.3% Triton X-100 for 30 min at room temperature, followed by respective primary antibody incubation in the same buffer overnight. The slides were then washed with 1x PBS containing 0.2% Triton X-100 and incubated with Alex fluor 488 or Alex fluor 594 conjugated donkey anti-rabbit, mouse or rat secondary antibodies (1:750; Invitrogen, Grand Island, NY) for 1 h. The slides were again washed with 1x PBS twice and covered with Fluoroshield mounting medium with DAPI. Immunofluorescence images shown in Figs.3, 4C, 7A, 9A, 10B and 10D were obtained with a fluorescence microscope (Nikon Eclipse TE-2000 U). The cells with both DAPI and expected fluorescence were defined positively stained cells. All immunofluorescence data were obtained in a minimum of 5-7 serial sections from the lumbar spinal cord of the same animal. For confocal analysis, sections were viewed on a Zeiss 710 confocal microscope and images were analyzed using Zen 2012 software (Carl Zeiss Microscopy, LLC, Thornwood, NY). We adjusted the PMT voltage until the brightest pixels in the collected image were at the saturation point but without seeing autofluorescence. The antibodies against COX-2 (Cayman Chemical, Ann harbor MI), TNF- α and activated leukocyte cell adhesion molecule (ALCAM) (Santa Cruz biotechnology, Santa Cruz, CA) were used in the diaminobenzidine (DAB) staining. After incubation with the primary antibodies mentioned above, the slides were subjected to

immunolabeling with a biotinylated secondary antibody. The expression of COX-2, TNF- α and ALCAM in spinal cord tissues was visualized after avidin-biotin horseradish peroxidase labeling and the subsequent DAB staining. F4-80, CD4, iNOS, TNF- α , NF- κ B, CC1, COX-2 and ALCAM were quantified by immunoreactive positive cells counting and results were given as mean cell numbers per mm². The negative controls were routinely performed in which the primary antibodies were omitted.

2.10. Luxol fast blue staining

Transverse spinal cord tissues were cut at 25 μ m using a cryostat and mounted onto Superfrost Plus slides. The sections were directly placed onto 1:1 ratio of alcohol/chloroform overnight and then hydrated back in 95% ethyl alcohol. The sections were left in luxol fast blue solution (containing luxol fast blue, ethyl alcohol and glacial acetic acid) at 56°C for 2 h and the excess stain was rinsed off with 95% ethyl alcohol. The grey and white matters were differentiated by dipping slides several times in 0.05% lithium carbonate solution and then in 70% ethyl alcohol. After washing, the slides were stained with cresyl echt violet solution for 10 min, dehydrated with absolute alcohol, and then switched to fresh xylene. Slides were covered with a permanent mounting media. Quantification of spinal cord demyelination was assessed as described (Esaki et al., 2010). Briefly, myelin breakdown was scored in a blinded manner as follows: score 0: no demyelination; score 1: mild demyelination; score 2: moderate demyelination; and score 3: severe demyelination. The average score from five spinal cord sections of each animal was used for counting.

2.11. Plasmid transfection

BV2 cells were trypsinized and plated into 24-well plate with about 80% confluence one day before transfection. On the day of transfection, the cultures were changed to serum-free medium, the NF- κ B reporter, a mixture of inducible NF- κ B-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct, and the negative control, a mixture of non-inducible firefly luciferase construct and constitutively expressing Renilla luciferase construct, (SABiosciences, Frederick, MD) were transfected by using FuGene HD transfection reagent (Promega, Madison, WI). For each transfection reaction, 125 μ g of the plasmid construct was mixed with 0.3 μ l transfection reagent in a final volume of 25 μ l serum-free medium. After sitting in the room temperature for 25 min, the transfection mixture was added into each well. The transfected cells were incubated for 16 h before being subjected to further treatment and luciferase activity assay.

2.12. Assay for luciferase activity

The luciferase activity assay was performed with Dual-Luciferase Reporter assay kit on the Synergy H1 Multi-Mode Reader (Biotech, Winooski, VT). Briefly, at 16 h after transfection, the cells were treated with lipopolysaccharide (LPS, 100 ng/ml) with/without WWL70 (10 μ M) for 3 h. The CB1R and CB2R antagonists AM281 (100 nM) and AM630 (100 nM) were added 15 min before the WWL70 treatment. The culture medium was removed from the transfected cells and the cells were rinsed with 1x PBS. Then, a certain amount of 1x passive lysis buffer was added into the cells followed by gentle shaking for 25 min at room temperature. Twenty μ l of cell lysates from each well was transferred to 96-well white polystyrene assay plate for further analysis according to the manufacturer's protocol. Firefly luciferase activity was quantified as

relative luciferase unit (RLU), normalized to Renilla luciferase activity of each well, and expressed as relative NF- κ B reporter activity.

2.13. Statistical analysis

Statistical analyses were performed using GraphPad InStat 3 software (GraphPad Software, Inc. La Jolla, CA). Analysis of variance (ANOVA) together with Bonferroni's multiple comparison post-test was used to compare the differences among the multiple groups, and the student's t-test was used to compare the results between two groups. Results were quantified and expressed as mean $\pm$ standard error of mean (SEM). Statistical difference was defined as $p \leq 0.05$.

3. Results

3.1. Inhibition of ABHD6 ameliorated clinical signs of EAE

Inhibition of ABHD6 has been suggested to fine-tune the 2-AG signaling in brain and shown to be effective in the animal models of traumatic brain injury and epileptic seizure (Marrs et al., 2010; Naydenov et al., 2014; Tchantchou and Zhang, 2013). **Very recently, enhancement of 2-AG signaling by inhibition of its major hydrolytic enzyme MAGL is also shown to attenuate neuroinflammation and neurodegeneration in the mouse model of mild traumatic brain injury (Katz et al., 2015; Zhang et al., 2015). In our previous studies, we have shown that a selective ABHD6 inhibitor WWL70 at 10 mg/kg has a better effect in blocking TBI-induced deficits in fine motor movement and motor coordination than WWL70 at 5 mg/kg (Tchantchou and Zhang, 2013). To determine the role of ABHD6 inhibition in the EAE mouse model, these two doses of WWL70 (5 mg/kg and 10 mg/kg) were given starting at disease onset and then once a day until**

day 21. As shown in figure 1A, treatment with WWL70 at 10 mg/kg significantly ameliorated the clinical signs of EAE. However, at 5 mg/kg, WWL70 failed to reduce the clinical symptoms. Using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), we measured the brain levels of 2-AG at 21 days after EAE induction and 12 days following WWL70 treatment. The 2-AG levels in control and EAE animals were 20.5 ± 1.2 pmol/mg and 26.7 ± 2.2 pmol/mg brain tissues, respectively (Fig. 1B). Although treatment with WWL70 at 5 mg/kg and 10 mg/kg both increased the brain levels of 2-AG (35.6 ± 2.8 pmol/mg and 47.4 ± 2.9 pmol/mg), the significant difference was only observed when the WWL70 at 10 mg/kg treated group was compared to the EAE-vehicle group (Fig. 1B. $p < 0.05$, $n=3$ /group). Thus, WWL70 at 10 mg/kg was used in our subsequent experiments. To explore the involvement of CB1 and CB2 receptors in the therapeutic effect of ABHD6 inhibition, the EAE mice were treated with the combination of WWL70 with the CB1R antagonist AM281 or the CB2R antagonist AM630 (Fig. 1C). The reduction of WWL70 on clinical scores was reversed by co-administration of AM630, but not AM281. The maximal average clinical score in the WWL70 and AM630 treated group was 3, which was similar to the vehicle-EAE group (average maximal score 3.5); whereas in the WWL70 and AM281 group, the maximal average clinical score was 1.9. Administration of the CB1 and CB2 receptor antagonists alone did not have any effect on the disease progression of EAE (Fig. 1D). These results suggest that the therapeutic effect of WWL70 on EAE is mostly mediated by activation of CB2 receptors.

3.2. WWL70 inhibits 2-AG hydrolysis in the EAE mouse spinal cord

Using the spinal cord tissue harvested from day 28 after EAE induction, we found that ABHD6 mRNA level in EAE mice was significantly increased as compared to that in control

mice (Fig. 2A). To further determine whether ABHD6 dependent 2-AG hydrolysis is altered in the EAE mouse spinal cord, we measured the degradation product ^3H -glycerol from ^3H -2-AG added into the spinal cord membrane fractions. The overall 2-AG hydrolysis is the same in both control and EAE mice spinal cords (Fig. 2B). Addition of WWL70 (10 μM) to the control spinal cord membrane fractions resulted in a 4% reduction of 2-AG hydrolysis, similar to the value reported in the mouse brain tissues (Blankman et al., 2007). However, when WWL70 was added into the EAE-vehicle membrane fractions, it caused a 12% reduction indicating that the ABHD6 dependent 2-AG hydrolysis was increased by 3-fold in the EAE mouse spinal cord. In order to determine the effect of WWL70 was indeed due to its inhibition of ABHD6, activity based protein profiling (ABPP) was examined using the spinal cord tissues from control, vehicle and WWL70 treated animals. ABPP assay is a functional proteomic technology that utilizes active site-directed probes to profile the functional state of enzymes in whole proteomes (Cravatt et al., 2008). ABPP probes label active enzymes, but not their inhibitor-bound forms, and thus can be used to detect the potency and selectivity of enzymatic inhibitors in vivo (Blankman and Cravatt, 2013). On day 28 after EAE induction, mice spinal cords were harvested and the membrane fractions were used to react with a novel serine hydrolase probe HT-01 that can specifically bind to ABHD6, but not the other serine hydrolases (Hsu et al., 2013). The membrane fraction from WWL70 treated EAE mice showed significantly reduced binding to the probe as compared to those from control and EAE-vehicle animals (Fig. 2C and D). This result suggested that administration of WWL70 to EAE mice could efficiently reduce the ABHD6 enzymatic activity in the spinal cord.

3.3. Treatment with WWL70 reduced immune cell infiltration and microglia/macrophages accumulation in EAE mouse spinal cord

During the symptomatic stage of the disease, proinflammatory mediators such as iNOS and TNF- α from activated microglia and macrophages can cause loss of oligodendrocytes and demyelination (De Groot et al., 1997; Gentile et al., 2013; Starossom et al., 2012). Recently, it has been reported that WWL70 can suppress inflammatory response in LPS activated macrophages (Alhouayek et al., 2013). In this study, we tested the role of WWL70 on microglia/macrophages activation and T cell infiltration in EAE mouse spinal cord. At 28 days post-immunization, a significant increase of microglia/macrophages and CD4⁺ T cell infiltrates in the EAE mouse spinal cord white matter was observed (Fig. 3A-D). The number of microglia/macrophages in the lumbar spinal cord white matter of EAE vehicle group is 300 ± 39 cells/mm² (Fig. 3B) and the number of CD4⁺ T cell is 308 ± 52 cells/mm² (Fig. 3D). These values are significantly greater than those in the WWL70 treated group in which the numbers of microglia/macrophages and CD4⁺ T are 78 ± 8 cells/mm² and 32 ± 12 cells/mm², respectively (Fig. 3B and D). It has been documented that the anti-inflammatory effects of cannabinoids can be mediated by CB1 and CB2 receptors activation (Mackie, 2008), and in the immune and inflammatory cells, the expression of CB2R is usually dominant compared to that of CB1R (Gong et al., 2006). To better understand the involvement of CB1 and CB2 receptors in the anti-inflammatory effect of WWL70, the accumulation of microglia/macrophages and the infiltration of CD4⁺ T cells were also examined in EAE mice treated with the combination of WWL70 with each of the cannabinoid receptors antagonists. When the EAE mice were treated with the combination of WWL70 and CB2R antagonist AM630, an increase in the number of microglia/macrophages and CD4⁺ T cells were seen in the spinal cord white matter compared to

the WWL70 alone treated group (Fig. 3A and C). The numbers of microglia/macrophages and CD4⁺ T cells in the WWL70 plus AM630 treated group are 238 ± 63 cells/mm² and 228 ± 31 cells/mm², respectively (Fig. 3B and D). These values are significantly greater than the numbers of microglia/macrophages ($p < 0.05$) and CD4⁺ T cells ($p < 0.01$) in the WWL70 alone treated group. On the other hand, in the WWL70 and AM281 treated group, the reduction of the numbers of microglia/macrophages and CD4⁺ T cells in the spinal cord white matter remained the same as that in the WWL70 treated group. Since CB2R are mostly expressed in immune and inflammatory cells, we hypothesized that the reduction of microglia/macrophages and T cells might also lead to the reduction of CB2R expression. Indeed, the mRNA levels of CB2 receptors were significantly increased in EAE vehicle mice and decreased by the treatment of WWL70 (Fig. 3E). Similar to our findings that combined treatment of AM630, but not AM281 with WWL70 significantly reversed the inhibitory effects of WWL70 on microglia and CD4 T cells, the reduced expression of CB2R mRNA by WWL70 was also reversed by CB2R antagonist, but not the CB1R antagonist.

3.4. Inhibition of 2-AG hydrolysis resulted in down-regulation of proinflammatory cytokines

An increasing amount of studies have shown that 2-AG exerts neuroprotective effects through suppressing the production of reactive oxygen/nitrogen species and proinflammatory cytokines in reactive microglia (Gallily et al., 2000; Panikashvili et al., 2001). In this study, through partial inhibition of 2-AG hydrolysis with WWL70, we sought to elucidate the potential therapeutic mechanisms of ABHD6 inhibition. We found a significant increase of TNF- α in EAE mice after 28 days immunization (Fig. 4A); the number of TNF- α positive cells in EAE mice is 170 ± 16 cells/mm², whereas the positive TNF- α stained cells in control mice are 20 ± 2 cells/mm²

($p < 0.001$; Fig. 4B). Administration of WWL70 reduced the number of TNF- α positive cells to 56 ± 7 cells/mm², which is significantly lower than the EAE-vehicle group ($p < 0.001$). Treatment with the combination of WWL70 and AM630 reversed the downregulation of TNF- α by WWL70, and the number of TNF- α positive cells increased to 121 ± 21 cells/mm² ($p < 0.05$). There was no difference in the number of TNF- α positive cells (46 ± 8 cells/mm²) in the EAE mice treated with WWL70 plus AM281 as compared to that in the WWL70 alone treated mice (Fig. 4B). In addition to the changes in the expression of TNF- α , the induction of iNOS and the increased expression of COX-2 were also observed in EAE mice and reduced by WWL70 treatment (Fig. 4C-F). Similarly, the effects of WWL70 on these parameters were diminished by co-administration of CB2R antagonist, but not CB1R antagonist. Consistently, a significant increase of the mRNA levels of TNF- α , iNOS, and IL-1 β was also observed in EAE mouse spinal cord and reduced by WWL70 treatment (Fig. 5). Co-administration of AM630 with WWL70 brought TNF- α , iNOS and IL-1 β mRNA levels back to those observed in the untreated EAE mice, whilst the addition of AM281 had no effect on WWL70 treatment.

3.5. The increased expression of activated leukocyte cell adhesion molecule (ALCAM) in EAE mouse spinal cord was reduced by WWL70 treatment

Previous studies have shown that in MS and other neuroinflammatory processes, blood-brain barrier (BBB) dysfunction was associated with increased leukocyte trafficking into the central nervous system (CNS), resulting in intense perivascular leukocytes infiltration (Lucchinetti et al., 2000; Noseworthy et al., 2000). The attachment of blood-borne leukocytes to the vascular bed and their migration into the brain parenchyma are the early events in CNS-directed immune responses (Engelhardt, 2006; Kebir et al., 2007). These processes are regulated by the interaction

of leukocyte adhesion molecules with their cognate ligands on endothelial cells. ALCAM was originally characterized as a ligand for CD6 and involved in lymphocyte activation and differentiation (Bowen et al., 1995; Gimferrer et al., 2004). A recent study has shown that ALCAM is expressed in human and mouse CNS microvascular endothelium and is involved in lymphocytes and macrophage infiltration across BBB (Cayrol et al., 2008). Similar to our previous findings (Vana et al., 2011), we found that in the EAE mouse spinal cord white matter, the increased expression of ALCAM was not only observed in the endothelial cells but also in regions with inflammatory cells infiltration (Fig. 6A). The positive ALCAM staining cells in the EAE vehicle group were significantly greater than those in the WWL70 treated group. The administration of AM630 reversed the inhibitory effect of WWL70 on ALCAM expression. Conversely, CB1R antagonist AM281 did not have any effect (Fig. 6B). This result suggested that WWL70 treatment blocks leukocyte recruitment in the brain by reducing ALCAM expression in the EAE mouse spinal cord.

3.6. NF- κ B activation was down-regulated in the spinal cord of EAE mouse treated with WWL70

Transcription factors provide the link between early membrane-proximal signaling events and the changes in gene expression. Several studies have shown that activation of NF- κ B can regulate the expression of multiple genes in glial and astrocyte cell lines, including murine primary astrocytes, astrocytoma cell lines, and microglia (Carter et al., 1996; Moynagh et al., 1993; Sparacio et al., 1992). Activation of NF- κ B is also implicated in the production of proinflammatory cytokines in mice with EAE (Wu et al., 2013). To further explore the underlying mechanisms of attenuation of neuroinflammatory response by WWL70, we

performed immunofluorescence assay to examine the activation of NF- κ B in control and drug treated EAE mice. The phosphorylation of NF- κ B was significantly increased in EAE mice at 28 days post immunization (Fig. 7A). Treatment with WWL70 reduced NF- κ B phosphorylation in the EAE mouse spinal cord white matter. In addition, we observed an increased activation of NF- κ B in the EAE mice treated with WWL70 together with the CB2R antagonist AM630 when compared to the WWL70 alone treated mice. Phosphorylation of NF- κ B in the EAE mice treated with WWL70 and CB1R antagonist AM281 was similar to that of the WWL70 alone treated animals. The quantitation of phosphorylated NF- κ B positive cells in each experimental group was shown in figure 7B. To determine whether the reduced NF- κ B activity by WWL70 also occurred in reactive microglia, we looked at the transcriptional regulation of NF- κ B in BV2 cells. As shown in figure 7C, the luciferase activity driven by NF- κ B promoter had a 3-fold increase when BV2 cells were activated by LPS and this increase was significantly reduced in the presence of WWL70. The combination of WWL70 with AM630, but not with AM281 reversed the inhibitory effect on luciferase activity, suggesting that suppression of NF- κ B activation by WWL70 in BV2 cells is also mediated by CB2 receptors.

3.7. ABHD6 was expressed in microglia/macrophages, but not in CD4⁺ T cells in the EAE mouse spinal cord

Our data showed that WWL70 treatment could significantly reduce the accumulation of microglia/macrophages, CD4⁺ T cell infiltrates and the production of proinflammatory molecules, such as iNOS, TNF- α , IL-1 β and COX-2 through CB2R mediated cannabinoid signaling pathways. We hypothesized that the expression of ABHD6 might be upregulated in the inflammatory cells, and its inhibition could boost the levels of 2-AG and reduce the

inflammatory response. To determine which cell types express ABHD6, we utilized immunofluorescence method to double stain the EAE spinal cord tissues with an anti-ABHD6 antibody together with an anti-F4/80 or an anti-CD4 antibody. Confocal images indicated that the ABHD6 was localized in F4/80 positive microglia/macrophages (Fig. 8A and C), but not in CD4 positive T cells (Fig. 8B and D). Since there is an increased expression of CB2R in microglia/macrophages from the EAE mouse spinal cord (Maresz et al., 2005), the increased expression of ABHD6 might suggest an enhanced 2-AG signaling mediated by CB2R in microglia/macrophages after EAE induction.

3.8. WWL70 reduced the loss of mature oligodendrocytes and demyelination in EAE mouse spinal cord

Treatment with WWL70 reduced the loss of mature oligodendrocytes in EAE mouse spinal cord as demonstrated by immunohistochemistry using an antibody against a mature oligodendrocyte marker CC1 (Fig. 9A). Meanwhile, luxol fast blue staining was used to detect the reduced demyelination in the spinal cord white matter of EAE mouse treated with WWL70 (Fig. 9C). The statistical analysis for CC1 and luxol fast blue staining was performed with CC1 counting and demyelination score in the spinal cord white matter (Fig. 9B and D). When EAE mice were treated with the combination of WWL70 and CB2R antagonist AM630, the CC1 positive cells were reduced and the demyelination was enhanced compared to the WWL70 alone treatment group. The effects of WWL70 on oligodendrocyte toxicity and demyelination were not affected by co-administration of CB1R antagonist (Fig. 9A-D). These results are in agreement with the anti-inflammatory effects of WWL70 through activation of CB2, but not CB1 receptors.

3.9. WWL70 did not have any protective effect in CB2R^{-/-} EAE mice

In order to further confirm our finding that WWL70 induced anti-inflammatory and neuroprotective effects in EAE mice is dependent on CB2 receptor, we examined the role of WWL70 in CB2R^{-/-} mice following EAE induction. Compared to the wild type mice in which treatment with WWL70 significantly reduced the clinical scores (from day 17 to day 26 after EAE induction, #, $p < 0.05$), the vehicle and WWL70 treated CB2R^{-/-} EAE mice exhibited the same degree of disease progression (Fig. 10A). Notably, there were increased clinical scores in the CB2R^{-/-} compared to the wild type control mice after EAE induction (Fig. 10A). On days 17, 20 and 21 after EAE induction, the clinical scores of CB2R^{-/-} EAE mice were significantly greater than those of the wild-type EAE mice ($p < 0.05$). When the transverse sections of the spinal cords from untreated and treated CB2R^{-/-} EAE mice were stained with a microglia/macrophages marker F4/80, we found the similar infiltration in both groups (Fig. 10B and C), unlike the inhibitory effect of WWL70 observed in wild type mice (Fig. 3A). The effects of WWL70 on demyelination and axonal injury were also examined in CB2R^{-/-} EAE mice using antibodies against myelin basic protein (MBP) and neurofilament heavy polypeptide (NF-200), respectively (Fig. 10D). Compared to the 7.8% the myelinated spinal cord areas, only 4.5% and 4.2% myelinated areas were observed in the spinal cords of the vehicle and WWL70 treated CB2R^{-/-} EAE mice (Fig. 10E). There were about 10% NF-200 positive stain areas of spinal cord in the control animal, whereas in the vehicle and WWL70 treated CB2R^{-/-} EAE mice, the NF-200 positive stain areas were 3.9% and 4.8%, respectively (Fig. 10F). These results demonstrated that the neuroprotective effects of WWL70 were lost in CB2R^{-/-} EAE mice.

4. Discussion

Administration of exogenous 2-AG has been shown to exhibit a therapeutic effect in both acute and chronic EAE mouse models (Lourbopoulos et al., 2011), but the role of augmentation of endogenous 2-AG in the treatment of EAE is unclear. The recent study has provided evidence that 2-AG hydrolysis in BV2 microglia is mainly controlled by ABHD6 (Marrs et al., 2010), although MAGL represents 85% of 2-AG hydrolytic activity in brain tissues (Blankman et al., 2007). These studies suggest that inhibition of individual 2-AG hydrolytic enzyme might allow differential control of endocannabinoid signaling at particular cell type and under different pathological conditions. In this study, we investigated the role of locally increased endogenous 2-AG through ABHD6 inhibition on neuroinflammation and neurodegeneration in an EAE mouse model. Post-symptom treatment with an ABHD6 inhibitor WWL70 significantly reduced clinical scores, CD4⁺ T cell infiltration, microglia/macrophages accumulation, production of inflammatory cytokines, loss of mature oligodendrocytes, demyelination and axonal injury in the EAE mouse spinal cord. The combination of WWL70 with the CB2R antagonist AM630, but not the CB1R antagonist AM281 remarkably attenuated the neuroprotective effect of WWL70, suggesting that the therapeutic effects of ABHD6 inhibition is attributable to CB2R activation. This conclusion was further supported by the diminished anti-inflammatory and neuroprotective effects of WWL70 in CB2R^{-/-} EAE mice.

ABHD6 is a membrane bound serine hydrolase and is expressed in postsynaptic neurons where the synthesis of 2-AG occurs (Savinainen et al., 2012). Although ABHD6 only contributes to 4% of 2-AG hydrolysis in brain homogenates, its inhibition is shown to fine-tune the cannabinoid signaling mediated by the local 2-AG increase (Blankman et al., 2007; Marrs et al.,

2010). In our study, we found that ABHD6 also contributes to 4% of 2-AG hydrolysis in control mouse spinal cord, but the 2-AG hydrolysis catalyzed by ABHD6 is increased by 3-fold in the EAE mouse spinal cord (Fig. 2B). Although MAGL is responsible for about 80% of 2-AG hydrolysis in the control mouse spinal cord, the MAGL activity remains unchanged in the spinal cord compared to the control and EAE mice (data not shown). Considering the expression of ABHD6 in microglia (Fig. 8A) and the increased ABHD6 activity in EAE spinal cord (Fig. 2B), we speculate that ABHD6 is likely to play an important role in the regulation of 2-AG signaling in microglia/macrophages, especially under inflammatory or pathological conditions.

Microglia represents a major cell type involved in the neuroinflammatory and neurodegenerative processes. Inhibition or depletion of microglia/macrophages has been shown to attenuate EAE progression (Berard et al., 2010a; Berard et al., 2010b; Bhasin et al., 2007; Tran et al., 1998). It has been well-documented that microglia express cannabinoid receptors and produce 2-AG and anandamide (Carrier et al., 2004; Carrier et al., 2005). In a mouse model of MS, 2-AG is shown to have immunoregulatory function and to promote tissue repair (Lourbopoulos et al., 2011). Therefore, control of microglia activation is believed to be the major step to limit leukocyte infiltration and constrain inflammation in EAE. In line with this assumption, we demonstrated that treatment with WWL70 reduced the accumulation of microglia/macrophages and the production of iNOS, COX-2, TNF- α and IL-1 β in EAE mouse spinal cord. The findings of reduced NF- κ B phosphorylation in WWL70 treated EAE mice and the inhibition of inducible NF- κ B-responsive firefly luciferase activity in BV2 cells also strongly support the role of WWL70 on the transcriptional regulation of proinflammatory cytokines. The reduced ALCAM expression in the spinal cords of WWL70-treated EAE mice

suggests that WWL70 treatment can also prevent the immune cell infiltration into the parenchyma of the brain and spinal cord.

A number of studies have shown that selective CB2R agonists reduce T cell proliferation and infiltration to the CNS, microglia activation and the production of inflammatory molecules (Arevalo-Martin et al., 2008; Dhopeswarkar and Mackie, 2014; Docagne et al., 2008; Kong et al., 2014; Rom and Persidsky, 2013). Our current study showed that administration of CB2R antagonist AM630, but not CB1R antagonist AM281 significantly blocked the effect of WWL70 on disease progression and neuroinflammation, suggesting the indispensable role of CB2R mediated endocannabinoid signaling in the amelioration of EAE through ABHD6 inhibition. Similar to a previous report showing CB2R mRNA was dramatically elevated in the EAE spinal cord tissues and the microglia cells (Maresz et al., 2005), we also found that at 28 days after EAE induction, the CB2R mRNA was significantly increased and reduced by WWL70 treatment (Fig. 3E). Because of the expression of ABHD6 (Fig. 8) and the upregulation of CB2R in microglia of the EAE mouse spinal cord (Maresz et al., 2005), we speculate that WWL70 can enhance endocannabinoid signaling in microglia by elevation of 2-AG and activation of CB2R in its close vicinity. Similar to the reports by others (Palazuelos et al., 2008), we also found that the clinical symptoms of EAE were exaggerated in the CB2R^{-/-} mice compared to their littermate control mice. Consistently, treatment with WWL70 had no effect on clinical scores, microglia/macrophages accumulation, demyelination and axonal injury in CB2R^{-/-} mice after EAE induction. These results further validated our findings that the therapeutic effect of WWL70 is mediated by activation of CB2 receptors. However, the studies from our lab and others have also demonstrated that the anti-inflammatory and neuroprotective effects of several cannabinoid

agonists in the EAE mouse model are mediated by CB1R, but not CB2R activation (de Lago et al., 2012; Pryce and Baker, 2007; Ribeiro et al., 2013). These results point to the complexity of various cannabinoid signaling pathways in the immune regulation under different conditions.

Besides being the major component of the endocannabinoid signaling pathway, 2-AG is also a main substrate for arachidonic acid production in brain and several peripheral tissues (Alhouayek et al., 2013; Nomura et al., 2011). Selective inhibition or targeted gene deletion of MAGL is protective in the animal models of Parkinson's disease and Alzheimer's disease via the reduction of the eicosanoid signaling (Chen et al., 2012; Nomura et al., 2011; Piro et al., 2012). Similarly, the studies from us (unpublished data) and others (Alhouayek et al., 2013) also found that ABHD6 could control both 2-AG hydrolysis and prostaglandin production in LPS activated microglia and macrophages, in which there is no or very limited expression of MAGL (Alhouayek et al., 2013; Marrs et al., 2010). Therefore, the reduced production of the inflammatory prostaglandins by inhibition of ABHD6 may also contribute to the therapeutic effect of WWL70 in the EAE mouse model. Interestingly, a recent study has shown that inhibition of ABHD6 protects against pentylenetetrazole-induced seizure in C57BL/6 mice via activation of GABA_A receptors, rather than activation of cannabinoid receptors (Naydenov et al., 2014). This finding is consistent with the report that 2-AG can directly activate GABA_A receptor (Sigel et al., 2011), and therefore is able to maintain the balance between the inhibitory and excitatory neurotransmitters and prevent glutamate induced excitotoxicity. It has been known that glutamate excitotoxicity is an important pathogenic mechanism of EAE (Pitt et al., 2000), therefore, the therapeutic effect of WWL70 on EAE might be also due to activation of GABA_A receptors by the increased production of 2-AG in microglia, probably also in neurons and

oligodendrocytes. These studies suggest that other mechanisms might be also involved in the protective effects of WWL70 in the EAE mouse model.

In conclusion, this is the first study to explore the role of ABHD6 inhibition in fine-tuning the 2-AG signaling in the EAE mouse model. Our results showed that inhibition of ABHD6 protected against neuroinflammation and neurodegeneration on EAE through CB2R activation. Taking the advantage of partial inhibition of 2-AG hydrolysis to avoid cannabinoid receptor desensitization and behavioral tolerance, development of selective ABHD6 inhibitors is likely to be a favorable approach for the treatment of MS and other inflammatory and neurodegenerative diseases.

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Figure Legends

Figure 1. Treatment with a selective ABHD6 inhibitor WWL70 dose-dependently ameliorated EAE progression via CB2 receptor activation

A. After the onset of clinical signs on day 10, EAE mice were given WWL70 at 5mg/kg and 10 mg/kg intraperitoneally. WWL70 at 10 mg/kg significantly ameliorated the clinical scores from day 13 until day 21 after EAE induction (**, $p < 0.01$ and ***, $p < 0.001$; $n=10/\text{group}$). The EAE mice treated with WWL70 at 5 mg/kg displayed the same clinical scores as that observed in the EAE vehicle group. WWL refers to WWL70 in this and the subsequent figures.

B. At 21 days after EAE induction, the 2-AG contents in cerebral cortices of vehicle and WWL70 treated groups were measured using LC-MS/MS. WWL70 at 10 mg/kg significantly increased the brain levels of 2-AG compared to the EAE-vehicle group (*, $p < 0.05$, $n=3/\text{group}$). Although there was a 33% increase in the brain levels of 2-AG after 5 mg/kg WWL70 treatment, the significant difference was not reached when it is compared to the EAE-vehicle group.

C. After the onset of clinical signs on day 10, EAE mice were administrated WWL70 at 10 mg/kg with or without the cannabinoid receptor antagonists. The suppression of WWL70 on clinical scores was reversed by co-administration of CB2R antagonist AM630 (###, $p < 0.001$; $n=10/\text{group}$), but not the CB1R antagonist AM281.

D. From day 10 after EAE induction until day 28, the EAE mice were treated with either CB1R antagonist AM281 or the CB2R antagonist AM630. The clinical scores were similar in vehicle, AM281 and AM630 treated EAE groups ($n=7/\text{group}$).

Figure 2 WWL70 inhibited 2-AG hydrolysis in EAE mouse spinal cord

A. Quantitative real-time PCR was used to assess ABHD6 expression in the spinal cord tissues of control and EAE mice. At 28 days after EAE induction, the mRNA level of ABHD6 in the spinal cord was significantly increased (**, $p < 0.001$, $n=4/\text{group}$).

B. Membrane fractions from the spinal cords of control and vehicle mice at 28 days after EAE induction were subjected to 2-AG hydrolysis assay. Addition of exogenous WWL70 at 10 μM caused a significant reduction of 2-AG hydrolysis in the EAE vehicle group, but not in the control group (*, $p < 0.05$; $n=4/\text{group}$).

C, D. Membrane fractions from the spinal cords of control, vehicle and WWL70 treated mice were subjected to ABPP assay to assess the selective inhibition of WWL70 on ABHD6 activity. The binding of ABHD6 to its specific probe HT-01 was reduced in the WWL70 treated mice compared to the control and EAE-vehicle animals. The results from two animals in each group were shown by in-gel scanning of the HT-01 labeled ABHD6, and the expression of calnexin was used as a loading control for membrane proteins by western blot (WB). Quantification of band intensity indicated that in-gel ABHD6 activity was significantly reduced in EAE mice treated with WWL70 compared to the vehicle controls (*, $p < 0.05$; $n=4/\text{group}$).

Figure 3. WWL70 treatment reduced microglia/macrophages accumulation, T cell infiltration and CB2R expression in the EAE mouse spinal cord

Immunofluorescence staining was done on transverse sections of the lumbar spinal cords from each experimental group at 28 days after EAE induction. The numbers of F4/80 positive microglia/macrophages (A, B; scale bar = 50 μm) and CD4^+ T cells (C, D; scale bar = 50 μm) were significantly reduced in WWL70 treated group compared to the EAE-vehicle group (**, $p < 0.01$ and ***, $p < 0.001$; $n=10/\text{group}$). Combined treatment with WWL70 and AM630 partially

attenuated the reduction of WWL70 on microglia/macrophages (*, $p < 0.05$; B) and $CD4^+$ T cells (**, $p < 0.01$; D), whereas the addition of CB1R antagonist AM281 to the WWL70 didn't have any effect on WWL70 treatment. Scale bar = 50 μm . The total mRNA from lumbar spinal cords of the each experimental group at 28 days after EAE induction was subjected to real-time quantitative PCR to assess CB2R expression. The CB2R mRNA expression level in EAE mice was significantly increased as compared to that in WWL70 or WWL70 and AM281 treated EAE mice (**, $p < 0.01$; E). The CB2R mRNA levels in the spinal cords of the mice treated with WWL70 and AM630 were the same to those in the EAE-vehicle mice (**, $p < 0.01$; E).

Figure 4. WWL70 treatment downregulated the expression of proinflammatory molecules in the EAE mouse spinal cord white matter

Immunofluorescence and DAB staining were performed with mouse lumbar spinal cord at 28 days after EAE induction (A-F). The upregulation of $\text{TNF-}\alpha$ (A, B), iNOS (C, D) and COX-2 (E, F) in EAE mouse spinal cord white matter was significantly reduced by WWL70, and this inhibitory effect was partially reversed by the CB2R antagonist, but not the CB1R antagonist. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ ($n=10/\text{group}$).

Figure 5. WWL70 attenuated mRNA expression of IL-1 β , $\text{TNF-}\alpha$ and iNOS in EAE mouse spinal cord through CB2R activation

Total RNA from spinal cords of the control, vehicle and WWL70 treated mice was subject to quantitative real-time PCR analysis. GAPDH was used as an internal control. The relative mRNA expression of each gene was calculated based on the value of its threshold cycle.

WWL70 significantly reduced the mRNA expression of TNF- α (A), iNOS (B) and IL-1 β (C). *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$ (n=5/group).

Figure 6. Treatment with WWL70 reduced the expression of activated leukocyte cell adhesion molecule (ALCAM) in the EAE mouse spinal cord

Anti-ALCAM antibody was employed for immunohistochemistry using spinal cord tissues from mice after 28 days EAE induction. Reduced number of positively stained cells was seen in the WWL70 treatment group compared to the EAE vehicle group. Co-administration of CB2R antagonist AM630, but not the CB1R antagonist AM281 with WWL70 enhanced ALCAM positive staining in the spinal cord white matter (A). The numbers of positive stained cells in the different treatment groups were shown (B, **, $p < 0.01$ and ***, $p < 0.001$; n=8/group).

Figure 7. WWL70 treatment downregulated NF- κ B activity in the EAE mouse spinal cord and BV2 microglia cells.

At 28 days after EAE induction, NF- κ B phosphorylation in the lumbar spinal cord sections from all the experimental groups was examined using immunofluorescence (A; scale bar = 50 μ m). NF- κ B phosphorylation was increased in the EAE-vehicle group, and reduced by WWL70 treatment. The phosphor-NF- κ B positive cells were increased in the WWL70 and AM630 treated groups compared to the WWL70 alone treated group, whereas the combined treatment with WWL70 and AM281 had no effect on NF- κ B phosphorylation (A and B, ***, $p < 0.001$; n = 8/group). BV2 cells were transfected with NF- κ B reporter construct for 16 h, and then treated with LPS for 3 h in the absence or presence of WWL70 and CBR antagonists. The increased NF- κ B activity activated by LPS was significantly reduced by WWL70 and this inhibitory effect was

reversed by CB2R antagonist, but not CB1R antagonist (C, **, $p < 0.01$ and ***, $p < 0.001$). The experiment was performed three times, and the representative data are shown.

Figure 8. ABHD6 expression was increased and co-localized with F4/80, but not with CD4 in EAE mouse spinal cord

Transverse sections from lumbar spinal cords of the EAE vehicle mice were subject to immunofluorescence double staining using anti-F4/80 and ABHD6 (A) or anti-CD4 and ABHD6 antibodies (B). CD4 and F4/80 positive cells were shown in red color, ABHD6 staining was shown in green color and nuclei were stained with DAPI shown in color blue (A, B; scale bar = 50 μm). The image with higher magnification of the F4/80, ABHD6 or CD4 and DAPI triple staining was shown (C, D; scale bar = 10 μm).

Figure 9. Treatment with WWL70 enhanced the survival of mature oligodendrocytes and reduced demyelination in EAE mouse spinal cord

Immunofluorescence and luxol fast blue staining were performed with transverse sections of lumbar spinal cord tissue from mice after 28 days EAE induction. The CC1 positive mature oligodendrocytes in the WWL70 treated group were obviously more than those in the EAE vehicle group. The combined treatment of WWL70 with AM630, but not AM281 reduced CC1 positive stained cells (A, B; *, $p < 0.05$ and ***, $p < 0.001$; $n=7/\text{group}$; scale bar = 50 μm). Less demyelination was observed in the WWL70 treatment group compared to the EAE vehicle group; Co-administration of CB2R antagonist AM630, but not CB1R antagonist AM281 attenuated the protective effect of WWL70, showing larger demyelinated areas in the spinal cord white matter (C, D; *, $p < 0.05$; $n=7/\text{group}$).

Figure 10. WWL70 didn't alleviate clinical scores, microglia/macrophages accumulation, demyelination and axonal injury in CB2R^{-/-} EAE mice

A. WWL70 was administrated to CB2R^{-/-} and the littermate control mice at the beginning of the disease onset (day 11 after EAE induction). On days 17, 20 and 21 after EAE induction, the clinical scores in the CB2R^{-/-} mice are significantly greater than that in the wild type EAE mice (*, $P < 0.05$, $n = 7/\text{group}$). However, different from the wild type mice in which WWL70 significantly reduced the clinical scores (#, $P < 0.05$, $n = 7/\text{group}$). WWL70 did not have any effect on clinical signs in CB2R^{-/-} EAE mice.

B-C. At 28 days after EAE induction, lumbar spinal cord sections from CB2R^{-/-} mice with/without WWL70 treatment were used to examine microglia/macrophages accumulation using an anti-F4/80 antibody (B; scale bar = 50 μm). Treatment with WWL70 did not reduce the intensity of microglia accumulation in CB2R^{-/-} mice (C). Microglia were stained in color green, nuclei were stained in color blue.

D-F. Lumbar spinal cord sections were stained with anti-MBP and anti-NF200 antibodies to detect the changes in myelination and axon density, respectively (D; scale bar = 50 μm). The similar degree of demyelination and axonal loss was observed in the vehicle and WWL70 treated CB2R^{-/-} EAE mice. The MBP and NF-200 positive staining areas were measured and expressed as the percentage of the whole area of the spinal cord white matter (*, $p < 0.05$ and **, $p < 0.01$; $n = 7/\text{group}$, in E and F).

Figure 1
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Figure 1

Figure 2
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A

B

C

D

Figure 3
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Figure 3

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