

## INTERACTION BETWEEN THE PROTECTIVE EFFECTS OF CANNABIDIOL AND PALMITOYLETHANOLAMIDE IN EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS IN C57BL/6 MICE

A. RAHIMI,<sup>a</sup> M. FAIZI,<sup>a</sup> F. TALEBI,<sup>b</sup> F. NOORBAKHS,<sup>c</sup> F. KAHRIZI<sup>a</sup> AND N. NADERI<sup>a\*</sup>

<sup>a</sup> Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>b</sup> Khatam-Al-Anbia Hospital, Shefa Neuroscience Research Center, Tehran, Iran

<sup>c</sup> Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

**Abstract**—Cannabinoids (CBs) have recently been approved to exert broad anti-inflammatory activities in experimental models of multiple sclerosis (MS). It has been demonstrated that these compounds could also have effects on neurodegeneration, demyelination, and autoimmune processes occurring in the pathology of MS. However, the clinical use of CBs is limited by their psychoactive effects. Among cannabinoid compounds, cannabidiol (CBD) and palmitoylethanolamide (PEA) have no psychotropic activities. We induced experimental autoimmune encephalomyelitis (EAE), a model of MS, by injecting myelin oligodendrocyte glycoprotein (MOG) to C57BL/6 mice. We assessed the effects of CBD, PEA, and co-administration of CBD and PEA on neurobehavioral scores, immune cell infiltration, demyelination, axonal injury, and the expression of inflammatory cytokines by using histochemistry methods and real-time RT-PCR. Treatment with either CBD (5 mg/kg) or PEA (5 mg/kg) during disease onset reduced the severity of the neurobehavioral scores of EAE. This effect of CBD and PEA was accompanied by diminished inflammation, demyelination, axonal damage and inflammatory cytokine expression while concurrent administration of CBD (5 mg/kg) and PEA (5 mg/kg) was not as effective as treatment with either drug per se. These results suggest that, CBD and

PEA, non-psychoactive CBs, attenuate neurobehavioral deficits, histological damage, and inflammatory cytokine expression in MOG-immunized animals. However, there is an antagonistic interaction between CBD and PEA in protection against MOG-induced disease. © 2015 IBRO. Published by Elsevier Ltd. All rights reserved.

**Key words:** cannabinoid, palmitoylethanolamide, cannabidiol, EAE, multiple sclerosis.

### INTRODUCTION

Multiple sclerosis (MS) is the most common immune-mediated demyelinating disorder of the central nervous system (CNS). The detrimental processes leading to neurological attenuation include infiltration of inflammatory leukocytes to CNS followed by axonal degeneration, oligodendrocyte cell death and autoimmune response against components of myelin (Steinman et al., 2002; Prat and Antel, 2005; Sospedra and Martin, 2005; Seehusen and Baumgärtner, 2010). These effects result in neurological deficits such as visual and sensory disturbances, bladder dysfunction, motor weakness, tremor, ataxia and progressive disability (Collin et al., 2007; Miller et al., 2008). Since the pathogenesis of MS is not fully understood and no definite treatment is yet available, researchers continue seeking pathological mechanisms and novel therapeutic approaches in MS.

In the last years, a vast literature has centered on the therapeutic potential of cannabinoid compounds. Cannabinoids (CBs) have a complex mechanism of action, but their effects are mainly mediated by two extracellular cannabinoid receptors, CB1 and CB2 (Croxford and Miller, 2003; Maresz et al., 2007). The anti-inflammatory and neuroprotective effects of CBs have been examined in several neurodegenerative disorders like Alzheimer's disease (Kim et al., 2012), Parkinson's disease (Baker et al., 2001), MS (Zajicek et al., 2003; Docagne et al., 2007; Buccellato et al., 2011), and amyotrophic lateral sclerosis (ALS; Ni et al., 2004). Cannabinoid drugs such as delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC) are now authorized as a treatment for the pain and spasticity in MS, but these medications may also have psychoactive effects (Rog et al., 2005, 2007). For this reason their indication is in some cases limited.

\*Corresponding author.

**Abbreviations:**  $\Delta^9$ -THC, delta-9-tetrahydrocannabinol; AEA, anandamide; ALS, amyotrophic lateral sclerosis; AMT, anandamide membrane transporter; ANOVA, analysis of variance; CBD, cannabidiol; CBs, Cannabinoids; CFA, complete Freund's adjuvant; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; GPR55, G-protein-coupled receptor 55; H & E, Hematoxylin and Eosin; IFN- $\gamma$ , interferon- $\gamma$ ; IL-17a, interleukin 17-a; LFB, Luxol Fast Blue; LPI, L-a-Lysophosphatidylinositol; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PBS, phosphate-buffered saline; PEA, palmitoylethanolamide; PPAR- $\alpha$ , peroxisome proliferator-activated receptor alpha; RT-PCR, reverse transcriptase-polymerase chain reaction; TMEV-IDD, Theiler's Murine Encephalomyelitis Virus-Induced Demyelinating Disease; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TRPV1, transient receptor potential cation channel subfamily V member 1.

Nevertheless, among cannabinoid compounds, cannabidiol (CBD) and palmitoylethanolamide (PEA) have no psychoactive effects (Arévalo-Martín et al., 2003; Loria et al., 2008). Cannabidiol is a nonpsychoactive phytocannabinoid that possesses anti-epileptic (Malfait et al., 2000), anti-hyperalgesic (Srivastava et al., 1998), anti-inflammatory (Pertwee, 2004), and neuroprotective properties (Bisogno et al., 2001). The precise mechanism by which CBD exerts its biological effects is still unclear. Cannabidiol displays a low affinity to CB receptors (Lee et al., 2008). It is also a G-protein-coupled receptor 55 (GPR55) antagonist (Mestre et al., 2009), a full, though weak agonist at transient receptor potential cation channel subfamily V member 1 (TRPV1; Watanabe et al., 1996), and inhibitor of endocannabinoid anandamide (AEA) uptake (Bisogno et al., 2001). PEA is an endogenously produced cannabinoid-like compound that prolongs and enhances the biological effects of AEA via a mechanism known as the 'entourage' effect (Lambert and Di Marzo, 1999; Smart et al., 2002). PEA has low affinity to cannabinoid receptors (Lambert et al., 1999) and it can induce activation of the peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ; Verme et al., 2005). It has also been proposed as an endogenous agonist for GPR55 (Ryberg et al., 2007). Available evidences suggest that PEA exerts anti-inflammatory effects in several animal models (Cerrato et al., 2010; Esposito et al., 2013). In addition to its anti-inflammatory activity, PEA has anti-nociceptive (D'Agostino et al., 2009; Naderi et al., 2012), anti-convulsant (Lambert et al., 2001), anti-depressant (Guan et al., 2011), and neuroprotective properties (Di Cesare Mannelli et al., 2013). Although PEA and CBD have shown therapeutic effects in animal models of MS (Arévalo-Martín et al., 2003; Loria et al., 2008), the effects of their co-administration have not been studied yet. As for the interactive effects of these two compounds on endocannabinoid (eCB) system and GPR55 receptor, we decided to investigate how co-administration of PEA and CBD will affect the neurological impairment, gene expression of inflammatory factors, immune-cells infiltration, demyelination, and axonal injury in an experimental autoimmune encephalomyelitis (EAE) model of MS.

## EXPERIMENTAL PROCEDURES

### Animals and experimental design

All experiments were performed on female C57BL/6 mice (Razi Institute, Karaj, Iran) weighing 20–25 g (12 weeks). The animals were kept under a 12-h-light/dark cycle in a temperature-controlled ( $22 \pm 2^\circ\text{C}$ ) environment with free access to food and water *ad libitum*. All studies and animal care procedures were accomplished in accordance with the local ethics committee for animal experimentation and in compliance with guidelines of Shahid Beheshti University of Medical Sciences. EAE was induced in mice with MOG<sub>35–55</sub> peptide emulsified in complete Freund's adjuvant (CFA) using Hooke kits (Hooke Laboratories, Lawrence, MA, USA) according to the manufacturer's instruction. All drugs and their respective vehicles were injected intraperitoneally. The intact (no EAE) group received CFA and pertussis toxin

without MOG (myelin oligodendrocyte glycoprotein) ( $n = 8$ ). The following groups of EAE-induced mice were used in this study: control group received cannabinoid vehicle (cremophor, ethanol and PBS (phosphate-buffered saline), v:v:v 1:1:18; EAE group,  $n = 8$ ), CBD (5 mg/kg; CBD/EAE group,  $n = 8$ ), PEA (5 mg/kg; PEA/EAE group,  $n = 8$ ) and co-administration of CBD and PEA (5 mg/kg; CBD + PEA/EAE group,  $n = 8$ ) were injected immediately at the onset of disease signs for three consecutive days.

The dose and duration of treatments were chosen based on previous studies using EAE and TMEV models of MS (Loria et al., 2008; Kozela et al., 2011). CBD (Tocris, Abingdon, UK) and PEA (Tocris, Abingdon, UK) solutions were prepared freshly on the day of injection.

### Neurological assessment

Mice were daily assessed to record behavioral and neurological signs until 28 days after the first immunization. The signs of EAE were scored as follows: 0, no signs; 0.5, half paralyzed tail; 1, fully paralyzed tail; 1.5, fully paralyzed tail and weak or altered gait; 2, fully paralyzed tail and hind limb weakness; 2.5, unilateral hind limb paralysis; 3, complete hind limb paralysis; 3.5, complete hind limb paralysis and forelimb weakness; 4, complete paralysis; 5, moribund state or death (Kafami et al., 2013). On twenty-eighth day, spinal cords were collected for further pathological and gene expression studies. Three weeks later the pathological and gene expression experiments were performed.

### Tissue preparation and histological assessment

Histological analysis of spinal cord sections was used to determine severity of inflammation, demyelination and axonal loss and the effect of treatments on these parameters. Twenty-eight days after the first immunization, mice were anesthetized and were intracardially perfused with freshly prepared 0.1 M PBS. Spinal cords were dissected and fixed in 10% buffered formalin, and embedded in paraffin. Then serial sections (10- $\mu\text{m}$  diameter) from lumbar spinal cord were obtained using a rotary microtome (Leica, Vienna, Austria), and mounted on glass slides. Subsequently, the sections were deparaffinized, rehydrated, and stained with Hematoxylin and Eosin (H & E; Sigma-Aldrich, Steinheim, Germany) to evaluate inflammatory infiltrates (Kim et al., 2012), Luxol Fast Blue (LFB; TAAB, Berks, UK) to assess demyelinated area (Mozafari et al., 2011), and Bielschowsky's Silver impregnation method for axonal loss (Tsutsui et al., 2005). Immunostaining for macrophage/microglia-specific Iba1 was performed on paraffin-embedded sections using rabbit anti-Iba1 (1:500; Wako, Tokyo, Japan), Goat Anti-Rabbit IgG-HRP (Life Technologies, NY, USA) were used together with the Vectastain ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA) (Tsutsui et al., 2004). For quantification of histological changes, the total area of spinal white matter was scanned and photographed using a camera mounted on microscope (Micros, Veit/Glan,

Austria), then the number of infiltrated cells was counted for H & E staining, the area of demyelination was measured for LFB staining, and the number of vacuoles was quantified for Silver staining by SCION Image software (Scion Corporation, MD, USA; Pomeroy et al., 2005; Kim et al., 2012).

### Real-time RT-PCR

For reverse transcriptase-polymerase chain reaction (RT-PCR) studies, the lumbar spinal cord from mice were rapidly removed and immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until required. Total RNA was extracted from tissues using the total RNA isolation solution (RiboEx<sup>TM</sup>; GeneAll, Seoul, Korea) according to the manufacturer's guidelines. The yield of RNA was determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., MA, USA), and 1- $\mu\text{g}$  RNA was used for the synthesis of complementary DNA and subsequent PCR (Oh et al., 2012). Real-time PCR was performed using 5 $\times$  HOT FIRPOL<sup>®</sup> EvAgreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and CFX96<sup>TM</sup> Real-time Detection System (Bio-Rad Laboratories, CA, USA). The following primers were used for amplification:  $\beta$ -ACTIN forward: 5'-ATGCTCCCCGGGCTGTAT-3',  $\beta$ -ACTIN reverse: 5'-CATAGGAGTCCTTCTGACCCATTC-3'; TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ) forward: 5'-CCAGTGTGGGAAGCTGTCTT-3', TNF- $\alpha$  reverse: 5'-AAGCAAAGAGGAGGCAACA-3'; IFN- $\gamma$  (interferon- $\gamma$ ) forward: 5'-ATGAACGCTACACACTGCACTC-3', IFN- $\gamma$  reverse: 5'-CCATCCTTTTGCCAGTTCTC-3'; IL-17a forward: 5'-TCATCCCTCAAAAGCTCAGCG-3', IL-17a reverse: 5'-TTCAATTGCCGTGGAGAGTCC-3' sequence. Semi-quantitative analysis was performed by monitoring in real-time increase of fluorescence of the EvAgreen dye on an i-Cycler (Bio-Rad Laboratories, CA, USA; Power et al., 2003; Sherafat et al., 2011). To affirm the single-band production, we performed melt-curve analysis and afterward confirmed it by electrophoresis and Red Safe staining (iNtRON Biotechnology, Kyungki-Do, Korea). All data were normalized against actin mRNA level and expressed as fold increases relative to controls.

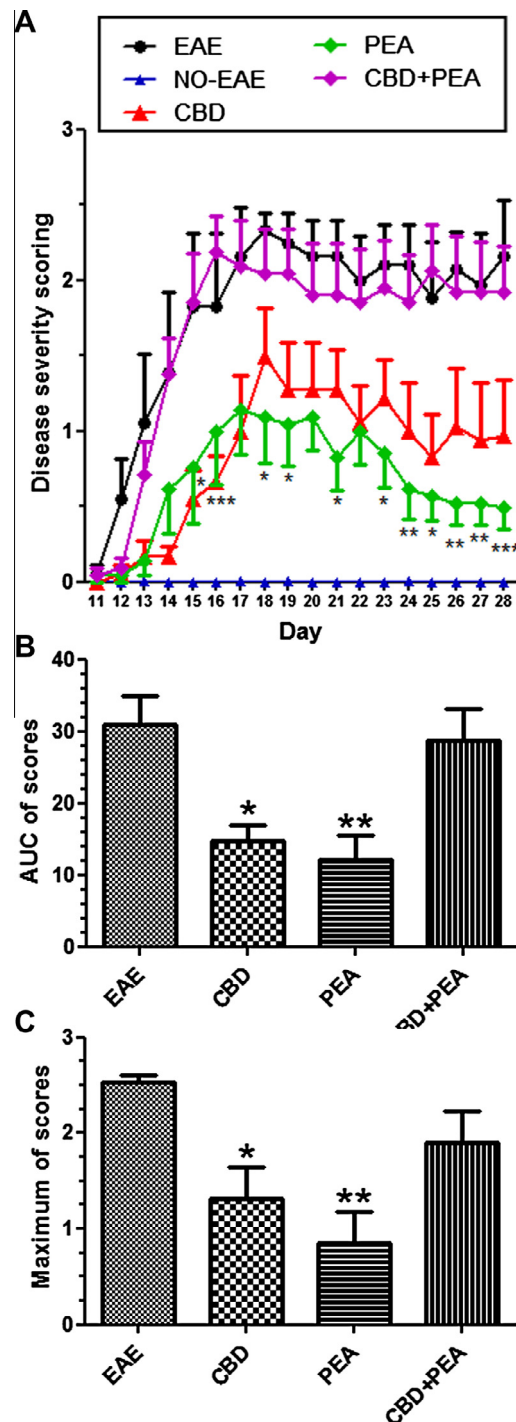
### Statistical analysis

The results are expressed as mean  $\pm$  SEM and were analyzed using GraphPad Prism (version 6, Graphpad Software Inc.). A one-way or two-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's tests were used for multiple comparison. For all statistical analyses,  $p < 0.05$  was considered significant.

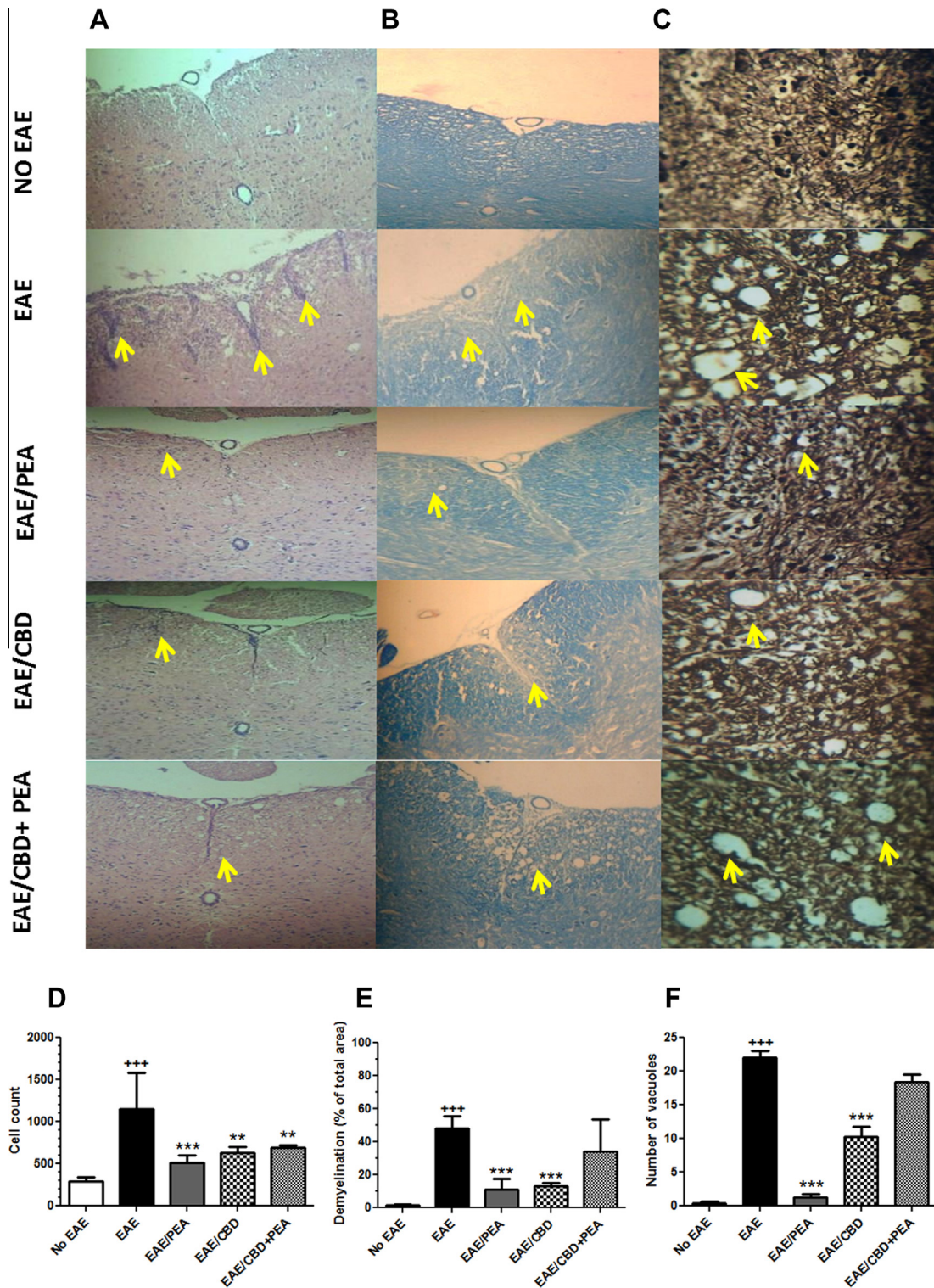
## RESULTS

### Both CBD and PEA but not their co-administration ameliorates neurological signs of MOG-induced EAE and disease progression

The results are shown in Fig. 1. A two-way ANOVA revealed a significant effect of treatment [ $F(3,396) = 70.67$ ,  $p < 0.001$ ; Fig. 1A]. Further analysis by Bonferroni's test revealed a significant reduction in



**Fig. 1.** Effects of cannabidiol and palmitoylethanolamide on neurological score of EAE. Treatment with both cannabidiol (CBD) and palmitoylethanolamide (PEA) per se ameliorated the neurobehavioral signs and progression of the disease in MOG-induced mice. However, co-administration of these two drugs did not have significant protection against EAE model. Behavioral scores were recorded daily until day 28 after induction of the disease. CBD, PEA or their vehicle (control group) were injected i.p. at the onset of the disease for three consecutive days. (A) Changes in the score during days after initiation of the treatments. Each point represents the mean behavioral scores  $\pm$  SEM. (B) Area under the curve (AUC), and (C) maximum of behavioral scores for each mouse depicted in panel (A). Each bar represents mean  $\pm$  SEM. Each group consisted of 6–7 mice. \* $p < 0.05$ , \*\* $p < 0.01$  significant difference compared to the control group.



**Fig. 2.** CBD and PEA ameliorate histological signs of MOG-induced EAE. (A, D) H & E staining of the spinal cord showed intense infiltration of inflammatory cells in control group (EAE) compared to the no-EAE group. Infiltration of immune cells has diminished in groups treated with PEA, CBD and co-administration of CBD and PEA. (B, E) LFB staining of sections showed irregular vacuolation and demyelination in the white matter of control group but no loss of myelin in no-EAE mice. The extent of demyelination decreased in mice treated with either CBD or PEA per se, but co-administration of CBD and PEA did not have significant protection against demyelination. (C, F) Bielschowsky silver staining of the same area shows the presence of irregular vacuolation and axonal loss in control group but no loss of axon in no-EAE mice. PEA and CBD could decrease the amount of vacuoles; however co-administration of CBD and PEA did not have significant protection against axonal loss. Magnification, X10 (H & E and LFB staining) and X40 (silver staining). The arrows indicate regions of lymphocyte infiltrates, demyelination, or axon loss. Each bar represents mean  $\pm$  SEM. Each group consisted of 5–6 mice. \*\*\* $p < 0.001$  significant difference compared to the control group (EAE). \*\*\*\* $p < 0.0001$  significant difference compared to the no-EAE group.

neurological signs of mice treated with PEA (5 mg/kg) on days 18, 19, 21, 23, 25 ( $p < 0.05$ ); 24, 26, 27 ( $p < 0.01$ ); and day 28 ( $p < 0.001$ ) compared to the control group. In addition, a significant reduction in neurological signs in mice treated with CBD (5 mg/kg) was observed on day 16 ( $p < 0.05$ ) compared to the control group. As shown in Fig. 1B, repeated i.p. administration of CBD (5 mg/kg) in three consecutive days during the onset of the signs of the disease, markedly ameliorated and delayed the disease progression. In addition, analyzing the AUC of the neurological scores showed that the i.p. administration of PEA (5 mg/kg) in three consecutive days reduced the severity of the neurological scores in MOG-induced mice compared to the control group ( $p < 0.01$ ; Fig. 1B). Interestingly, co-administration of CBD (5 mg/kg) and PEA (5 mg/kg) did not produce any significant changes in neurological score in the mice treated by combination of CBD and PEA (5 mg/kg each) compared to the control group. A significant decrease in the maximum disease score was also shown in CBD- ( $p < 0.05$ ) and PEA- ( $p < 0.01$ ) treated group compared to the control group (Fig. 1C).

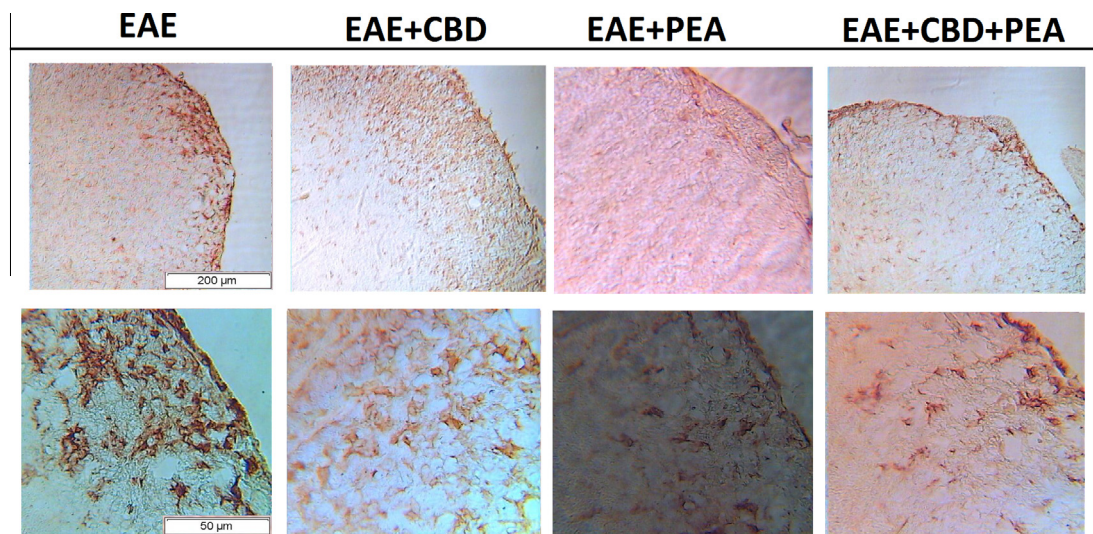
#### CBD and PEA ameliorate histological signs of MOG-induced EAE

The H & E staining was performed for evaluating the evidence of active inflammation and inflammatory infiltration (Fig. 2A). H & E staining revealed that MOG-induced EAE caused an increase in immune cell infiltration into the spinal cord of treated mice compared to the intact (no EAE) group ( $p < 0.001$ ; Fig. 2D). The immune cell infiltration into the anterior white matter of the spinal cords in both EAE mice treated with CBD ( $p < 0.01$ ) and PEA ( $p < 0.001$ ) was lower than the EAE mice with no treatment (Fig. 2D). Also, co-administration of CBD and PEA could

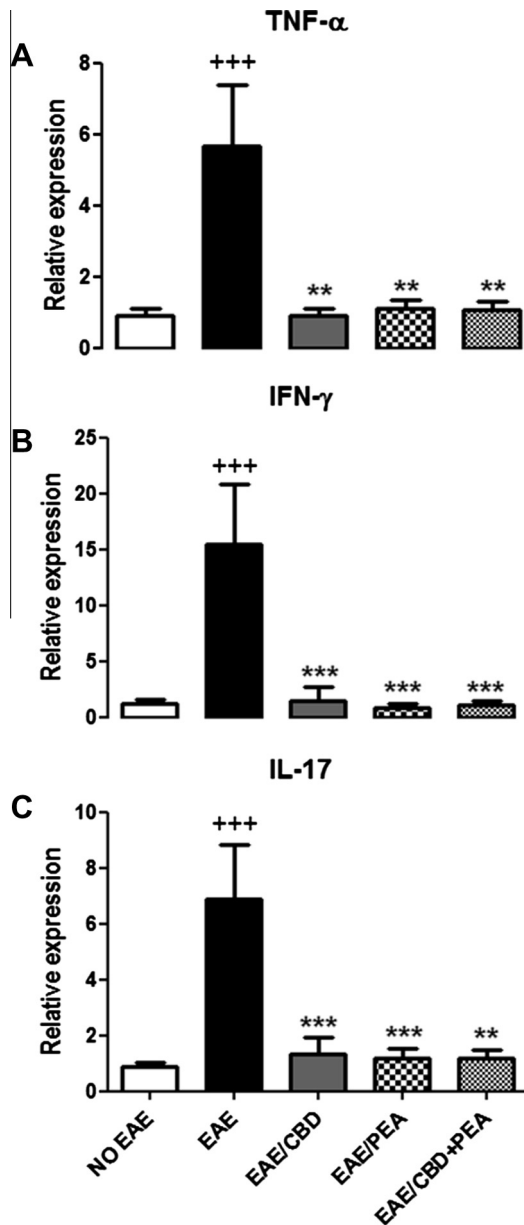
significantly attenuate the spinal cord inflammation compared to the EAE mice without treatment ( $p < 0.01$ ; Fig. 2D). To assess demyelination, we stained axonal myelin sheath with LFB (Fig. 2B). Mice in control group (EAE) showed profound demyelination in spinal cord compared to the intact (no EAE) group ( $p < 0.001$ ; Fig. 2E). Treatment with both CBD and PEA per se decreased the number of irregular vacuoles as well as demyelination severity in the white matter of spinal cord ( $p < 0.001$ ; Fig. 2E). Nonetheless, no significant change in spinal cord demyelination was observed in mice treated with co-administration of CBD and PEA compared to the EAE mice without treatment (Fig. 2E). Axonal degeneration was evaluated by Bielschowsky Silver staining that stains axons dark brown (Fig. 2C). Bielschowsky Silver staining of lumbar spinal cord sections showed irregular vacuolation and intense axon loss in the demyelinated regions in the control group (EAE) compared to the intact (no EAE) group ( $p < 0.001$ ; Fig. 2F). CBD and PEA markedly ameliorated axonal integrity and extent of irregular vacuoles ( $p < 0.001$ ; Fig. 2F), but co-administration of CBD and PEA could not improve axonal damage compared to the EAE mice without treatment (Fig. 2F).

#### CBD and PEA affects microglial/macrophage responses after MOG-induced EAE induction

The EAE induction activated microglia, as displayed in intensified Iba-1 immunostaining density in the lumbar area of the spinal cord. Compared with the control group, both CBD and PEA treatment attenuated the morphological changes to the activated-form of microglia. Also, co-administration of CBD and PEA attenuated EAE-induced microglia activation in the spinal cord (Fig. 3).



**Fig. 3.** Representative low and high magnification photomicrographs of lumbar spinal cord sections from control, CBD, PEA and CBD + PEA treated mice allowed 28 days survival after EAE induction and stained for anti-Iba1. Scale bars: low magnification: 200  $\mu\text{m}$  and high magnification: 50  $\mu\text{m}$ .



**Fig. 4.** The expression level of cytokine genes in the spinal cord of mice were assessed by EvAGreen RT-PCR. Data represent the relative expression of mRNA after normalization to actin expression. (A) TNF- $\alpha$  expression was significantly increased in control group (EAE) compared to the no-EAE group. CBD, PEA and co-administration of CBD and PEA could decrease the level of TNF- $\alpha$  expression. (B) IFN- $\gamma$  expression was significantly increased in control group (EAE) compared to the no-EAE group. CBD, PEA, and co-administration of CBD and PEA significantly reduced the level of IFN- $\gamma$  expression. (C) The expression of IL-17 was significantly increased in control group (EAE) compared to the no-EAE group. CBD, PEA, and concurrent administration of CBD and PEA significantly diminished the level of IL-17 expression. Each bar represents mean  $\pm$  SEM. Each group consisted of 8–10 mice. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  significant difference compared to the control group (EAE). +++ $p < 0.001$  significant difference compared to the no-EAE group.

#### CBD, PEA, and co-administration of CBD and PEA ameliorate the expression of inflammatory cytokines of MOG-induced EAE

We investigated the expression of TNF- $\alpha$ , IFN- $\gamma$  and IL-17 mRNA in the spinal cord of mice in the EAE model of MS

by real-time RT-PCR. TNF- $\alpha$  expression was increased in the control group (EAE) compared to the no-EAE group ( $p < 0.001$ ; Fig. 4A). CBD, PEA and concurrent administration of CBD and PEA were able to decrease the level of TNF- $\alpha$  expression ( $p < 0.01$ ; Fig. 4A). We observed that in MOG-induced EAE, IFN- $\gamma$  expression was increased compared to the intact (no EAE) group ( $p < 0.001$ ; Fig. 4B). CBD, PEA, and co-administration of CBD and PEA significantly diminished the expression of IFN- $\gamma$  ( $p < 0.001$ ; Fig. 4B). The expression of IL-17 was significantly increased in the EAE group compared to the intact (no EAE) group ( $p < 0.001$ ; Fig. 4C). However, the expression of this cytokine was greatly reduced by treatment of MOG-induced mice with CBD ( $p < 0.001$ ), PEA ( $p < 0.001$ ), and co-administration of CBD and PEA ( $p < 0.01$ ; Fig. 4C).

## DISCUSSION

Current therapeutic strategies for MS include anti-inflammatory, immunosuppressive and immunomodulatory drugs. These treatments are not considered an effective cure for this disease and often produce severe adverse effects. Several studies reported a therapeutic role of CBs in MS (Mestre et al., 2005; Docagne et al., 2007). In spite of the emerging evidence regarding putative therapeutic activities of CBs in neurodegenerative diseases, their indication in the clinical use is still controversial and strongly limited by inevitable psychoactive effects, exhibited by many of them. CBD and PEA are destitute of such psychotropic effects and their neuroprotective and immunosuppressive activities are the most relevant to the complicated pathology of MS.

It has been evidenced that CBD provide neuroprotection against hydroperoxide-induced oxidative neuronal damage (Hampson et al., 1998), 6-hydroxydopamine-induced (Lastres-Becker et al., 2005), and  $\beta$ -amyloid-induced (Harvey et al., 2012) neurodegeneration. On the other hand, PEA has been frequently studied for its anti-inflammatory and neuroprotective effects (Franklin et al., 2003). PEA was able to reduce  $\beta$ -amyloid evoked neuroinflammation and attenuate its neurodegenerative consequences (Scuderi et al., 2012).

CBD and PEA exert their protective effects against neurological disorders via multiple mechanisms. CBD is generally known to have a very low affinity for the cannabinoid CB1 and CB2 receptors (Pertwee, 2008). It was found that CBD inhibits both AEA hydrolysis by fatty acid amide hydrolase (FAAH) and AEA uptake by anandamide membrane transporter (AMT) (Bisogno et al., 2001). CBD exerts immunosuppressive actions on macrophages and microglial cells by diminishing the production of proinflammatory cytokines including TNF $\alpha$ , IL-1b, IL-2, IL-6, IL-12 and IFN- $\gamma$  (Rieder et al., 2010). Finally, CBD acts as an antagonist at GPR55 receptors (Izzo et al., 2009).

On the other hand, several pathways have been suggested to mediate the effects of PEA. PPAR- $\alpha$  is thought to be one of the main targets of PEA (Hesselink, 2012). Moreover, PEA is an agonist with high affinity for GPR55 receptor (Ryberg et al., 2007) and

potentiate the effect of AEA on cannabinoid or VR1 receptor (Lambert and Di Marzo, 1999; De Petrocellis et al., 2001).

The dual effects of CBD and PEA on eCB and GPR55 receptor systems could give rise to different pharmacological profiles in inflammatory conditions. Although both of these two compounds potentiate the effects of endocannabinoids, they exert opposite effects on GPR55 receptor. Yet, the interaction between these two compounds in attenuation of MS-related neurological and pathological parameters in experimental models of MS has not been verified. In the present study, we evaluated the co-administration of CBD and PEA in the animal model of MS for the first time. In this study, we used the EAE model of MS to evaluate the effects of peripherally administered CBD and PEA at the onset of the disease for three consecutive days. As the results showed, Both CBD and PEA ameliorated neurobehavioral signs of MOG-induced EAE and disease progression but their co-administration had no effect. Our observations related to the effect of CBD and PEA on behavioral signs and histological changes are in agreement with previous studies in EAE as well as in Theiler's Murine Encephalomyelitis Virus-Induced Demyelinating Disease (TMEV-IDD) models of MS (Loría et al., 2008; Kozela et al., 2011). Conversely, our findings in behavioral scores are in contrast with the results reported by Maresz et al. in which various doses of CBD (0.5, 5, 10, and 25 mg/kg) could not improve behavioral scores (Maresz et al., 2007). The inconsistency between our results and those of Maresz might be due to the type of EAE induction (MOG35–55 peptide vs. spinal cord homogenate) and/or animals used (C57BL/6 mice vs. ABH mice).

The results obtained from histological analysis imply that although CBD and PEA reduced immune cell infiltration, demyelination, and axonal damage in the lumbar spinal cord of EAE-induced mice, co-administration of these two compounds only could decrease inflammatory infiltration and did not affect demyelination and axonal loss.

In gene expression study, the expression of TNF- $\alpha$ , IFN- $\gamma$ , and IL-17 were assessed. It is generally considered that CD4+ Th1 and Th17 cells and their related cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , and IL-17 are the primary mediators in autoimmune neuroinflammation and EAE development (Bettelli et al., 2006; El-behi et al., 2010). TNF- $\alpha$  is made by both CNS-resident microglia and infiltrating macrophages during EAE, and its production is controlled by cytokines secreted by infiltrating CD4+ T cells (Renno et al., 1995). CBD and PEA reduce TNF- $\alpha$  expression in retinal damage (Liou et al., 2008) collagen-induced arthritis and chronic pain (Costa et al., 2008; Paterniti et al., 2013). In this study, we observed that TNF- $\alpha$  expression was increased in the control group (EAE) compared to the no-EAE group. In addition, CBD, PEA and co-administration of CBD and PEA could significantly reduce the level of TNF- $\alpha$  expression. Our results are consistent with the previous study in which PEA could decrease the level of TNF- $\alpha$  expression in the TMEV model of MS (Loría et al., 2008).

IL-17 is produced by Th17 cells and has been linked to many autoimmune diseases (Jin and Dong, 2013). Elevation in IL-17 levels was seen in lymphocytes derived from mice with EAE and may contribute to the development of this model (Komiyama et al., 2006). In the present study, the level of IL-17 was increased in the control group (EAE group) compared to the no-EAE group. CBD, PEA, and concurrent administration of CBD and PEA reduced the expression of this cytokine.

IFN- $\gamma$  has a pathological role in the development of this autoimmune disease (Hammarberg et al., 2000). Our results show that the MOG-induced EAE causes significant increases in IFN- $\gamma$  expression compared to the intact (no EAE) mice. CBD, PEA, and co-administration of CBD and PEA significantly diminished the expression of this cytokine.

The antagonistic interaction between CBD and PEA could highlight the role of GPR55 in pharmacological effects of these CBs in the EAE model of MS. GPR55 is a new candidate receptor for cannabinoid, but has a unique response profile differing from CB1 to CB2 (Bonica, 1979). The prominent expression of GPR55 within the brain, dorsal root ganglion of spinal cord, lymphoid organs, and primary microglial cells suggests an important role of GPR55 in the regulation of inflammatory and neuropathic pain (Lauckner et al., 2008; Oka et al., 2010). Moreover, it was reported that GPR55 signaling can up-regulate certain cytokines and this may contribute to the lack of inflammatory mechanical hyperalgesia in the GPR55-/- mice (Staton et al., 2008).

## CONCLUSION

Our results provide further evidence to suggest the effectiveness of PEA and CBD as nonpsychoactive CBs against MS disease. Moreover, our report also suggests the possible involvement of GPR55 receptor as part of the protective system that could be considered for the development of new therapeutic approaches. Further experiments using a selective GPR55 antagonist or GPR55-/- knockout animals would be required to confirm the GPR55-mediated anti-nociceptive and anti-inflammatory actions of CBD and PEA.

## REFERENCES

- Arévalo-Martin Á, Vela JM, Molina-Holgado E, Borrell J, Guaza C (2003) Therapeutic action of cannabinoids in a murine model of multiple sclerosis. *J Neurosci* 23:2511–2516.
- Baker D, Pryce G, Croxford JL, Brown P, Pertwee RG, Makriyannis A, Khanolkar A, Layward L, Fezza F, Bisogno T (2001) Endocannabinoids control spasticity in a multiple sclerosis model. *FASEB J* 15:300–302.
- Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235–238.
- Bisogno T, Hanuš L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R, Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br J Pharmacol* 134:845–852.

- Bonica J (1979) The need of a taxonomy. *Pain* 6:247–248.
- Buccellato E, Carretta D, Utan A, Cavina C, Speroni E, Grassi G, Candeletti S, Romualdi P (2011) Acute and chronic cannabinoid extracts administration affects motor function in a CREAE model of multiple sclerosis. *J Ethnopharmacol* 133:1033–1038.
- Cerrato S, Brazis P, Della Valle M, Miolo A, Puigdemont A (2010) Effects of palmitoylethanolamide on immunologically induced histamine, PGD<sub>2</sub> and TNF $\alpha$  release from canine skin mast cells. *Vet Immunol Immunopathol* 133:9–15.
- Collin C, Davies P, Mutiboko I, Ratcliffe S (2007) Randomized controlled trial of cannabis-based medicine in spasticity caused by multiple sclerosis. *Eur J Neurol* 14:290–296.
- Costa B, Comelli F, Bettoni I, Colleoni M, Giagnoni G (2008) The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB<sub>1</sub>, TRPV1 and PPAR $\gamma$  receptors and neurotrophic factors. *Pain* 139:541–550.
- Croxford JL, Miller SD (2003) Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R (+) WIN55, 212. *J Clin Invest* 111:1231–1240.
- D'Agostino G, La Rana G, Russo R, Sasso O, Iacono A, Esposito E, Mattace Raso G, Cuzzocrea S, LoVerme J, Piomelli D (2009) Central administration of palmitoylethanolamide reduces hyperalgesia in mice via inhibition of NF- $\kappa$ B nuclear signalling in dorsal root ganglia. *Eur J Pharmacol* 613:54–59.
- De Petrocellis L, Davis JB, Di Marzo V (2001) Palmitoylethanolamide enhances anandamide stimulation of human vanilloid VR1 receptors. *FEBS Lett* 506:253–256.
- Di Cesare Mannelli L, D'Agostino G, Pacini A, Russo R, Zanardelli M, Ghelardini C, Calignano A (2013) Palmitoylethanolamide is a disease-modifying agent in peripheral neuropathy: pain relief and neuroprotection share a PPAR- $\alpha$ -mediated mechanism. *Mediators Inflamm* 2013:1–12. <http://dx.doi.org/10.1155/2013/328797>. Article ID: 328797.
- Docagne F, Muñetón V, Clemente D, Ali C, Loria F, Correa F, Hernangómez M, Mestre L, Vivien D, Guaza C (2007) Excitotoxicity in a chronic model of multiple sclerosis: neuroprotective effects of cannabinoids through CB1 and CB2 receptor activation. *Mol Cell Neurosci* 34:551–561.
- El-behi M, Rostami A, Ciric B (2010) Current views on the roles of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *J Neuroimmune Pharmacol* 5:189–197.
- Esposito G, Capoccia E, Turco F, Palumbo I, Lu J, Steardo A, Cuomo R, Sarnelli G, Steardo L (2013) Palmitoylethanolamide improves colon inflammation through an enteric glia/toll like receptor 4-dependent PPAR- $\alpha$  activation. *Gut*. <http://dx.doi.org/10.1136/gutjnl-2013-305005>.
- Franklin A, Parmentier-Batteur S, Walter L, Greenberg DA, Stella N (2003) Palmitoylethanolamide increases after focal cerebral ischemia and potentiates microglial cell motility. *J Neurosci* 23:7767–7775.
- Guan L-P, Sui X, Deng X-Q, Zhao D-H, Qu Y-L, Quan Z-S (2011) N-palmitoylethanolamide derivatives: synthesis and studies on anticonvulsant and antidepressant activities. *Med Chem Res* 20:601–606.
- Hammarberg H, Lidman O, Lundberg C, Eltayeb S, Gielen A, Muhallab S, Svenningsson A, Lindå H, van Der Meide P, Cullheim S (2000) Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *J Neurosci* 20:5283–5291.
- Hampson A, Grimaldi M, Axelrod J, Wink D (1998) Cannabidiol and (–)  $\Delta$ 9-tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci* 95:8268–8273.
- Harvey BS, Ohlsson KS, Määg JL, Musgrave IF, Smid SD (2012) Contrasting protective effects of cannabinoids against oxidative stress and amyloid- $\beta$  evoked neurotoxicity in vitro. *NeuroToxicology* 33:138–146.
- Hesselink JMK (2012) New targets in pain, non-neuronal cells, and the role of palmitoylethanolamide. *Open Pain J* 5.
- Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R (2009) Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol Sci* 30:515–527.
- Jin W, Dong C (2013) IL-17 cytokines in immunity and inflammation. *Emerg Microbes Infect* 2:e60.
- Kafami L, Etesami I, Felfeli M, Enayati N, Ghiaghi R, Aminian A, Dehpour A (2013) Methadone diminishes neuroinflammation and disease severity in EAE through modulating T cell function. *J Neuroimmunol* 255:39–44.
- Kim BS, Jin Y-H, Meng L, Hou W, Kang HS, Park HS, Koh C-S (2012) IL-1 signal affects both protection and pathogenesis of virus-induced chronic CNS demyelinating disease. *J Neuroinflamm* 9:1–13.
- Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, Sudo K, Iwakura Y (2006) IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177:566–573.
- Kozela E, Lev N, Kaushansky N, Eilam R, Rimmerman N, Levy R, Ben-Nun A, Juknat A, Vogel Z (2011) Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice. *Br J Pharmacol* 163:1507–1519.
- Lambert DM, Di Marzo V (1999) The palmitoylethanolamide and oleamide enigmas two fatty acid amides cannabimimetic? *Curr Med Chem* 6:757–773.
- Lambert DM, DiPaolo FG, Sonveaux P, Kanyonyo M, Govaerts SJ, Hermans E, Bueb J-L, Delzenne NM, Tschirhart EJ (1999) Analogues and homologues of N-palmitoylethanolamide, a putative endogenous CB2 cannabinoid, as potential ligands for the cannabinoid receptors. *Biochim Biophys Acta (BBA) Mol Cell Biol Lipids* 1440:266–274.
- Lambert DM, Vandevoorde S, Diependaele G, Govaerts SJ, Robert AR (2001) Anticonvulsant activity of N-palmitoylethanolamide, a putative endocannabinoid, in mice. *Epilepsia* 42:321–327.
- Lastres-Becker I, Molina-Holgado F, Ramos JA, Mechoulam R, Fernández-Ruiz J (2005) Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: relevance to Parkinson's disease. *Neurobiol Dis* 19:96–107.
- Lauckner JE, Jensen JB, Chen H-Y, Lu H-C, Hille B, Mackie K (2008) GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc Natl Acad Sci* 105:2699–2704.
- Lee C-Y, Wey S-P, Liao M-H, Hsu W-L, Wu H-Y, Jan T-R (2008) A comparative study on cannabidiol-induced apoptosis in murine thymocytes and EL-4 thymoma cells. *Int Immunopharmacol* 8:732–740.
- Liou GI, Auchampach JA, Hillard CJ, Zhu G, Yousufzai B, Mian S, Khan S, Khalifa Y (2008) Mediation of cannabidiol anti-inflammation in the retina by equilibrative nucleoside transporter and A2A adenosine receptor. *Invest Ophthalmol Vis Sci* 49:5526–5531.
- Loria F, Petrosino S, Mestre L, Spagnolo A, Correa F, Hernangómez M, Guaza C, Di Marzo V, Docagne F (2008) Study of the regulation of the endocannabinoid system in a virus model of multiple sclerosis reveals a therapeutic effect of palmitoylethanolamide. *Eur J Neurosci* 28:633–641.
- Malfait A, Gallily R, Sumariwalla P, Malik A, Andreaskos E, Mechoulam R, Feldmann M (2000) The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritis therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci* 97:9561–9566.
- Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, Shriver LP, Ledent C, Cheng X, Carrier EJ, Mann MK (2007) Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nat Med* 13:492–497.
- Mestre L, Correa F, Arévalo-Martín A, Molina-Holgado E, Valenti M, Ortas G, Di Marzo V, Guaza C (2005) Pharmacological modulation of the endocannabinoid system in a viral model of multiple sclerosis. *J Neurochem* 92:1327–1339.
- Mestre L, Docagne F, Correa F, Loria F, Hernangomez M, Borrelli J, Guaza C (2009) A cannabinoid agonist interferes with the



- progression of a chronic model of multiple sclerosis by downregulating adhesion molecules. *Mol Cell Neurosci* 40:258–266.
- Miller D, Weinshenker B, Filippi M, Banwell B, Cohen J, Freedman M, Galetta S, Hutchinson M, Johnson R, Kappos L (2008) Differential diagnosis of suspected multiple sclerosis: a consensus approach. *Mult Scler* 14:1157–1174.
- Mozafari S, Javan M, Sherafat MA, Mirnajafi-Zadeh J, Heibatollahi M, Pour-Beiranvand S, Tiraihi T, Ahmadiani A (2011) Analysis of structural and molecular events associated with adult rat optic chiasm and nerves demyelination and remyelination; possible role for 3rd ventricle proliferating cells. *NeuroMol Med* 13:138–150.
- Naderi N, Majidi M, Mousavi Z, Tusi SK, Mansouri Z, Khodagholi F (2012) The interaction between intrathecal administration of low doses of palmitoylethanolamide and AM251 in formalin-induced pain related behavior and spinal cord IL1- $\beta$  expression in rats. *Neurochem Res* 37:778–785.
- Ni X, Geller EB, Eppihimer MJ, Eisenstein TK, Adler MW, Tuma RF (2004) Win 55212-2, a cannabinoid receptor agonist, attenuates leukocyte/endothelial interactions in an experimental autoimmune encephalomyelitis model. *Mult Scler* 10:158–164.
- Oh S-s, Kim D, Kim D-H, Chang HH, Sohn K-C, Kim KH, Jung SH, Lee BK, Kim JH, Kim KD (2012) NDRG2 correlated with favorable recurrence-free survival inhibits metastasis of mouse breast cancer cells via attenuation of active TGF- $\beta$  production. *Carcinogenesis* 33:1882–1888.
- Oka S, Kimura S, Toshida T, Ota R, Yamashita A, Sugiura T (2010) Lysophosphatidylinositol induces rapid phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor 2 in HEK293 cells expressing GPR55 and IM-9 lymphoblastoid cells. *J Biochem* 147:671–678.
- Paterniti I, Impellizzeri D, Di Paola R, Navarra M, Cuzzocrea S, Esposito E (2013) A new co-ultramicrosized composite including palmitoylethanolamide and luteolin to prevent neuroinflammation in spinal cord injury. *J Neuroinflamm* 10:91.
- Pertwee R (2008) The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids:  $\Delta^9$ -tetrahydrocannabinol, cannabidiol and  $\Delta^9$ -tetrahydrocannabivarin. *Br J Pharmacol* 153:199–215.
- Pertwee RG (2004) The pharmacology and therapeutic potential of cannabidiol. In: *Cannabinoids*. Kluwer Academic/Plenum Publishers. p. 32–83.
- Pomeroy IM, Matthews PM, Frank JA, Jordan EK, Esiri MM (2005) Demyelinated neocortical lesions in marmoset autoimmune encephalomyelitis mimic those in multiple sclerosis. *Brain* 128:2713–2721.
- Power C, Henry S, Del Bigio MR, Larsen PH, Corbett D, Imai Y, Yong VW, Peeling J (2003) Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases. *Ann Neurol* 53:731–742.
- Prat A, Antel J (2005) Pathogenesis of multiple sclerosis. *Curr Opin Neurol* 18:225–230.
- Renno T, Krakowski M, Piccirillo C, Lin J, Owens T (1995) TNF- $\alpha$  expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J Immunol* 154:944–953.
- Rieder SA, Chauhan A, Singh U, Nagarkatti M, Nagarkatti P (2010) Cannabinoid-induced apoptosis in immune cells as a pathway to immunosuppression. *Immunobiology* 215:598–605.
- Rog DJ, Nurmikko TJ, Friede T, Young CA (2005) Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. *Neurology* 65:812–819.
- Rog DJ, Nurmikko TJ, Young CA (2007) Oromucosal  $\Delta^9$ -tetrahydrocannabinol/cannabidiol for neuropathic pain associated with multiple sclerosis: An uncontrolled, open-label, 2-year extension trial. *Clin Ther* 29:2068–2079.
- Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, Elebring T, Nilsson K, Drmota T, Greasley P (2007) The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* 152:1092–1101.
- Scuderi C, Valenza M, Stecca C, Esposito G, Carratù MR, Steardo L (2012) Palmitoylethanolamide exerts neuroprotective effects in mixed neuroglial cultures and organotypic hippocampal slices via peroxisome proliferator-activated receptor- $\alpha$ . *J Neuroinflamm* 9:131.
- Seehusen F, Baumgärtner W (2010) Axonal pathology and loss precede demyelination and accompany chronic lesions in a spontaneously occurring animal model of multiple sclerosis. *Brain Pathol* 20:551–559.
- Sherafat MA, Javan M, Mozafari S, Mirnajafi-Zadeh J, Motamedi F (2011) Castration attenuates myelin repair following lysolecithin induced demyelination in rat optic chiasm: an evaluation using visual evoked potential, marker genes expression and myelin staining. *Neurochem Res* 36:1887–1895.
- Smart D, Jonsson KO, Vandevoorde S, Lambert DM, Fowler CJ (2002) 'Entourage' effects of N-acyl ethanolamines at human vanilloid receptors. Comparison of effects upon anandamide-induced vanilloid receptor activation and upon anandamide metabolism. *Br J Pharmacol* 136:452–458.
- Sospedra M, Martin R (2005) Immunology of multiple sclerosis. *Annu Rev Immunol* 23:683–747.
- Srivastava MD, Srivastava B, Brouhard B (1998)  $\Delta^9$  tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. *Immunopharmacology* 40:179–185.
- Staton PC, Hatcher JP, Walker DJ, Morrison AD, Shapland EM, Hughes JP, Chong E, Mander PK, Green PJ, Billinton A (2008) The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain. *Pain* 139:225–236.
- Steinman L, Martin R, Bernard C, Conlon P, Oksenberg JR (2002) Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Annu Rev Neurosci* 25:491–505.
- Tsutsui S, Noorbakhsh F, Sullivan A, Henderson AJ, Warren K, Toney-Earley K, Waltz SE, Power C (2005) RON-regulated innate immunity is protective in an animal model of multiple sclerosis. *Ann Neurol* 57:883–895.
- Tsutsui S, Schnermann J, Noorbakhsh F, Henry S, Yong VW, Winston BW, Warren K, Power C (2004) A1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis. *J Neurosci* 24:1521–1529.
- Verme JL, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D (2005) The nuclear receptor peroxisome proliferator-activated receptor- $\alpha$  mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol* 67:15–19.
- Watanabe K, Kayano Y, Matsunaga T, Yamamoto I, Yoshimura H (1996) Inhibition of anandamide amidase activity in mouse brain microsomes by cannabinoids. *Biol Pharmaceut Bull* 19:1109–1111.
- Zajicek J, Fox P, Sanders H, Wright D, Vickery J, Nunn A, Thompson A (2003) Cannabinoids for treatment of spasticity and other symptoms related to multiple sclerosis (CAMS study): multicentre randomised placebo-controlled trial. *Lancet* 362:1517–1526.