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Study of the regulation of the endocannabinoid system in a virus model of multiple sclerosis reveals a therapeutic effect of palmitoylethanolamide

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Abstract

Cannabinoids have recently been approved as a treatment for pain in multiple sclerosis (MS). Increasing evidence from animal studies suggests that this class of compounds could also prove efficient to fight neurodegeneration, demyelination, inflammation and autoimmune processes occurring in this pathology. However, the use of cannabinoids is limited by their psychoactive effects. In this context, potentiation of the endogenous cannabinoid signalling could represent a substitute to the use of exogenously administrated cannabinoid ligands. Here, we studied the expression of different elements of the endocannabinoid system in a chronic model of MS in mice. We first studied the expression of the two cannabinoid receptors, CB_1 and CB_2, as well as the putative intracellular cannabinoid receptor peroxisome proliferator-activated receptor-α. We observed an upregulation of CB_2, correlated to the production of proinflammatory cytokines, at 60 days after the onset of the MS model. At this time, the levels of the endocannabinoid, 2-arachidonoylglycerol, and of the anti-inflammatory anandamide congener, palmithoylethanolamide, were enhanced, without changes in the levels of anandamide. These changes were not due to differences in the expression of the degradation enzymes, fatty acid amide hydrolase and monoacylglycerol lipase, or of biosynthetic enzymes, diacylglycerol lipase-α and *N*-acylphosphatidylethanolamine phospholipase-D at this time (60 days). Finally, the exogenous administration of palmitoylethanolamide resulted in a reduction of motor disability in the animals subjected to this model of MS, accompanied by an anti-inflammatory effect. This study overall highlights the potential therapeutic effects of endocannabinoids in MS.

Introduction

Multiple sclerosis (MS) is the most common chronic demyelinating disease of the CNS in humans. Its pathological signs range from sensory deficits to tremor, bladder dysfunction and ataxia. The deleterious processes leading to neurological decline include chronic inflammation, infiltration of T-cells and macrophages, axonal damage, oligodendrocyte cell death and autoimmune response against myelin.

Among the various therapeutic targets for MS, the endocannabinoid system raises considerable interest, as molecules that interact with this system potentially modulate the inflammatory, neurodegenerative, immunological and demyelinating components of MS (Bisogno & Di Marzo, 2007; Centonze *et al.*, 2007). Such molecules include active components of the plant *Cannabis sativa*, synthetic compounds and endogenous ligands (endocannabinoids). Their effects are mediated by two extracellular cannabinoid receptors, termed CB_1 and CB_2, and several other, still not identified receptors (Brown, 2007), which could include members of the peroxisome proliferator-activated receptor (PPAR) family of intracellular receptors and of the transient receptor potential (TRP) family of channels (De Petrocellis *et al.*, 2007; O'Sullivan, 2007; Starowicz *et al.*, 2007). Cannabis extracts, in the form of an oromucosal spray, are now authorized as a treatment for pain in MS, but their indication is in some cases limited by their psychoactive effects, likely mediated by CB_1. For this reason, a great effort is given to investigate potential therapeutic effects of CB_2 agonists. However, long-term treatment could induce harmful secondary effects, such as immunodeficiency or deleterious effects on haematopoiesis.

Alternative strategies would consist in prolonging the lifespan of endocannabinoids, by inhibiting their degradation, or by potentiating non-CB_1/non-CB_2 receptors for endocannabinoids. Palmitoylethanolamide (PEA) is an endogenously produced cannabinoid-like molecule that is thought to potentiate the effects of the endocannabinoid anandamide (AEA) at cannabinoid and TRPV1 receptors through a mechanism known as the 'entourage' effect (Lambert & Di Marzo, 1999). Alternatively, this molecule could also induce its own effects, preferentially through binding to PPAR-α (LoVerme *et al.*, 2005). PEA levels have been shown to be affected in animal models of Huntington's disease (Bisogno *et al.*, 2007), MS (Baker *et al.*, 2001), neuropathic pain (Petrosino *et al.*, 2007) and cerebral ischaemia (Franklin *et al.*, 2003). Beneficial effects have been described for this cannabinoid in models of excitotoxicity (Skaper *et al.*, 1996), epilepsy

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(Sheerin *et al.*, 2004), MS (Baker *et al.*, 2001) and pain (Haller *et al.*, 2006). Furthermore, no psychoactivity has been reported for this compound, which makes it a particularly interesting substance with promising therapeutic properties.

In this study, we investigated, in relation to inflammation, the expression of the classical cannabinoid receptors CB_1 and CB_2 and the putative cannabinoid receptor PPAR-α in the spinal cord of mice subjected to a viral model of MS. We then measured in the spinal cord the concentration of the endocannabinoids AEA, 2-arachidonoylglycerol (2-AG) as well as of PEA and evaluated the expression of different enzymes implicated in the metabolism of endocannabinoids. Finally, we estimated for the first time the potential therapeutic effects of a peripheral treatment with PEA in this model of MS.

Materials and methods

Reagents

Theiler's virus (DA strain) was a kind gift from Dr M. Rodriguez at Foundation Mayo Clinic (Rochester, MN, USA). All other products were from Sigma, unless stated elsewhere in the text.

Animals and Theiler's virus inoculation

We used female SJL/J mice, susceptible to TMEV-IDD (Harlan Iffa-Credo, France), maintained on food and water *ad libitum* in a 12-h light/dark cycle. Four-week-old mice were inoculated intracerebrally in the right cerebral hemisphere with 10^6 p.f.u. of DA TMEV strain in 30 μL Dulbecco's modified essential medium supplemented with 10% foetal calf serum (FCS), as previously described (Lledo *et al.*, 1999). Handling of animals was performed in compliance with the guidelines of animal care set by the European Union (86/609/EEC) and the Spanish regulations (BOE67/8509-12; BOE1201/2005) on the use and care of laboratory animals, and approved by the local Animal Care and Ethics Committee of the CSIC.

Animals' sub-chronic treatments

Sham or TMEV-IDD mice were injected intraperitoneally with vehicle or PEA (5 mg/kg) between Days 60 and 70 post-infection. The duration of treatment was chosen on the basis of two previous studies using inhibitors of AEA reuptake in the same model of MS (Mestre *et al.*, 2005; Ortega-Gutierrez *et al.*, 2005). The treatment was initiated at a time when clinical signs of motor impairment were clearly present in the animals. Animals were killed by the injection of an overdose of anaesthetic at Day 70 post-infection and processed for tissue collection.

Tissue collection and RNA extraction

After saline perfusion, tissue for reverse transcriptase-polymerase chain reaction (RT-PCR) studies was rapidly removed. Tissue samples were frozen in dry ice and stored at −70°C until required. Total RNA was extracted using RNeasy mini columns (Qiagen, UK). Contaminating genomic DNA was degraded by a treatment with DNaseI (Qiagen, UK). The yield of RNA was determined using a Nanodrop® spectrophotometer (Nanodrop technologies, USA).

RT and TaqMan PCR

Total RNA (1 μg in 20 μL) was reverse transcribed into cDNA using the Promega reverse transcription kit (Promega, Spain) with poly-dT primers. TaqMan primers and probes for tumour growth factor (TGF)-β, CB_1 and CB_2 were designed using the PRIMER EXPRESS software (Applied Biosystems, UK) from sequences published in the NCBI database. Primer and probe sequences are detailed in Table 1. Where possible, probes were designed to span an intron–exon boundary in order to avoid amplification of genomic DNA. Primers for monacylglycerol lipase (MAGL) were obtained from Chon *et al.* (2007). Primer sequences for interleukin (IL)-6 were kindly provided by Dr Colm Cunningham (Trinity college of Dublin, Republic of Ireland). Probes were labelled at the 5′ end with a 6′-carboxyfluorescein (FAM) reporter dye and at the 3′ end with a 6′-carboxy-tetramethyl rhodamine (TAMRA) quencher dye. Primers and probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were supplied by Applied Biosystems, UK. For the other genes studied in this work, we used specific TaqMan gene expression assays (Applied Biosystems). TaqMan PCR was performed from 1 μL of cDNA (corresponding to 50 ng RNA input) using Universal TaqMan Mastermix with 100 nM primers and a 50 nM probe. In the case of IL-6, TGF-β and MAGL, no probe was used, as the PCR reaction was set up using the Power SYBR Green PCR Master Mix (Applied Biosystems) to detect double-stranded amplified DNA. Cycling conditions were: 50°C for 2 min; 95°C for 10 min; followed by 40 cycles of amplification (95°C for 15 s, 60°C for 1 min). Samples were assayed on the Applied Biosystems PRISM 7700 sequence detection system. Each sample was assayed in duplicate, and a six-point standard curve run in parallel. To ensure the absence of genomic DNA contamination, a control sample of non-reverse-transcribed RNA was run for each set of

TABLE 1. Primers and probes used in TaqMan PCR

Primer or probe	Sequence (5′–3′)	Position (bp)	Amplicon size (bp)
CB_1 forward primer	AGCTTTGTTGACTTCCACGTGTT	1068–1090	56
CB_1 reverse primer	CTGCCCACAGATGCTGTGAA	1168–1149	
CB_1 probe	CCGCAAAGATAGTCCCAATGTGTTTCTGTTC	1094–1124	
CB_2 forward primer	AGGAGCTGTCAGCTCAGGGTAT	1716–1737	60
CB_2 reverse primer	CTGCGCCCCTAAGGACCTA	1816–1798	
CB_2 probe	AGGGCCCTCCCGACATCCTGATAAG	1752–1776	
TGF-β forward primer	TAAAGAGGTCACCCGCGTGCTAAT	1182–1206	116
TGF-β reverse primer	ACTGCTTCCCGAATGTCTGACGTA	1298–1274	

TaqMan probe oligonucleotides were labelled at the 5′ end with the reporter dye 6′-carboxyfluorescein (FAM) and at the 3′ end with the quencher dye 6′-carboxy-tetramethyl rhodamine (TAMRA). In the case of tumour growth factor (TGF)-β, no probe was used as the reaction was set up by using the SybrGreen dye to detect amplified DNA. Primers and probes for PPAR-α, FAAH, NAPE-PLD and DGL-α were obtained from Applied Biosystems (Applied biosystems gene expression assays). Primers for IL-6 were obtained from Dr Colm Cunningham (Trinity college of Dublin), primers for MAGL were earlier published (Chon *et al.*, 2007). CB, cannabinoid receptor.

RNA extractions. Relative quantification was obtained by calculating the ratio between the values obtained for each gene of interest and the house-keeping gene GAPDH. Results are expressed as a percentage of sham animals for each time point, following the formula: $(X_t/GAPDH_t)/(X_s/GAPDH_s)*100$, where X_s and X_t are the values for the genes of interest, respectively, in sham and TMEV-IDD animals.

Measurement of endocannabinoids and PEA levels

The extraction, purification and quantification of endocannabinoids and PEA require a set of different biochemical steps (Di Marzo *et al.*, 2001a). First, tissue was dounce-homogenized and extracted with chloroform : methanol : Tris–HCl 50 mM pH 7.5 (2 : 1 : 1, v/v) containing internal standards (200 pmol each of $[^2H]_8$AEA, $[^2H]_5$2-AG and $[^2H]_4$PEA). The lipid-containing organic phase was dried down and weighed. The extract was pre-purified by open bed chromatography on silica columns eluted with increasing concentrations of methanol in chloroform. Fractions for AEA, PEA and 2-AG measurement were obtained by eluting the column with 9 : 1 (by vol.) chloroform : methanol and then directly analysed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) using a Shimadzu high-pressure liquid chromatography (HPLC) apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface. LC-APCI-MS analyses were carried out in the selected ion-monitoring mode, as described previously (Di Marzo *et al.*, 2001a, b). The temperature of the APCI source was 400°C, the HPLC column was a Phenomenex (5 μm, 150 × 4.5 mm) reverse phase column, eluted as described. AEA, PEA and 2-AG quasi-molecular ions were quantified by isotope dilution with the above-mentioned deuterated standards. Endocannabinoid levels were normalized to the total weight of wet tissue (pmol/g tissue for AEA and PEA; pmol/mg tissue for 2-AG).

Evaluation of motor coordination

To evaluate motor neurological deficits of mice, we used the rotarod test, which measures balance, coordination and motor control. The rotarod apparatus (Ugo Basile, Comerio, Italy) consists of a suspended rod able to run at constant or accelerating speed. All mice were exposed to a 5-min training period, at constant speed (4.5 rpm), immediately before the test to familiarize them with the apparatus. The test was performed at gradually accelerating rod speed. Data were collected from mouse rotarod performance 1 day after the end of the period of sub-chronic PEA treatment, which corresponds to 69 days after TMEV infection. The trial was terminated when mice fell from the apparatus or after a maximum of 5 min.

Spontaneous motor activity

The screening for locomotor activity was performed using an activity cage (Activity Monitor System Omnitech Electronics, Columbus, OH, USA) coupled to a Digiscan Analyser. This test was performed 3 h after PEA treatments to eliminate any direct effect of this compound on locomotor activity. Vertical and horizontal activities were measured for each animal during the last 5 min of a session of 10 min.

Splenic cell isolation and proliferation assay

Spleens from naïve mice were minced in a glass homogenizer and cell suspension was incubated for 1–2 min in a red cell lysis buffer (from Sigma). After removing the erythrocytes, the remaining mononuclear cells were resuspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (10 U/mL)-streptomycin (10 μg/mL), 10 mM Hepes, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 0.1 mM non-essential amino acids (Gibco, Invitrogen). Cells were plated at the density of 2 millions/mL and stimulated with Concanavalin A (ConA; Sigma) at the dose of 0.6 μg/mL alone or in combination with PEA (0.1–1–10 μM). After 3 days cells were counted and viability assessed by trypan blue exclusion. Splenic cells isolated from TMEV- or sham-infected mice with or without PEA treatment were stimulated *in vitro* with ConA (0.6 μg/mL). After 4 days, cell proliferation was assessed by bromodeoxyuridine (BrdU) staining following the manufacturer's instructions (Colorimetric BrdU kit assay, Calbiochem).

Immunohistochemistry

For routine histological examination, serial sections (20 μm) were stained with Iba-1. Briefly, free-floating sections were washed with 0.1 M phosphate buffer (PB) for 10 min, and incubated with 0.2% Triton X-100 and 5% normal goat serum for 30 min (Vector Laboratories, Burlingame, CA, USA). The sections were then incubated overnight at 4°C with rabbit anti-Iba1 antibody (ionized calcium-binding adapter molecule 1; Wako, Richmond, VA, USA). The following day, the sections were rinsed and incubated for 2 h using goat anti-rabbit conjugated with Alexa 594 (Molecular Probes, Eugene, OR, USA). Sections were rinsed with 0.1 M PB for 10 min and mounted with fluorescent mounting medium. Specificity of staining was confirmed by omitting the primary antibody. Quantification of staining was performed using the IMAGE J software designed by National Institutes of Health.

Statistical analysis

All results are expressed as mean + SEM. Unless stated in the figure legend, statistical analysis consisted of Student's *t*-test. Mann–Whitney's *U*-test was applied when the Levene's test for homogeneity of variances gave $P < 0.05$. One-way ANOVA followed by Bonferroni–Dunn's test was used for multiple comparisons. For correlation studies, Pearson's product-moment correlation coefficients were calculated and referred to the corresponding critical value to estimate their significance.

Results

CB_2 is upregulated during the course of TMEV-IDD and correlated to the production of pro-inflammatory molecules

First, we investigated by real-time RT-PCR the expression of CB_1, CB_2 and PPAR-α mRNA in the spinal cord of mice subjected to the TMEV-IDD model of MS. Total RNA were extracted at 35, 60, 90 and 180 days post-infection. The most significant observation was a dramatic upregulation of CB_2 at 60 days post-infection, that progressively went back to basal levels with no variation at 180 days (Fig. 1a and b). CB_1 nevertheless showed a slight upregulation at 35 and 60 days, that reached significance at 90 days post-infection (Fig. 1a), while in the late stage of the disease (180 days post-infection) its expression was decreased (Fig. 1a). However, no change was observed in the expression of PPAR-α at any of the times studied (Fig. 1c).

Our next step was to investigate if these changes in CB_2 could be linked to the production of the typical proinflammatory cytokines,

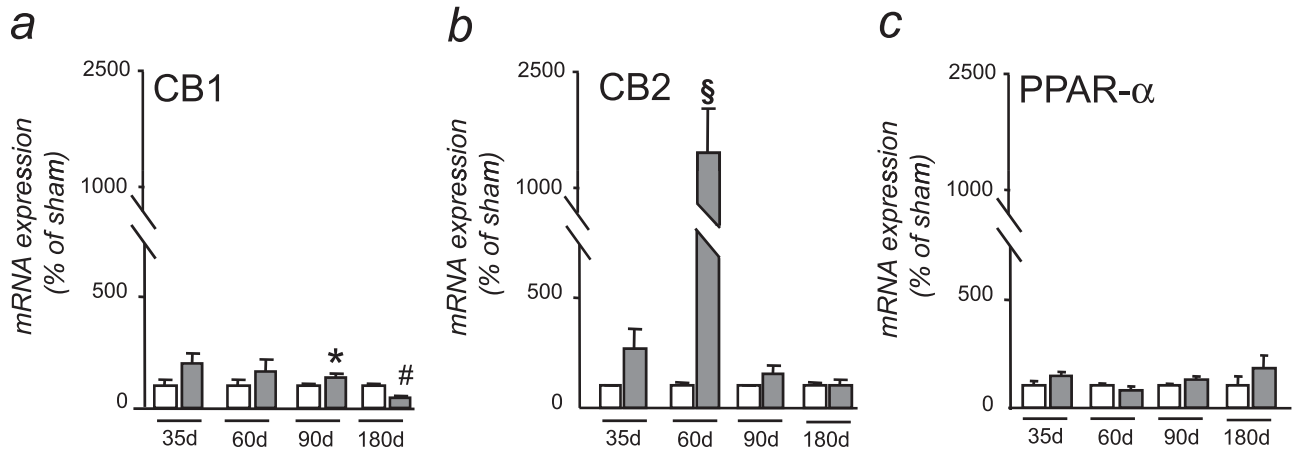

FIG. 1. Cannabinoid receptor $(CB)_2$ mRNA is upregulated at 60 days post-infection in the TMEV-IDD model. Histograms show mean values + SEM of (a) CB_1, (b) CB_2 and (c) peroxisome proliferator-activated receptor-α (PPAR-α) mRNA expression, assessed by TaqMan RT-PCR at the indicated times, in sham (white bar) or TMEV-infected (grey bar) spinal cord. Values represent the relative expression of mRNA, normalized to GAPDH expression for each time point (n = 5–9). *Significantly ($P < 0.05$) enhanced as compared with sham animals at the same time point. §Significantly ($P < 0.05$) enhanced as compared with sham animals at the same time point (Mann–Whitney's U-test). #Significantly ($P < 0.05$) reduced as compared with sham animals at the same time.

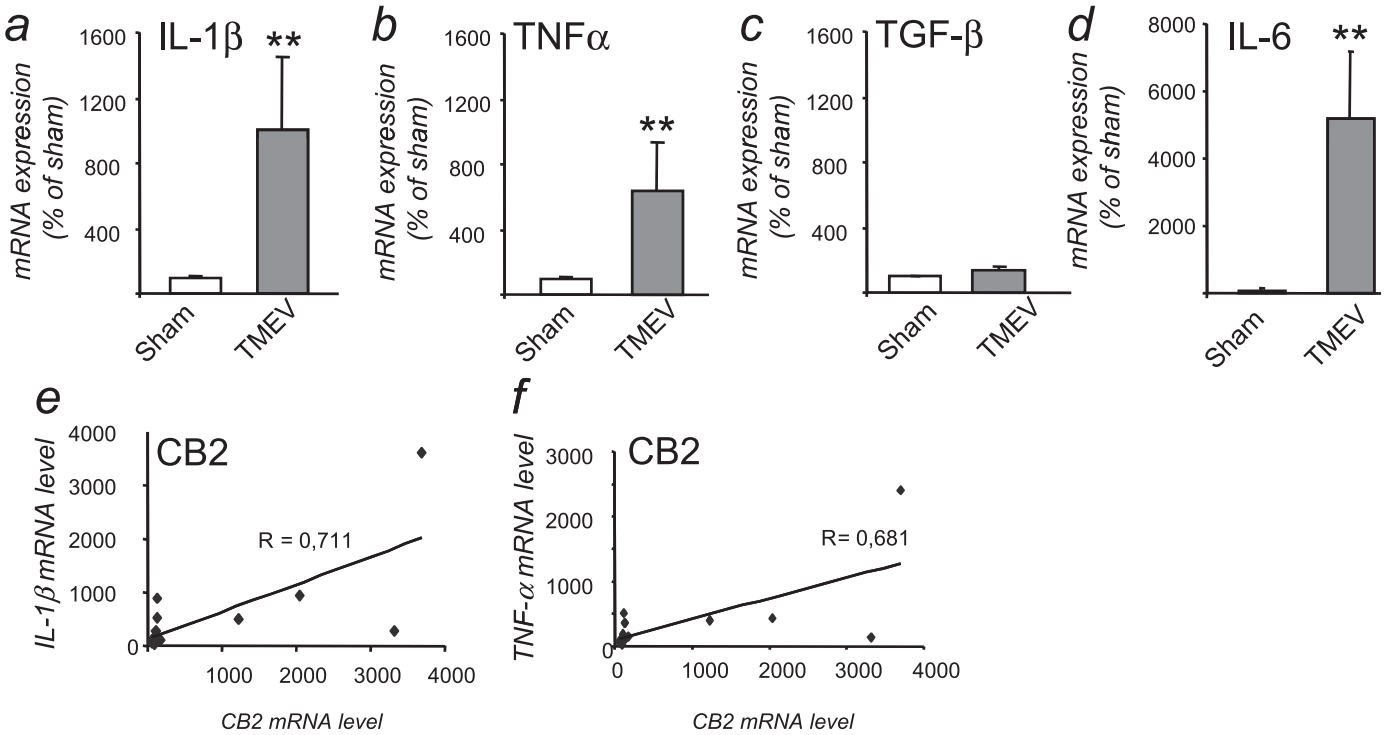

FIG. 2. Cannabinoid receptor $(CB)_2$ mRNA expression is correlated to interleukin (IL)-1β and tumour necrosis factor (TNF)-α expression at 60 days post-infection. Histograms show mean values + SEM of (a) IL-1β, (b) TNF-α, (c) tumour growth factor (TGF)-β and (d) IL-6 mRNA expression, assessed by TaqMan RT-PCR, in sham (white bar) or TMEV-infected (grey bar) spinal cord. Values represent the relative expression of mRNA, normalized to GAPDH expression for each time point (n = 5–9). CB_2 mRNA expression was correlated to (e) IL-1β and (f) TNF-α expression (n = 16, sham and TMEV-infected animals; $P = 0.002$ and $P = 0.004$, respectively). **Significantly ($P < 0.005$) enhanced as compared with sham animals.

IL-1β and tumour necrosis factor (TNF)-α. The level of expression of the mRNA for these two molecules at 60 days post-infection was estimated by real-time RT-PCR. First of all, we observed that both cytokines displayed elevated expression (Fig. 2a and b), while TGF-β, which is considered as an anti-inflammatory cytokine, remained unchanged (Fig. 2c) and IL-6, a classical IL-1 target gene (Tsakiri *et al.*, 2008) was upregulated in TMEV-IDD animals (Fig. 2d). Then, the relative levels of cannabinoid receptors were plotted against the relative levels of IL-1β and TNF-α. Interestingly, while no correlation was observed between the levels of CB_1 or PPAR-α mRNA and the levels of IL-1β or TNF-α mRNA (data not shown), there was a positive correlation between CB_2 expression and the expression of both proinflammatory cytokines IL-1β and TNF-α (CB_2 vs. IL-1β: $R = 0.71$; Fig. 2e; CB_2 vs. TNF-α: $R = 0.68$; Fig. 2f). On the other hand, no correlation was observed between the expression of CB_2 and the anti-inflammatory cytokine, TGF-β (data not shown).

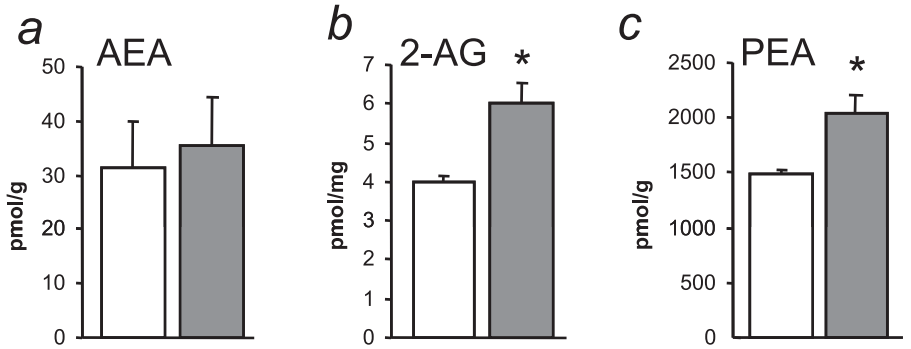

FIG. 3. 2-Arachidonoylglycerol (2-AG) and palmitoylethanolamide (PEA) spinal cord levels are increased in TMEV-IDD model. Histograms show mean values + SEM (n = 5–6) of endocannabinoid measurements relative to total tissue weight. Quantification was performed in the spinal cord at 60 days post-infection in sham (white bars) and TMEV-infected (grey bars) animals. (a) Anandamide (AEA) levels were unchanged, whereas (b) 2-AG and (c) PEA levels were significantly increased. *Significantly ($P < 0.05$) increased as compared with sham animals.

The production of 2-AG and PEA are enhanced concomitantly with the upregulation of CB_2 and independently of the expression of the synthesis and degradation enzymes

The above data suggest an enhanced capacity of response to endocannabinoids in the spinal cord of TMEV-IDD mice at 60 days post-infection. We thus measured the levels of the endocannabinoid molecules, AEA, 2-AG and PEA in the spinal cord of sham-operated and TMEV-IDD mice at 60 days. No change was observed in the levels of AEA (Fig. 3a). However, 2-AG and PEA levels were both enhanced at this time point (Fig. 3b and c) in TMEV-IDD mice. As the increase in these molecules could be due to changes in the expression of the enzymes responsible for their synthesis and degradation, we investigated by real-time RT-PCR the expression of the two endocannabinoid synthesis enzymes, diacylglycerol lipase-α (DGL-α) and N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD), more specific for 2-AG and AEA, respectively, the degradation enzyme, fatty acid amide hydrolase (FAAH), responsible for the degradation of AEA and PEA, and monoacylglycerol lipase (MAGL), the enzyme responsible for the degradation of 2-AG. No significant changes could be observed in the expression of these enzymes at 60 days post-infection (Fig. 4a–d). Furthermore, no correlation was observed between the mRNA levels of these four enzymes and the expression of IL-1β or TNF-α (data not shown).

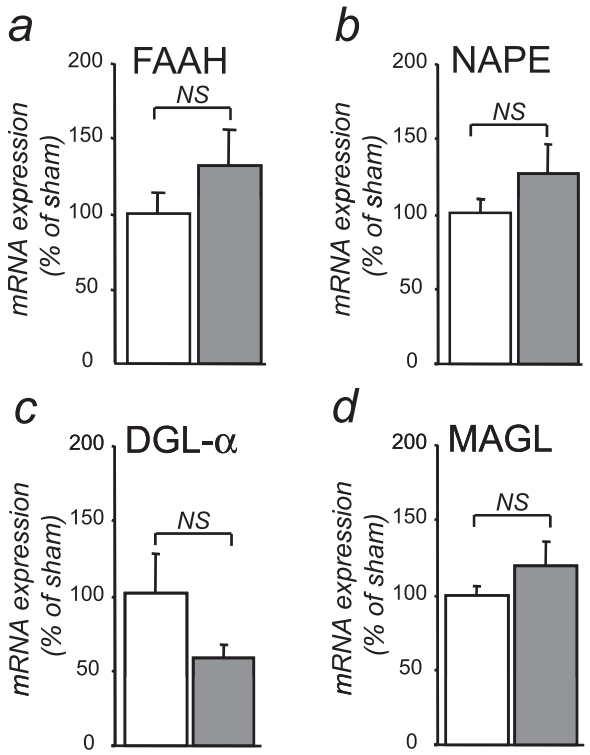

FIG. 4. Unmodified expression of the enzymes implicated in the metabolism of endocannabinoids. Histograms show mean values + SEM (n = 5–9) of (a) fatty acid amide hydrolase (FAAH), (b) N-acylphosphatidylethanolamine phospholipase-D (NAPE-PLD), (c) diacylglycerol lipase-α (DGL-α) and (d) monoacylglycerol lipase (MAGL) mRNA expression, assessed by TaqMan RT-PCR at 60 days post-infection, in sham (white bar) or TMEV-infected (grey bar) spinal cord. Values represent the relative expression of mRNA, normalized to GAPDH expression for each time point. NS, non-significantly different from sham animals (P = 0.25, P = 0.57, P = 0.26 and P = 0.34, respectively).

PEA ameliorates motor deficits during established pathology in TMEV-IDD animals through an anti-inflammatory effect

The above results would suggest that 2-AG and PEA are produced in response to injury in this model of MS. However, the increases in these molecules are relatively modest. We therefore became interested in studying what might be the consequences of a supplementary application of these compounds on the pathology of TMEV-IDD. The fact that no psychoactive effects have been reported for PEA makes it a particularly interesting substance with promising therapeutic properties. We thus chose to test the effects of this compound on the behavioural impairments induced by TMEV-IDD. As previously observed (Docagne *et al.*, 2007; Pachner *et al.*, 2007), the performance of the animals of the TMEV group in the rotarod test was greatly reduced (Fig. 5a). Interestingly, when TMEV-IDD animals were subchronically treated with PEA (one daily i.p. injection of 5 mg/kg during 10 days) between 60 and 70 days post-infection, their score in the rotarod was significantly improved (Fig. 5a). The horizontal activity of the TMEV-IDD animals, measured in the activity cage, was also greatly reduced as compared with sham animals (Fig. 5b). PEA treatment completely reversed this motor impairment (Fig. 5b). Finally, although vertical activity was practically abolished in the TMEV-IDD animals, the treatment with PEA improved motor deficits (Fig. 5c).

As peripheral leukocytes express CB_2, and CB_2 expression by encephalitogenic T-cells is critical for controlling CNS inflammation (Maresz *et al.*, 2005), clinical improvement after PEA treatment could be due to an immunomodulatory effect. We thus performed proliferation assay using BrdU on cultured splenic cells extracted from sham-operated and TMEV-infected mice subchronically injected with PEA

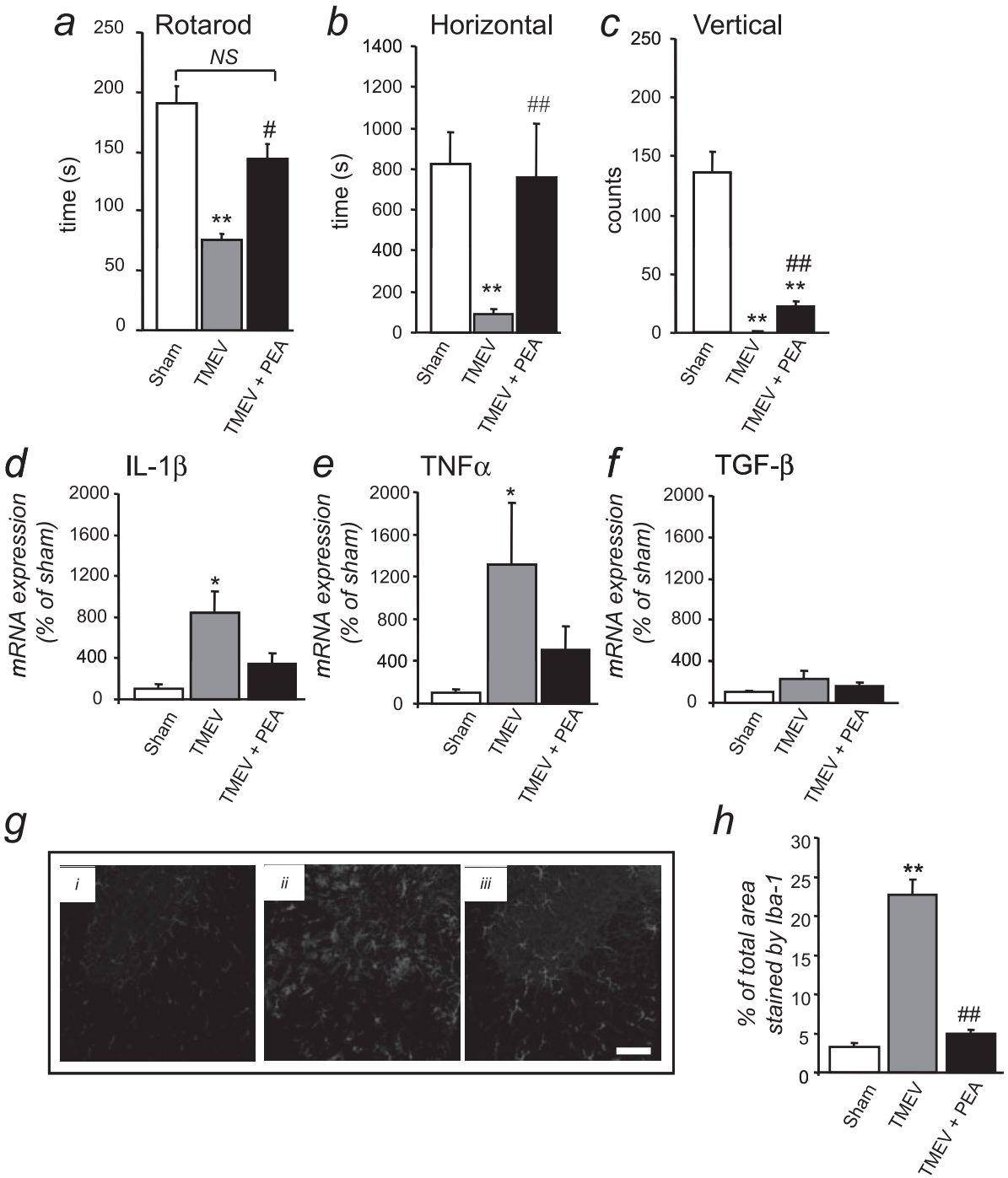

FIG. 5. Palmitoylethanolamide (PEA) treatment ameliorates motor impairment in TMEV-IDD mice. Animals were treated with PEA (5 mg/kg) between Days 60 and 70 post-infection, and submitted to tests for motor activity and coordination. White bars: sham; grey bars: TMEV-infected; black bars: PEA-treated, TMEV-infected (control: $n = 3$, other groups: $n = 6$). (b) Histograms show mean values + SEM of total time of horizontal movement in the last 5 min of a 10-min activity cage test. (c) Histograms show mean values + SEM of total number of vertical movements in the last 5 min of a 10-min activity cage test. (a) Histograms show mean scores + SEM in the rotarod test, as a measure of motor coordination. Results are expressed as total latency time spent by the animals on the wheel. **Significantly ($P < 0.01$) different from sham animals. $^{\#}$, $^{\#\#}$Significantly ($P < 0.01$ and $P < 0.05$, respectively) different from TMEV animals. (d)–(f) Histograms show mean values + SEM of (d) interleukin (IL)-1β, (e) tumour necrosis factor (TNF)-α and (f) tumour growth factor (TGF)-β mRNA expression, assessed by TaqMan RT-PCR, in the spinal cord of sham (white bar), TMEV-infected (grey bar) or TMEV-infected, PEA-treated (black bar) animals. Values represent the relative expression of mRNA. (g) Confocal photomicrographs show representative staining of microglial cells after immunohistochemistry for the microglial marker Iba1 in the spinal cord of (i) sham animals, (ii) TMEV-IDD animals treated with vehicle and (iii) TMEV-IDD animals treated with PEA. Scale bar: 100 μm. (h) Quantification of microglial staining in the spinal cord of sham (white bar), TMEV-infected (grey bar) or TMEV-infected, PEA-treated (black bar) animals. Histograms show mean values + SEM (20 sections/group) of fraction of tissue stained by the Iba1 antibody. *$P < 0.05$ and **$P < 0.01$, compared with sham animals. $^{\#\#}$Significantly ($P < 0.01$) different from TMEV animals.

or vehicle. Cells were incubated with or without Con A (0.6 μg/mL) for 4 days to induce proliferation. Cells responded with the same intensity to Con A, whether they were extracted from vehicle- or PEA-treated animals (data not shown). We also assessed the possible toxic effect of PEA on naïve splenic cells *in vitro*. PEA (0.1–10 μM) did not induce any effect on cell survival, as assessed by trypan blue exclusion (data not shown). Finally, immunostaining for CD4 and CD8 has been performed in the spinal cord of TMEV-IDD animals treated with vehicle or PEA, and no difference was observed between these two groups (data not shown).

In an attempt to further determine the mechanisms responsible for the effects of PEA, we studied the expression of the proinflammatory cytokines, IL-1β and TNF-α, in the spinal cord of sham and TMEV-IDD animals treated with PEA and its vehicle. While the levels of expression of both cytokines were enhanced in TMEV animals, as already described earlier in this study, no significant difference could be observed between TMEV animals treated with PEA and sham animals (Fig. 5d and e). However, PEA did not induce any change in the levels of TGF-β (Fig. 5f). Finally, we assessed the effect of PEA on microglial activation, which, as a central component of neuroinflammation, may parallel the expression of IL-1β and TNF-α. Microglia was immunostained using the Iba1 antibody. First, we observed a weak Iba1 staining in sham animals, with a typical morphology of resting microglia (Fig. 5gi). TMEV infection resulted in a dramatic activation of microglial cells, as observed both by the increased intensity of Iba1 staining and the morphological characteristics of Iba1-positive cells (Fig. 5gii). PEA treatment considerably reduced microglial activation (Fig. 5giii). These different levels of microglial activation were quantified by measuring the total area of staining (Fig. 5h), confirming that microglial activation was dramatically enhanced in TMEV-IDD animals and reduced by PEA treatment (Fig. 5h).

Taken together, these data show that the improvement of motor deficits by PEA in this model of MS is accompanied by a reduction of inflammation.

Discussion

This study describes the pattern of expression of various components of the endocannabinoid system during the time course of the TMEV-IDD model of MS. Our first observation was that the CB_2 receptor was upregulated at 60 days post-infection. Focusing on this particular time point, we report here a positive correlation between the expression of this receptor and the production of the proinflammatory cytokines, IL-1β and TNF-α. These modifications were accompanied by an enhanced production of the endocannabinoid 2-AG and of the anti-inflammatory AEA congener, PEA. Finally, we observed an improvement in the motor function of the animals subjected to this model of MS, when treated with PEA.

Our observation that CB_2 expression is enhanced in the TMEV-IDD model in relation with inflammation is in agreement with previous studies in the experimental autoimmune encephalomyelitis (EAE) model of MS (Maresz *et al.*, 2005), in which the expression of CB_2 has been identified in microglial progenitors (Palazuelos *et al.*, 2008), and suggested in autoreactive T-cells (Maresz *et al.*, 2007). In *post-mortem* specimen from patients with MS, CB_2 was expressed by microglial cells of the spinal cord (Yiangou *et al.*, 2006), as well as in T-lymphocytes, astrocytes and perivascular and activated microglia in the brain (Benito *et al.*, 2007). This receptor was thus defined as a specific marker of plaque subtypes, with an even distribution within active plaques and at the periphery of chronic plaques (Benito *et al.*, 2007). Overall, our study reinforces the idea that CB_2 is upregulated in response to CNS inflammation. The fact that the expression of CB_2 is positively correlated to the expression of the proinflammatory cytokines IL-1β and TNF-α in the TMEV-IDD model, but is not correlated to the expression of TGF-β, which is generally considered as an anti-inflammatory cytokine, is also in agreement with this idea. Our data suggest that CB_2 upregulation is part of the response of the spinal cord tissue to inflammation during TMEV-IDD.

Few reports are available concerning the modulation of CB_1 receptors in human MS or in animal models of this pathology. This receptor has been detected in active and chronic MS plaques, particularly in neurons and oligodendrocytes (Benito *et al.*, 2007). Our data are in accordance with this, as we report here a first phase of progressive CB_1 upregulation between Days 35 and 90 of the TMEV-IDD. Downregulation of CB_1 has also been reported, and was likely related to neuronal loss, for instance in models of cerebral ischaemia (Amantea *et al.*, 2007). Accordingly, in our hands, CB_1 expression was decreased at 180 days post-infection, which could be related to the neuronal and axonal injury that can be observed at late stages in this model (Ure & Rodriguez, 2000; Tsunoda *et al.*, 2003).

Several studies have described the modulation of endocannabinoid ligands in MS as well as in EAE in mice. As previously reported (Mestre *et al.*, 2005), we describe here that AEA levels were not modified in the TMEV-IDD model. These data complement previous observations of Witting *et al.* (2006) that AEA levels remained unchanged in the EAE model. Interestingly, the form of EAE used in this last study (chronic progressive EAE) results in a progressive worsening of the symptoms in the absence of remission episodes, so that this form of EAE and the TMEV-IDD model are considered as more specific models for the primary-progressive form of MS. Conversely, in a model considered to be more closely related to relapsing–remitting MS (chronic relapsing EAE), AEA has been reported to be increased (Baker *et al.*, 2001; Centonze *et al.*, 2007), although this increase is only observed during the phases of relapse and not during remission (Baker *et al.*, 2001). This is in agreement with the report of an increase in AEA levels in the cerebrospinal fluid of patients with MS during relapse (Centonze *et al.*, 2007). Taken together, our data and these previous reports in the literature would indicate that AEA increase might be a more specific marker of the relapsing phase of relapsing–remitting MS. Further studies in humans are needed to investigate this hypothesis.

Fewer data are available concerning the production of PEA in animal models of MS or in the human disease. We report here that this endocannabinoid-like molecule is increased in the spinal cord of the TMEV-IDD model, which supplements previous observations from the chronic relapsing (Baker *et al.*, 2001) and chronic progressive (Muccioli & Stella, 2007) forms of EAE in mice.

In the present study, no regulation at 60 days post-infection was observed for NAPE-PLD or FAAH, two enzymes respectively implicated in the synthesis and degradation of both AEA and PEA. This correlates with stable levels of AEA, but fails to explain the increase in PEA levels. This difference between AEA and PEA might be accounted for by different metabolic pathways for these two molecules. For instance, while the degradation of AEA is mostly attributed to FAAH, PEA is preferentially hydrolysed by *N*-acyleth-anolamine acid amidase (Tsuboi *et al.*, 2005). Furthermore, differences in the availability of the respective *N*-acyl-phosphatidyl-ethanolamine biosynthetic precursors for AEA and PEA could also explain the differential regulation of AEA and PEA levels.

Concerning 2-AG, we show here an increase in the levels of this endocannabinoid in the spinal cord, in agreement with that observed in chronic relapsing EAE (Baker *et al.*, 2001), but contrasts with other reports from chronic progressive EAE (Witting *et al.*, 2006) or the relapsing–remitting form of human MS (Centonze *et al.*, 2007). Here,

2-AG levels were increased without any modification of DGL-α, the enzyme mostly responsible for its biosynthesis, or MAGL, which degrades 2-AG. We can postulate that the increase in 2-AG could thus be related to a regulation of the activity, rather than expression, of DGL-α or MAGL. Finally, as stated above for PEA, the increase in 2-AG could also be due to a modification in the levels of its phospholipid precursors.

Two distinct pathways have been proposed to mediate the effects of PEA. This molecule, acting as a substrate for the FAAH enzyme, might potentiate the effects of AEA by a mechanism known as the 'entourage' effect (Di Marzo *et al.*, 2001b). Alternatively, this molecule may induce effects *per se* by entering the cytoplasm thanks to its lipophilic nature and binding to the intracellular receptor PPAR-α (O'Sullivan, 2007). In the present study, the enhancement of PEA levels at 60 days post-infection was not accompanied by a modification of AEA levels, a result that would not favour the occurrence of an 'entourage' effect of PEA in these conditions. We also observed that TMEV animals express PPAR-α at the same level as sham animals at 60 days post-infection, which makes this receptor a possible mediator of the PEA effect. Indeed, we show here that, when injected at this time point in TMEV-IDD animals, PEA is able to ameliorate the motor deficits occurring in this model of MS. In these conditions of treatments, in which the concentration of PEA in the inflamed tissue is enhanced, we cannot exclude that exogenous PEA might also act through the 'entourage' effect. Indeed, in earlier studies, we (Mestre *et al.*, 2005; Ortega-Gutierrez *et al.*, 2005) and others (Cabranes *et al.*, 2005) showed that the potentiation of AEA effects, through the inhibition of its inactivation, induced comparable therapeutic effects. The fact that PPAR-α agonists, mainly by counteracting inflammatory and immune processes, show beneficial effects in mice with EAE (Cunard *et al.*, 2002; Heneka *et al.*, 2007) is in favour of the idea that PEA might induce its beneficial effect through the activation of this receptor. Further studies are needed to address the question of the mechanism of action of PEA in MS models, whether through the 'entourage' effect, the activation of PPAR-α or both mechanisms.

Here, despite higher levels of PEA in TMEV-IDD mice as compared with controls, their rotarod and activity cage performances were significantly worse than controls. This could appear as a paradox regarding the reduction of motor impairments in animals treated with PEA. However, while the increase in endogenous PEA during TMEV-IDD likely induces protection in these animals, this increase is probably too modest to more efficiently reverse the clinical signs. This means that the TMEV-IDD animals would probably be more affected if this increase in PEA did not occur. This idea is closely related to the broadly accepted concept that the endocannabinoid system might consist in a 'self-defence' mechanism against CNS injury (Bisogno & Di Marzo, 2007).

Our data somehow overlap previous observations by Baker *et al.* (2000). This study reported a reduction in spasticity in CREAE mice after a single treatment with PEA. Two differences, however, exist between this last work and the present one. First, the dose (10 mg/kg vs. 5 mg/kg in the present study) and more importantly the regimen of treatment (acute vs. chronic in the present study) are distinct. Second, the assessment of spasticity and motor deficits were performed within 1 h in the Baker *et al.* study, whereas we performed our tests the day after the last injection, which excludes immediate effects such as pain relief or others. Rather than a discrepancy, this would account for two distinct, but complementary mechanisms: the rapid, short-lasting effects of PEA, observed on spasticity would be usefully completed by a longer-lasting effect that would more likely be due to a modification of the course of the disease. This is interestingly related to the situation that occurs in the management of cannabinoid-related drugs in the treatment of MS: while cannabis-derived oromucosal spray is already available to treat spasticity and offer palliative effects such as pain relief to patients with MS, a new challenge now arises to study the possible longer-lasting, curative effects that these drugs may offer. We believe that our study provides new clues in this direction.

Here, we show that PEA treatment reduces the expression of the typical pro-inflammatory cytokines IL-1β and TNF-α, without affecting the levels of TGF-β. This was accompanied by a reduction in microglial activation. However, PEA remained without effect on splenic cell proliferation or survival *in vitro*, and did not modify lymphocytes infiltration in TMEV-IDD animals. These effects account for an anti-inflammatory effect of PEA that is likely responsible for the improvement in motor performance in TMEV-IDD animals treated with this compound, without peripheral, immunomodulatory effects. This is in accordance with the recently reported anti-inflammatory effects of PEA or its analogues in spinal cord injury (Genovese *et al.*, 2008), as well as in chronic inflammation (De Filippis *et al.*, 2008). Demyelination and axonal damage are two of the principal hallmarks of MS pathology, and co-exist at the stage of the TMEV-IDD model considered in this study (Tsunoda *et al.*, 2003). In the present study, no modification was observed in the extent of demyelination in PEA-treated animals (data not shown), despite clinical ameliorations with this compound. As demyelination does not necessarily correlate with the severity of neurological decline in human pathology, and as demyelination can be secondary to axonal damage in this model of MS (Tsunoda *et al.*, 2003), one hypothesis would be that PEA, as a result of its anti-inflammatory effect, could promote therapeutic effects by reducing axonal damage. Indeed, we described in an earlier study (Docagne *et al.*, 2007) a reduction of axonal damage after treatment with cannabinoids in the same model of MS.

Overall, considering the fact that no undesired psychoactive effect has been reported for PEA, our study points to this endocannabinoid-like molecule as a potentially interesting template for the development of new therapeutic strategies in the treatment of MS. Our report also highlights the fact that the endocannabinoid system is greatly influenced by pathological conditions mimicking MS. Indeed, our data reinforce the concept that this system represents an endogenous mechanism of defence against diseases such as MS.

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Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, anandamide; BrdU, bromodeoxyuridine; CB, cannabinoid receptor; Con A, Concanavalin A; DGL-α, diacylglycerol lipase-α; EAE, experimental autoimmune encephalomyelitis; FAAH, fatty acid amide hydrolase; FAM, 6′-carboxyfluorescein; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-pressure liquid chromatography; IL, interleukin; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; MAGL, monoacylglycerol lipase; MS, multiple sclerosis; NAPE-PLD, N-acylphosphatidylethanolamine phospholipase-D; PB, phosphate buffer; PEA, palmitoylethanolamide; PPAR-α, peroxisome proliferator-activated receptor-α; RT-PCR, reverse transcriptase-polymerase chain reaction; TAMRA, 6′-carboxy-tetramethyl rhodamine; TGF, tumour growth factor; TNF, tumour necrosis factor; TRP, transient receptor potential.

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