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ORIGINAL ARTICLE

A Cannabigerol Quinone Alleviates Neuroinflammation in a Chronic Model of Multiple Sclerosis

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Abstract Phytocannabinoids like Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) show a beneficial effect on neuroinflammatory and neurodegenerative processes through cell membrane cannabinoid receptor (CBr)-dependent and -independent mechanisms. Natural and synthetic cannabinoids also target the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ), an attractive molecular target for the treatment of neuroinflammation. As part of a study on the SAR of phytocannabinoids, we have investigated the effect of the oxidation modification in the resorcinol moiety of cannabigerol (CBG) on CB_1, CB_2 and PPARγ binding affinities, identifying cannabigerol quinone (VCE-003) as a potent anti-inflammatory agent. VCE-003 protected neuronal cells from excitotoxicity, activated PPARγ transcriptional activity and inhibited the release of pro-inflammatory mediators in LPS-stimulated microglial cells. Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis (MS) was used to investigate the anti-inflammatory activity of this compound in vivo. Motor function performance was

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evaluated and the neuroinflammatory response and gene expression pattern in brain and spinal cord were studied by immunostaining and qRT-PCR. We found that VCE-003 ameliorated the symptoms associated to TMEV infection, decreased microglia reactivity and modulated the expression of genes involved in MS pathophysiology. These data lead us to consider VCE-003 to have high potential for drug development against MS and perhaps other neuroinflammatory diseases.

Keywords Cannabinoids · Cannabigerol · PPARγ · Neuroinflammation · Multiple sclerosis

Abbreviations

CBD	Cannabidiol
CBG	Cannabigerol
EAE	Experimental autoimmune encephalomyelitis
Foxp3	Forkhead box P3
Icam-1	Intercellular adhesion molecule 1
IDD	Induced Demyelinating Disease
PGE_2	Prostaglandin E2
PPAR	Peroxisome proliferator-activated receptor
RSZ	Rosiglitazone
THC	Δ9-tetrahydrocannabinol
TMEV	Theiler's Murine Encephalomyelitis Virus
VLA-4	Very late antigen 4

Introduction

Cannabinoids are a group of chemicals exemplified by Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive component of *Cannabis sativa* (Mechoulam and Gaoni 1967). Over 500 secondary metabolites have been characterized from *C. sativa*, and over 100 of them are cannabinoids. The cannabinoid profile of *C. sativa* is under genetic and environmental control, with Δ^9-THC, cannabidiol (CBD) and their precursor cannabigerol (CBG) being the most abundant ones (Hill et al. 2011). While cannabinoids such as Δ^9-THC elicit diverse central and peripheral effects mainly by activating G protein–coupled cannabinoid receptors CB_1 and CB_2 (Howlett et al. 2002), other cannabinoids may act on different cell surface receptors. In vitro studies have revealed that CBG is a α2-adrenoceptor agonist and antagonizes $5HT_{1A}$ receptors (Cascio et al. 2010). Other findings indicate that CBG can activate TRPV1, TRPV2, and TRPA1 and is an antagonist of TRPM8 in vitro (De Petrocellis et al. 2011; De Petrocellis et al. 2008).

Several lines of evidence also show that phytocannabinoids bind and activate the nuclear receptor superfamily of peroxisome proliferator-activated receptors (PPARs) (O'Sullivan 2007). Three PPAR isoforms have been identified (PPARγ, PPARα and PPARδ) and they have been reported to control the expression of genes related to lipid and glucose metabolism as well as inflammatory responses (Bensinger and Tontonoz 2008). Within these receptors, PPARγ can be activated by phytocannabinoids such as Δ^9-THC and CBD (Esposito et al. 2011; O'Sullivan et al. 2005), as well as by synthetic derivatives (Liu et al. 2003; Mestre et al. 2009). Due, in part, to their PPARγ-activating properties, these cannabinoids may exert anti-inflammatory activities, thus showing a therapeutic potential for the treatment of inflammatory diseases (O'Sullivan et al. 2009).

PPARγ receptors have been shown to be involved in the modulation of inflammation, as PPARγ agonists down-regulate the expression of several proinflammatory cytokines (Jiang et al. 1998). Interestingly, PPARγ has been detected in neurons and glial cells (Moreno et al. 2004) and participates in mechanisms that control activation of microglia (Mrak and Landreth 2004). Microglia-driven inflammatory response includes modulation of cytokines and chemokines expression, neuronal dysfunction, and neurodegeneration (Perry et al. 2007). Microglia appear to be the initiator of such a response by secreting inflammatory mediators, such as IL-1β, IL-6, TNF-α and prostaglandin E_2 (PGE_2) that can act on astrocytes to induce secondary inflammatory responses (Saijo et al. 2009). A sustained neuroinflammatory response results in neuronal disfunction and neurodegeneration, contributing to disease progression (Perry et al. 2007). In fact, it has been demonstrated that neuroinflammation is an integral component to disorders such as Alzheimer's disease, Parkinson's disease, stroke and multiple sclerosis (MS) (Infante-Duarte et al. 2008).

MS is the most common chronic inflammatory-demyelinating CNS disease that is driven by myelin-specific auto-reactive T cells that infiltrate the CNS and activate other inflammatory cells (McFarland and Martin 2007). Microglial activation contributes to MS pathology through antigen presentation and secretion of pro-inflammatory mediators (Benveniste 1997). In addition, macrophages and microglial cells are involved in demyelination and phagocytosis of the degraded myelin (Bauer et al. 1994). Demyelination and axonal damage are responsible of clinical symptoms such as functional impairment, neuropathic pain and disability (reviewed in (Hauser and Oksenberg 2006)). Despite being the most common human primary demyelinating disease of the CNS, there is no satisfactory treatment as yet for MS, and there is a clear need for the development of agents able to treat this progressive disorder. PPARγ agonists have been shown to reduce the incidence and severity of disease in experimental models of MS such as experimental autoimmune encephalomyelitis (EAE) (Mestre et al. 2009; Niino et al. 2001; Feinstein et al. 2002), and a small clinical trial suggest that PPARγ could be a pharmacological target for the management of MS (Pershadsingh et al. 2004).

Here we present data demonstrating that cannabigerol quinone (VCE-003) binds to and activates PPARγ, acting as a partial agonist, showing negligible activity at the CB_1 receptor and a modest binding capacity to CB_2 receptors. VCE-003 inhibits cytokine production in activated primary microglia cultures and presents neuroprotective effects in cell lines and primary neuronal cultures. In the TMEV-IDD model of MS, VCE-003 improved the motor function performance, and reduced microglia reactivity and the expression of the adhesion molecule VCAM-1. In addition, we also showed that that VCE-003 treatment modified the expression of several immunomodulatory molecules associated to MS.

Methods

Cell lines and reagents The HEK293 cell line was obtained from American Type Culture Collection (LGC Standards, Barcelona, Spain), and the HT22 cell line was a kindly gift from Dr. Dave R. Schubert (Salk Institute, San Diego, CA, USA). All cell lines were maintained in exponential growth in DMEM supplemented with 10 % heat-inactivated foetal calf serum plus 0.5 %v/v penicillin/streptomycin. Rosiglitazone and HU-331 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Phytocannabinoids were isolated as described previously (Appendino et al. 2008; Tubaro et al. 2010). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless indicated. The synthesis and structure of VCE-003 is described in supplementary information.

PPARγ binding assay and transcriptional activity PPARγ binding activity was determined by using PolarScreen™ PPAR Competitor Assay kit (Life Technologies), according to the manufacturer's specifications. Relative affinity for PPARγ (percentage of polarization) is plotted against the concentration of the test compound. Concentration of the test compounds resulting in a half maximal shifts in polarization value determines the EC_{50} of each compound. To determine the effects on PPARγ transcriptional activity HEK293 or HT22 cells were transiently co-transfected with the expression vector GAL4-PPARγ and the luciferase reporter vector GAL4-luc using Roti©-Fect (Carl Roth, Karlsruhe, Germany) following the manufacturer's instructions. Twenty-four hours after transfection the cells were treated for 6 h with the indicated compounds and luciferase activity was determined in the cell lysates by using a luciferase assay kit (Promega, Madison, WI, USA). Specific transactivation was expressed as fold induction over the control (untreated cells).

Binding studies The binding assays were performed following a procedure previously described (Cumella et al. 2012). Membranes from human CB_1 or CB_2 receptor-transfected cells (RBHCB1M400UA and RBXCB2M400UA, respectively) were purchased to Perkin-Elmer Life and Analytical Sciences (Boston, MA). The protein concentration for the CB_1 and CB_2 receptor membranes was 8.4 mg/ml and 3.6 mg/ml respectively. The commercial membranes were diluted (1:20) with the binding buffer (50 mM TrisCl, 5 mM $MgCl_2.H_2O$, 2.5 mM EDTA, 0.5 mg/mL BSA and pH=7.4 for CB_1 binding buffer; 50 mM TrisCl, 5 mM $MgCl_2.H_2O$, 2.5 mM EGTA, 1 mg/mL BSA and pH=7.5 for CB_2 binding buffer). The radioligand was [^{3}H]-CP55940 (144 Ci/mmol; PerkinElmer) used at a concentration of 0.14 nM, and the final volume was 200 μL for CB_1binding and 600 μL for CB_2 binding. 96-Wells plates and the tubes necessary for the experiment were previously siliconized with Sigmacote (Sigma). Membranes were resuspended in the corresponding buffer and were incubated with the radioligand and each compound (10^{-4}–10^{-11} M) for 90 min at 30 °C. Non-specific binding was determined with 10 μM WIN55212-2 and 100 % binding of the radioligand to the membrane was determined by its incubation with membrane and without any compound. Filtration was performed by a Harvester® filtermate (Perkin-Elmer) with Filtermat A GF/C filters pretreated with polyethylenimine 0.05 %. After filtering, the filter was washed nine times with binding buffer, dried and a melt-on scintillation sheet (Meltilex™A, Perkin Elmer) was melted onto it. Then, radioactivity was quantified by a liquid scintillation spectrophotometer (WallacMicroBetaTrilux, Perkin-Elmer). Competition binding data were analyzed by using GraphPad Prism® version 5.01 (GraphPad Software Inc., San Diego, CA, USA) and *Ki* values are expressed as mean±SEM of at least three experiments performed in triplicate for each point.

Primary neuronal cell cultures Cortical neuronal cultures were prepared from embryos (E17-E18) of mice as previously described (Rose et al. 1990). Then, 1.25×10^5 cells were seeded on 15μg/ml poly-D-lysine-coated 24 well culture plates for 3 h. After this, medium was replaced to remove non-adherent cells, and neurobasal medium was added, containing antibiotics, 2 mM Glutamax and B-27 supplement (Life Technologies). After 3 days in vitro, non-neuronal cell division was halted by exposure to 10 μM cytosine-d-arabinofuranoside. Cells were grown in a humidified environment containing 5 % CO_2 at 37 °C for 9 days.

Cytotoxicity assays Neuronal death was quantified by measurement of lactate dehydrogenase (LDH) release from damaged neurons into the bathing medium. Cells were exposed to A23187 (0.5μM) and 24 h later supernatants were collected and analysed. Background of LDH levels was determined in paralleled cultures subjected to sham washes and subtracted from experimental values. Percentage of cell death in experimental conditions was referred to the value of A23187 alone (set as 100 % of cytotoxicity). Each experimental condition

was compared to this value to calculate relative cell death. HT22 cell viability was determined by the MTT assay. Briefly, cultured HT22 cells were pre-incubated for 1 h, as indicated, and then treated with 15 mM glutamate in the presence or absence of both CBG and VCE-003 during 24 h. Control cells were set as 100 % and data were referred to that value.

Cytokine and PGE_2 release measurement in primary microglia cells Rat microglial cells were purified from forebrains and cultured as previously described (Molina-Holgado et al. 2001). Cells were seeded on poly-D-lysine-coated plaques at a density of 50.000 cells/cm^2 and maintained for 3 days in DMEM containing 5 % horse serum. Then, the cells were incubated for 18 h with 0.5μg/ml LPS, in the presence of different concentrations of CBG or VCE-003, and supernatants were collected. Supernatants were spun down at 2,000 rpm for 10 min, 4 °C, and assayed by Bio-PlexPro™ Rat Cytokine assay (Bio-Rad) for the detection of IL-1 β, IL-6, TNFα, and MIP-1α, following the manufacturer's instructions. Bio-Plex™ 200 System was used for data acquirement, and Bio PlexManager™ software (Bio-Rad) was used for data analysis. PGE_2in microglia was analysed as previously described (de Oliveira et al. 2012) and quantified by using the prostaglandin E_2 EIA Kit (Cayman chemicals) following provider recommendations.

Animal and Theiler's virus inoculation and clinical evaluation TMEV-induced demyelinating disease (TMEV-IDD) in SJL/J mice was performed as previously described (Mestre et al. 2009). Sixty days after TMEV infection, mice were treated daily for 14 consecutive days with VCE-003 (5 mg/kgi.p.) or appropriate vehicle (5 % BSA and 0.2 % DMSO in phosphate-buffered saline). Handling of animals was performed in compliance with the guidelines of animal care set by the European Union guidelines 86/609/EEC, and approved by the local Animal Care and Ethics Committee of the CSIC. General health conditions (weight and clinical score) and motor function of animals were periodically evaluated, from day 60 until day 75 post-infection. Clinical scores after TMEV infection were assigned based on a scale of 0–5: score 1, mice show waddling gait; score 2, mice show more severe waddling gait; score 3, mice had a loss of righting ability associated with spastic hind limbs; score 4, mice had paralysis of hind limbs; and score 5, mice were moribund. The screening for locomotor activity (LMA) was performed using an activity monitor system coupled to a Digiscan Analyser (Omnitech Electronics, Columbus, OH, USA). Data for the following variables of LMA for a session of 10 min were collected: horizontal activity, as the total number of beam interruptions of horizontal area sensors, and vertical activity, as total number of beam interruptions in the vertical sensor. Another set of experiments, using the same protocol schedule of VCE-003 treatment, was performed, but administering doses of 1 mg/kg or 10 mg/kg.

Immunohistochemistry (IHC) Mice were transcardially perfused with PBS. Spinal cords were fixed in 4 % paraformaldehyde in 0.1 M PBS, washed in 0.1 M PBS, cryoprotected with a 7 %, 15 % and later 30 % solution of sucrose in 0.1 M PBS and frozen at −80 °C until used. Free-floating brain and spinal cord sections (30 μm thick) were processed as described previously (Mestre et al. 2009). For IHC, a primary rat anti-mouse VCAM-1 antibody (BD Biosciences, ref: 550547) was used to detect VCAM-1 expression on spinal cord sections. For immunofluorescence, microglia cells were stained with a rabbit anti-mouse Iba-1 antibody (Wako Chemical Pure Industry, Osaka, Japan, ref: 019–01974) on spinal cord sections. Six spinal cord sections per animal from at least 4 animals per group were analysed and two microphotographs per section were taken. Quantification of staining was performed using the Image J software (NIH, Bethesda, MD, USA).

qRT-PCR arrays Total RNA was isolated from brain and spinal cord tissue from control and treated animals, as described above. One μg of RNA was retrotranscribed using the iScript™cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and the cDNA was analysed by real-time PCR, using the qPCR SYBR Green PCR Kit (Promega). The expression profile of 84 key genes involved in multiple sclerosis (supplemental information) was studied by real-time PCR using the mouse multiple sclerosis RT^2Profiler™ PCR Array (Qiagen, Hilden, Germany), following manufacturer's instructions. Brain and spinal cord sections from at least 4 animals per group were analyzed.

Statistical analysis Every assay was performed in duplicate and at least three independent experiments were performed. In every assay performed, the mean and the standard deviation were calculated and plotted to visualize differentiates between the average values of the experimental populations. Sample population means were compared against control population means in an unpaired two-tailed Student's *t* test. The p value obtained from the Student's *t* test analysis marks the probability of rejecting the null hypothesis, that is, the events are independent, and they may be: $p \leq 0.05$, significant (represented as *), $p \leq 0.01$, very significant (represented as **) and $p \leq 0.005$, highly significant (represented as ***).

Results

Oxidation to quinols increases the PPARγ binding activity of cannabinoids Although the ability of several cannabinoids to bind to and modulate PPARγ transactivation has been

previously investigated (O'Sullivan 2007), the effects of CBG on PPARγ activity have not been investigated yet. Thus, we first wanted to investigate whether CBG was able to bind to PPARγ, and compare its binding capacity to other cannabinoids such as Δ^9-THC, CBC, CBD and HU-331, a quinol derivative of CBD. Using a PPARγ competitor-binding assay, we found that all cannabinoids tested were able to bind to PPARγ. CBD and Δ^9-THC, two well-known PPARγ ligands, showed an EC_{50} of 20.1 μM and 21.2 μM, respectively. CBC bound to the nuclear receptor with lower affinity EC50 >25 μM, and CBG was the most potent PPARγ agonist, showing an EC_{50} of 12.7 μM. Interestingly, HU-331 showed an EC_{50} of 5μM, thus presenting four times higher binding affinity than its parent molecule CBD (Fig. 1). This finding provided a rationale to investigate the biological translation of the resorcinyl-to-quinone oxidation also in CBG, the most potent PPARγ agonist within the phytocannabinoids we had screened. The quinol analogue of CBG (VCE-003) (Fig. 1b) was prepared by base-accelerated atmospheric oxidation of CBG (Fig. 1c), and showed a significantly enhanced binding affinity for PPARγ (EC50=2.2 μM) compared to CBG (Fig. 2a).

VCE-003 activates PPARγ transcriptional activity Next, we analysed the ability of CBG and VCE-003 to activate PPARγ transcriptional activity. For this purpose, HEK293 cells were transfected with a GAL4-PPARγ expression plasmid plus a GAL4-luc reporter plasmid. Our results showed that VCE-003 induced PPARγ transactivation in a concentration dependent manner and it was more potent than CBG, which transactivated PPARγ only at 25 μM (Fig. 2b). To further analyse the effects of CBG and VCE-003 at this receptor, we studied the behaviour of these compounds in the presence of rosiglitazone (RSZ), a very potent activator of PPARγ (Lehmann et al. 1995). To achieve this, GAL4-PPARγ/GAL4-luc transfected HEK293 cells were pre-incubated with increasing concentrations of CBG or VCE-003, and then treated with 1μM RSZ. Both CBG and VCE-003 were able to decrease the RSZ-induced PPARγ transactivation in a dose dependent manner (Fig. 2c), thus suggesting that CBG, VCE-003 and RSZ may bind to the same binding site on PPARγ. However, we found that VCE-003 and CBG are less adipogenic than RSZ (supplemental information).

CBG and VCE-003 show anti-inflammatory and neuroprotective effects To study the impact of CBG and VCE-003 in microglia response to LPS, we analysed their effects on the secretion of IL-1β, IL-6, TNF-α, the chemokine, MIP-1α and prostaglandin E_2 (PGE_2) by primary microglia. After

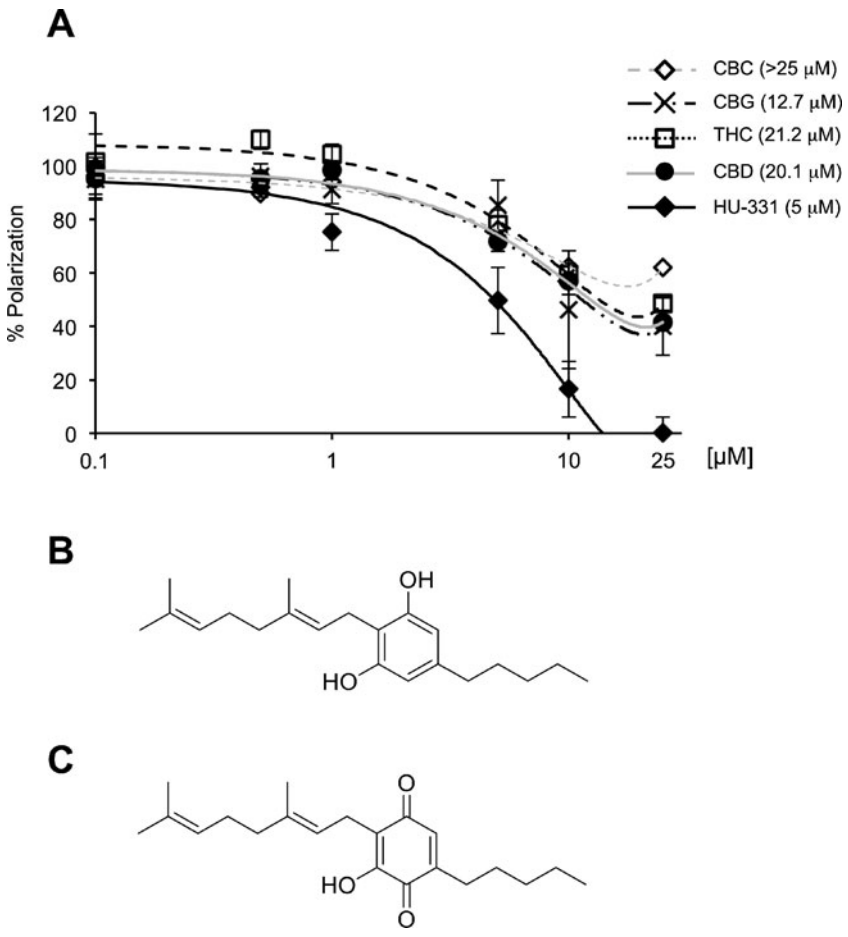

Fig. 1 Cannabinoids bind to PPARγ (**a**). Relative PPARγ affinity of the phytocannabinoids Cannabichromene (CBC), Cannabigerol (CBG), Tetrahydrocannabinol (THC), Cannabidiol (CBD) and HU-331 (Cannabidiol-quinone) was measured by using a ligand competition assay. The indicated concentrations were tested in three independent experiments, and results were plotted. Mean ± S.D. are shown for each concentration, and the polynomial trend on a logarithmic range was obtained and used to calculate the EC_{50} values (shown on the right). Chemical structures of CBG (**b**) and VCE-003 (**c**). Skeletal formulas representing the molecular structure of CBG and VCE-003 are shown

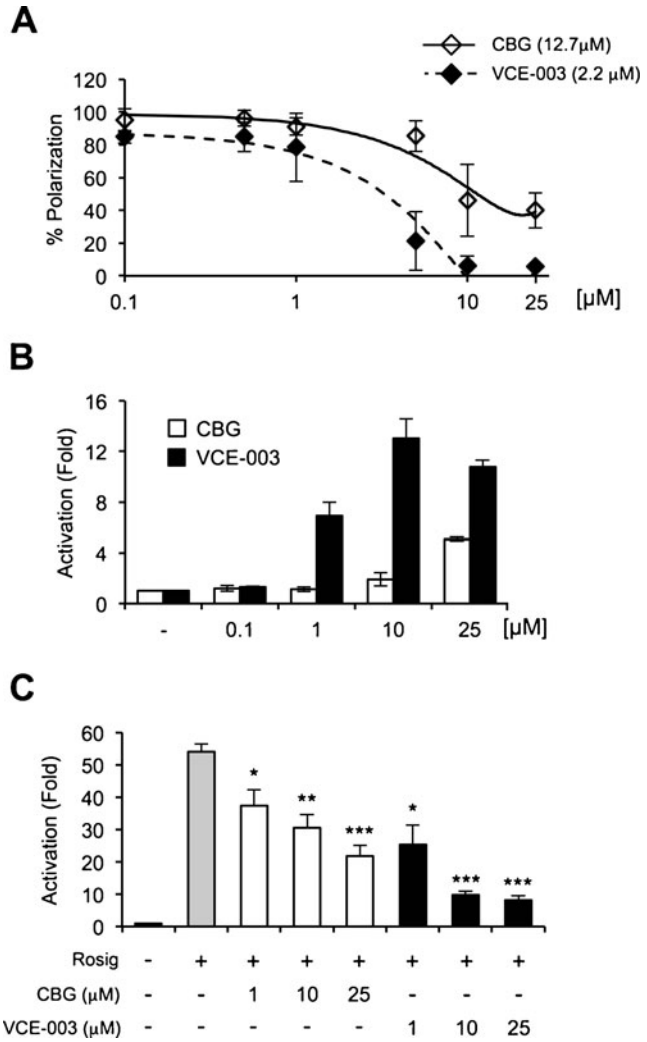

Fig. 2 VCE-003 is a partial PPARγ agonist. **a** Relative PPARγ binding affinity of CBG and VCE-003 was measured. The indicated concentrations were tested and results were plotted to obtain the polynomial trend on a logarithmic range, and to calculate the EC_{50} values (shown within the graphs). **b** Effect of CBG and VCE-003 on PPARγ transcriptional activity. HEK293 cells were co-transfected with GAL4- PPARγ and GAL4-luc. Cells were incubated overnight in the presence of CBG or VCE-003 at the indicated concentrations, and protein lysates were prepared and analysed for luciferase activity. Fold activation level was calculated, taking the control sample as reference. Data are expressed as mean ± S.D. of at least three independent experiments. (**c**) Effect of CBG and VCE-003 on PPARγ transcriptional activity in the presence of Rosiglitazone. HEK-293 cells were co-transfected with GAL4-PPARγ and GAL4-luc. Cells were pre-incubated for 1 h with the indicated doses of CBG or VCE-003, and then incubated for 6 h with 1 μM Rosiglitazone. Protein lysates were prepared and analysed for luciferase activity. Data are expressed as mean ± S.D. of at least three independent experiments. Three independent experiments were performed, and data are presented as mean ± S.D. *$p<0.05$, **$p<0.01$ and ***$p<0.005$ in an unpaired two-tailed Student's *t* test

LPS stimulation, microglial cells became fully activated and both CBG and VCE-003 inhibited the release of the above pro-inflammatory mediators to a different extent (Fig. 3). CBG strongly inhibited the synthesis of IL-1β (Fig. 3a), IL-6 (Fig. 3b) and PGE_2 (Fig. 3e) in a dose-dependent manner, but only inhibited partially the expression of TNF-α (Fig. 3c) and MIP-1α (Fig. 3d) at the highest concentration used (25 μM). Interestingly, VCE-003 outperformed CBG in terms of capacity to inhibit the release of cytokines even at doses of 1 μM in the case of IL-1β and IL-6 (Fig. 3a, b) and at 10 μM in the case of TNF-α or MIP-1α (Fig. 3c, d). However, VCE-003 was as efficient as CBG in inhibiting the release of PGE_2 (Fig. 3e).

Evidence suggests that neuroinflammation contributes to the development of neurodegenerative diseases (Glass et al. 2010). Since both CBG and VCE-003 present a strong anti-inflammatory profile, we decided to explore their neuroprotective potential. To assess this, we used HT22 hippocampal cells lacking the ionotrophic glutamate receptors. In this way, excitotoxicity does not contribute to the cell death in response to glutamate, making this cell line a useful model of glutamate-induced oxidative stress (Li et al. 1997). HT22 resting cells showed a 100 % viability that was reduced to 35 % after glutamate treatment. However, when the cells were pre-treated with CBG or VCE-003 before addition of glutamate, cell viability was rescued on a dose-dependent manner with VCE-003, showing higher efficacy in protecting cells from glutamate-induced cytotoxicity (Fig. 4a). We also found that RSZ (Fig. 4b), CBG and VCE-003 (Fig. 4c) also transactivated PPARγ in HT22 cells, although RSZ failed to protect HT22 cells from glutamate-induced cell death (Fig. 4a), suggesting that the protective effect of CBG and VCE-003 are not mediated by PPARγ. Moreover, the neuroprotective activity of VCE-003 and CBG was not abrogated by the presence of the PPARγ antagonist T0070907 (Supplemental information). These data indicate that these two cannabinoids protect neuronal cells from glutamate-induced cytotoxicity through PPARγ-independent mechanisms. To further demonstrate the neuroprotective effects of VCE-003, we studied its activity on mouse primary neuronal cells. Primary neuronal cultures were exposed to the calcium ionophore A23187 and lactate dehydrogenase (LDH) secretion was quantified as a cell death read-out. Exposure to A23187 induced the LDH release to a level that we set as the highest neuronal death reached (100 %). When the cells were pre-treated with 1μM or 10μM VCE-003, calcium-induced cell death was diminished to 50 %, thus showing that VCE-003 also exerted a strong neuroprotective activity in primary neurons (Fig. 5d). We also found that RSZ did not protect from A23187-induced cytotoxicity in primary neurons (data not shown).

VCE-003 binds CB_2 receptor It has been shown recently that CBG may bind to CB_1 and CB_2 receptors and behaves as a CB_1 receptor competitive antagonist in vitro (Cascio et al. 2010). Thus, we studied the binding affinity of VCE-003 and

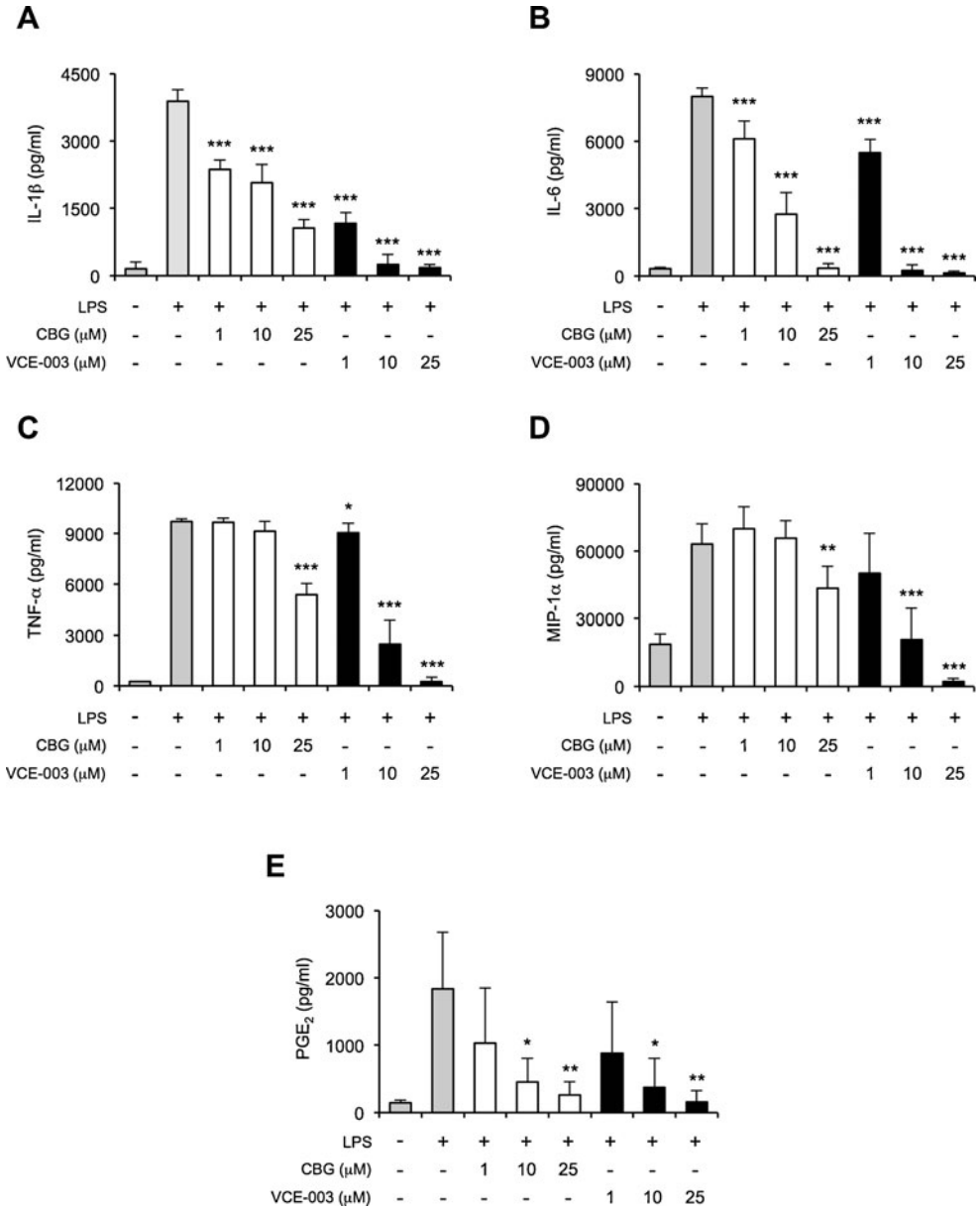

Fig. 3 Effects of CBG and VCE-003 on the secretion of pro-inflammatory molecules by primary microglia. Rat primary microglial cells were incubated with LPS for 18 h, in the presence of the indicated concentrations of CBG or VCE-003, and supernatants were collected and assayed by using a BioPlex Pro Multiple Cytokine assay for the detection of IL-1β (**a**), IL-6 (**b**), TNF-α (**c**), MIP-1α (**d**). Supernatants were also analysed by ELISA to quantify PGE_2 (**e**). Results are shown as mean ± S.D. from at least three independent experiments, and expressed as the concentration in picograms per millilitre (pg/ml). Asterisks indicate significant differences for each treatment compared to the LPS positive control. *$p<0.05$, **$p<0.01$ and ***$p<0.005$ in an unpaired two-tailed Student's *t* test

CBG to CB_1 and CB_2 receptors using membranes from human CB_1 or CB_2 receptor-transfected cells. We found that in contrast to WIN55,212-2 neither CBG nor VCE-003 bound to hCB_1receptor. Interestingly VCE-003 showed ability to displace [^{3}H]CP55,940 from specific binding sites in HEK-293-hCB_2 cell membranes and it was almost 10 fold more potent than CBG in the binding to hCB_2 receptors, although its *Ki* value was in the low micromolar range (Table 1). However, VCE-003 and CBG were almost equally potent to protect HT22 cells from glutamate-induced cytotoxicity (Fig. 4a).

VCE-003 ameliorates neurological deterioration in TMEV-IDD Next, we studied the effect of VCE-003 in a well-defined model of murine primary progresive multiple sclerosis. Hence, 60 days after TMEV infection the mice were treated with VCE-003 (5 mg/kg) or an equivalent amount of vehicle, during 14 days. Then, mice were examined and clinical scores were assigned to all of them (Fig. 5a). Sham mice (inoculated with vehicle) exhibited good health conditions and all were assigned a clinical score of 0. As expected, the mice infected with TMEV receiving vehicle showed clinical score increased to an average of 3.5, meaning that almost every animal presented spastic hind limbs (score 3) or paralysis of such limbs (score 4) at 75 days post-infection. However, TMEV-infected mice treated with VCE-003, showed the clinical score reduced to an average 2.2, meaning that most of the animals presented waddling gait (score 2) but no overall signs of spastic or paralyzed limbs. Furthermore, motor activity was assessed in an activity cage, and horizontal activity (Fig. 5b) and vertical activity

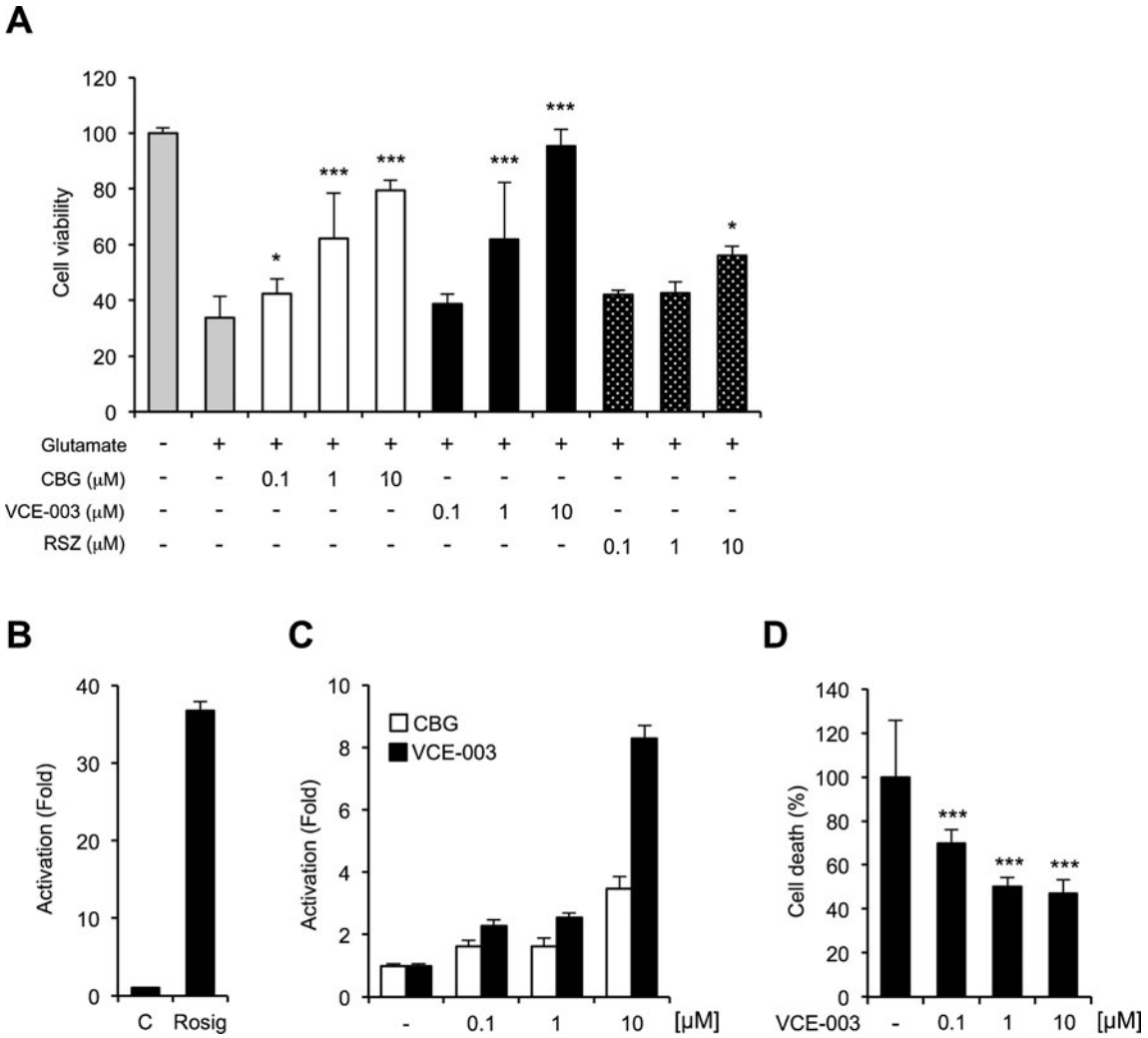

Fig. 4 Neuroprotective activity of CBG and VCE-003. HT22 mouse hippocampal cells were cultured and incubated with 15 mM glutamate to induce oxidative toxicity. **a** Neuroprotective capacity of CBG, VCE-003 and Rosiglitazone was tested by MTT. Cells were incubated with the indicated doses of the compounds, and cell viability was quantified. Results are shown as mean ± S.D. from at least three independent experiments, and expressed as percentage of cell viability against the negative control (100 %). HT22 neuronal cells were transfected with the constructs GAL4-PPARγ and GAL4-luc and the cells were incubated overnight with 1 μM rosiglitazone (**b**) or with CBG or VCE-003 (**c**), and protein lysates were prepared and analysed for luciferase activity. Fold activation was calculated, taking the control sample as reference. Data are expressed as mean ± S.D. of at least 3 independent experiments. **d** Primary neurons from forebrains were incubated with the indicated doses of VCE-003 and excitotoxicity was induced with 0.5 μM A23187 during 24 h. Cell death was calculated as percentage taking A23187 in the absence of VCE-003 as reference (100 % death). Asterisks indicate significant differences for each treatment compared to A23187 alone. *$p<0.05$, **$p<0.01$ and ***$p<0.005$ in an unpaired two-tailed Student's *t* test

(Fig. 5c) were analysed. Sham animals exhibited normal activity levels; 1,443 bean interruptions (average) for horizontal activity and 147 times (average) standing up for vertical activity. As expected in the chronic phase of the model, TMEV infection dramatically reduced both horizontal and vertical activities to very low levels. Strikingly, treatment with VCE-003 completely abrogated the decreased motor activity, recovering motor activities to normal levels. These data indicate that VCE-003 treatment recovers motor activity function in TMEV-infected mice. To perform dose studies, in a set of experiments we administered 1 mg/kg or 10 mg/kg of VCE-003 to TMEV-infected mice following the same schedule of treatment as the used above. The dose of 1 mg/kg resulted ineffective and did not modify TMEV-induced motor deficits, but mice receiving 10 mg/Kg of VCE-003 show the same profile of response than that observed in mice treated with the dose of 5 mg/kg.

During the course of MS, demyelination and axonal damage accounts for the impairment of motor activity, making oligodendrocyte-mediated remyelination a key event for recovery of motor activity (Franklin and Ffrench-Constant 2008). Thus, we studied whether oligodendrocytes could be a target for VCE-003 actions. At a first approach we evaluated the effects of VCE-003 and CBG on oligodendrocyte

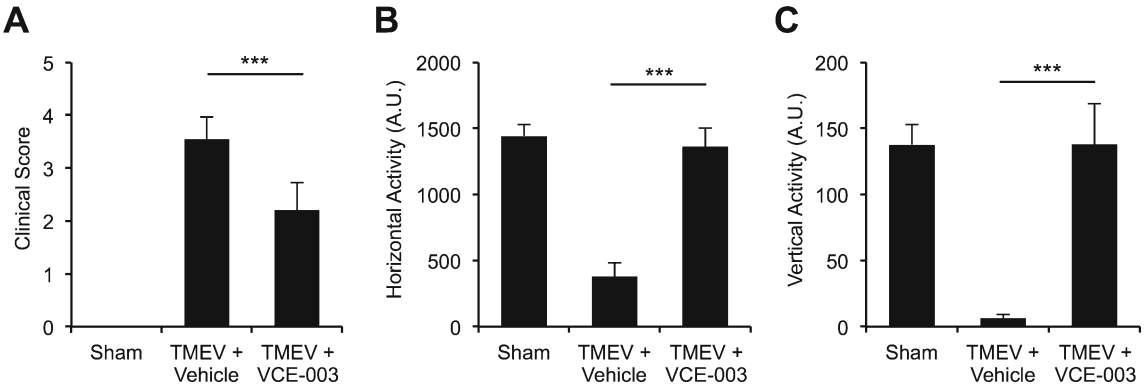

Fig. 5 Effects of VCE-003 in motor function of TMEV-infected mice. SJL/J mice were inoculated intra-cranially with TMEV or Sham-operated. After 60 days, mice were treated intraperitoneally with VCE-003 or a similar amount of vehicle for 15 days (5 mg/kg, once a day). The last day of treatment, mice were examined and clinical scores were assigned (**a**). Mice were also assessed for motor activity in an activity cage, where horizontal (**b**) and vertical (**c**) activities were studied. Results represent means ± SEM. $n=9$ for Sham and TMEV + vehicle, and $n=12$ for TMEV + VCE-003. ***$p<0.005$ in an unpaired two-tailed Student's t test

differentiation. Primary oligodendrocyte precursors (OPCs) were cultured in the presence of 10μM CBG or 10μM VCE-003 and the expression of mature oligodendrocyte markers, myelin binding protein (MBP) and myelin associated glycoprotein (MAG), were analysed. Neither CBG nor VCE-003 promoted oligodendrocyte differentiation as measured by changes in the expression of MBP or MAG (Supplemental information).

VCE-003 reduces microglia reactivity and expression of adhesion molecules in vivo Microglial/macrophage cell activation plays a pivotal role in TMEV-IDD (Kim et al. 2005). Thus, we analysed the effect of VCE-003 on the expression of Iba-1, a marker of microglia, in the spinal cord of TMEV-infected mice. Fluorescent staining revealed that TMEV-infection increased the intensity of fluorescence of Iba-1^+ in the spinal cord showing the cells an activation state (Fig. 6a). Microglia activation was greatly prevented by VCE-003 treatment, which led to a significant reduction of the intensity of microglia activation in the spinal cord to levels close to those quantified in the Sham group (Fig. 6a, right panel). In TMEV-IDD the blockade of adhesion molecules impaired the development of disease (Inoue et al. 1997). Thus, we analysed whether VCE-003 could be affecting the expression of the adhesion molecule VCAM-1. TMEV infection strongly induced the expression of VCAM-1, which was clearly inhibited in VCE-003-treated mice (Fig. 6b). Collectively, these data indicate that VCE-003 treatment induces a down-regulation of VCAM-1 as well as a reduction in microglia activity in the spinal cord of TMEV-infected mice.

Table 1 Binding affinity of VCE-003 and CBG to hCB_1 and hCB_2 receptors, using WIN55,212-2 as a positive control

Compound	hCB_1-*Ki* (nM)[a]	hCB_2-*Ki* (nM)[a]
VCE-003	>40,000	1,759 ± 649
CBG	>40,000	16,075 ± 4,835
WIN55,212-2	46 ± 9	4 ± 0.2

[a] Values obtained from competition studies using [^{3}H]CP55,940 as radioligand for hCB_1 and hCB_2 receptors. They are expressed as the mean ± SEM of at least three different experiments each performed in triplicates

VCE-003 and the expression of MS-related genes in the brain and spinal cord of TMEV-infected mice In order to understand the molecular mechanisms underlying the beneficial effects of VCE-003 in TMEV-IDD, we analysed the impact of VCE-003 on gene expression pattern during TMEV infection. mRNA was isolated from the brain and spinal cord of TMEV-infected mice subjected or not to VCE-003 treatment, and the expression of 83 genes involved in MS physiopathology was analysed by qRT-PCR. Fold up- or down-regulation was calculated for each gene in the spinal cord and in the brain (supplementary information). We found that the expression of many genes involved in myelination, cellular stress and apoptosis were not affected in TMEV-infected mice treated with VCE-003. However, the expression of several genes involved in the inflammatory response, cell adhesion and T cell activation was modulated by VCE-003 suggesting its interest as immunomodulatory agent. It is worth noting that, although the expression pattern of those genes in the spinal cord and in the brain shared some similarity, the impact of VCE-003 was slightly different on those tissues. As shown in Fig. 7, the most striking result was that obtained for the chemokine Ccl12, a chemotactic agent for monocytes and lymphocytes, which was strongly upregulated after TMEV-infection in both spinal cord (Fig. 7a) and brain (Fig. 7b). Treatment with VCE-003

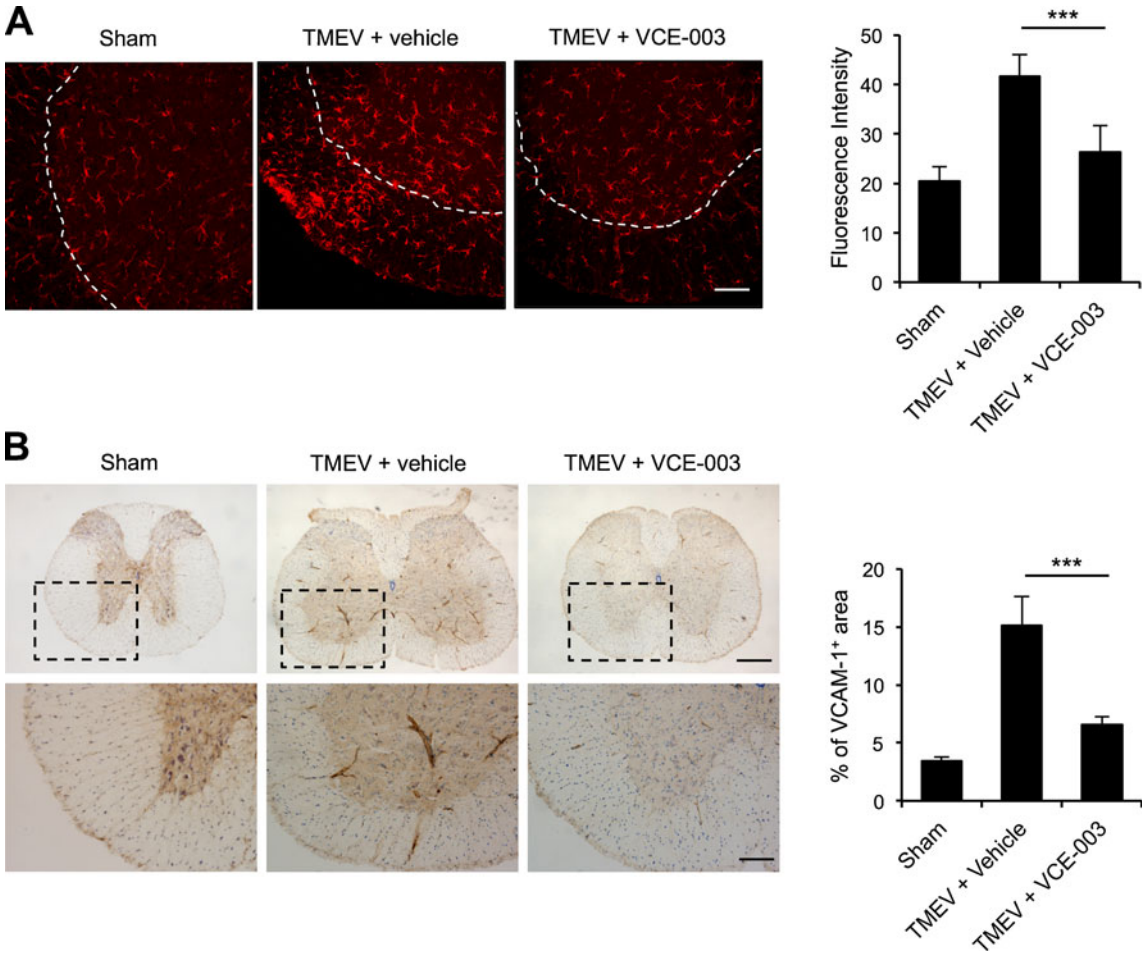

Fig. 6 Effect of VCE-003 on microglia activation and expression of adhesion molecules on TMEV-infected mice. Spinal cord and forebrain tissue sections were taken from control, infected, and infected and treated mice. **a** Sections were stained with an anti-Iba-1 mAb and analysed by confocal microscopy. Representative images from triplicate experiments are shown. Fluorescence intensity was measured by using Image J software and plotted to show quantification of the analysed images, mean ± S.D. $^{**}p<0.01$ in an unpaired two-tailed Student's *t* test. Scale bar=100 μm. **b** Sections were stained with an anti-VCAM-1 mAb and images were acquired by using light microscopy. Representative images from triplicate experiments are shown (top row, scale bar=600 μm) and magnified image sections (bottom row, scale bar=150 μm). VCAM-1-stained area (VCAM-1^{+}) intensity was measured by using Image J software and plotted to show quantification of the analysed images, mean ± S.D. $^{**}p<0.01$ and $^{***}p<0.005$ in an unpaired two-tailed Student's *t* test

notably decreased Ccl12 over-expression in both tissues. Moreover, other chemokines and chemokine receptor genes upregulated during TMEV-IDD were modulated by VCE-003. For instance, Ccr1 and Ccr5 chemokine receptors, which are involved in leukocyte recruitment to inflammatory sites, were upregulated in both spinal cord and brain of TMEV-infected mice. VCE-003 treatment significantly reduced Ccr1 expression level in both tissues, but Ccr5 expression was only reduced in the brain. In addition, the T cell chemotactic Cxcl11 and its high affinity receptor gene Cxcr3 were upregulated after TMEV infection, and VCE-003 was able to significantly reduce their expression in the spinal cord, but not in the brain. Furthermore, expression of the monocyte/macrophage chemokine Ccl7 gene was also upregulated in the brain of infected mice, and treatment with VCE-003 strongly reduced its expression. By contrast, the T cell chemoattractant Cxcl9 was upregulated in both areas in TMEV-infected mice but VCE-003 had no effect on it, thus showing that VCE-003 modulated the chemokine expression pattern in a selective way. Expression of CD4 and CD28 was also analysed. VCE-003 had no effect on the expression of both genes in the spinal cord (Fig. 7a), but it was able to reduce their expression in the brain of TMEV-infected mice (Fig. 7b). The expression of the integrin fibronectin (Fn1) and the intercellular adhesion molecule-1 (ICAM-1) were also analysed. In the spinal cord (Fig. 7a) both Fn1 and ICAM-1 were upregulated by viral infection, and VCE-003 significantly reduced the expression level of both molecules. In the brain we detected a slight increase of ICAM-1 expression after TMEV infection that was counteracted by VCE-003 (Fig. 7b). It is worth noting that the expression of the T cell transcription factor Foxp3 was affected by both TMEV

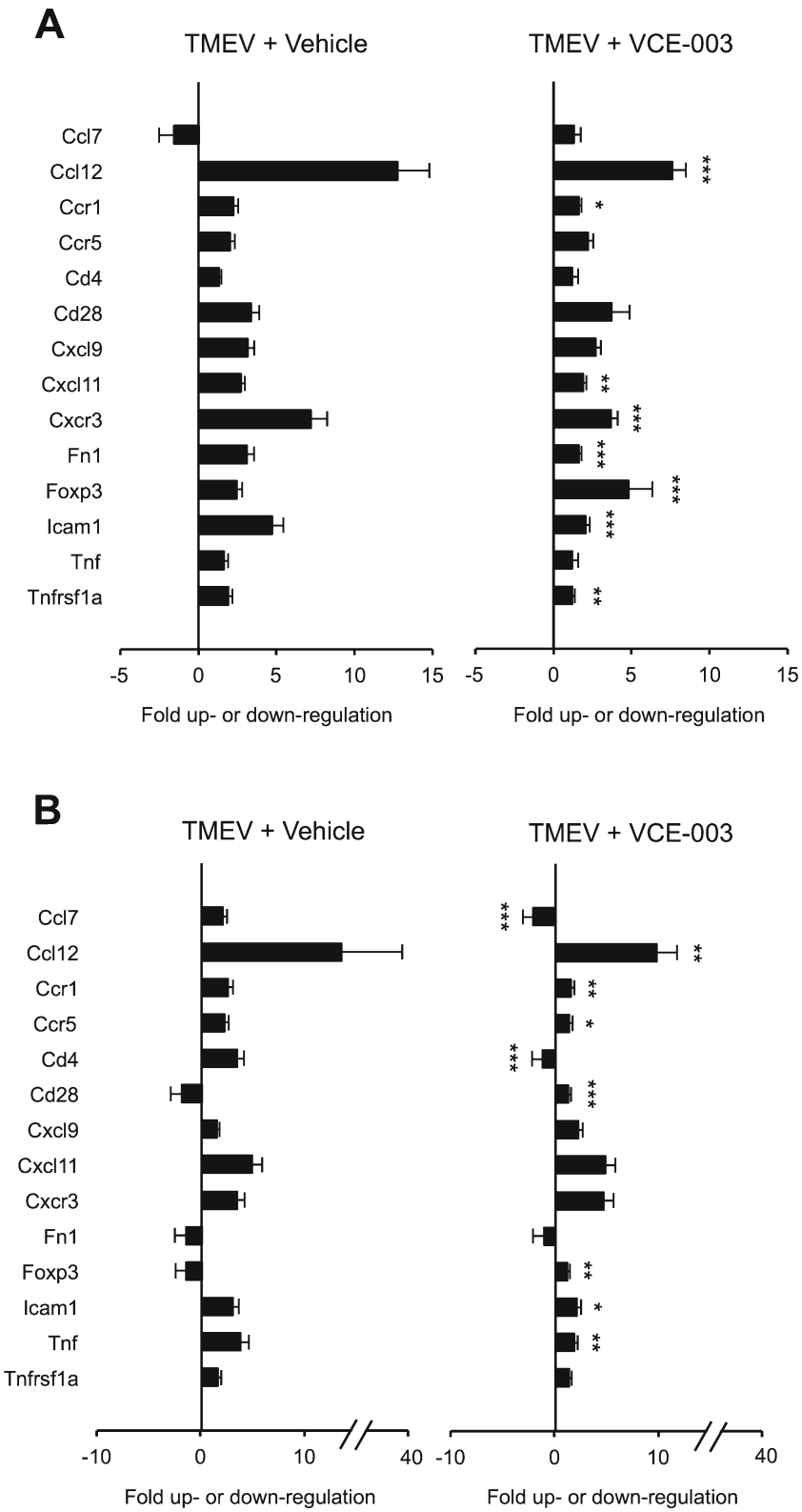

Fig. 7 VCE-003 modulates gene expression in the brain and spinal cord in TMEV-infected mice. Spinal cord and forebrain tissues were taken from control, infected, and infected and treated mice and total RNA was isolated. 1 μg of RNA was retrotranscribed and the resulting cDNA was analysed in a mouse multiple sclerosis PCR array. Gene expression levels are expressed as fold up- or down-regulation during sickness (TMEV + vehicle) and treatment (TMEV + VCE-003) compared against sham-operated animals. Five housekeeping genes contained on the experimental system were used to standardize the mRNA expression levels in every sample. Those genes showing a relevant change in the expression pattern are shown for spinal cord (**a**) and brain (**b**). Statistical significance in the change of the expression levels is noted with asterisks, where*$p<0.05$, **$p<0.01$ and *** $p<0.005$ in an unpaired two-tailed Student's *t* test

infection and VCE-003 treatment. Interestingly, VCE-003 moderately upregulated Foxp3 expression levels in the brain of infected animals, and dramatically up-regulated Foxp3 mRNA levels in the spinal cord.

Discussion

Some cannabinoids have been shown to mediate their anti-inflammatory effects through the activation of PPARγ

(O'Sullivan 2007). In this work we show that oxidation of the resorcinyl moiety of CBG to a quinol increases the binding affinity for PPARγ and its transcriptional activity. In LPS-stimulated microglia, VCE-003 dramatically inhibited the secretion of the major inflammatory mediators TNF-α, IL-1β, IL-6, MIP-1α and PGE_2. Although CBG significantly diminished the secretion of these inflammatory mediators, VCE-003 showed a much stronger inhibitory activity, suggesting that oxidation of the resorcinyl moiety enhances the potential anti-inflammatory of the parent compound. These findings support the view that PPARγ may play a role in the anti-inflammatory activity of phytocannabinoids, as previously suggested for CBD (Esposito et al. 2011). The safety and toxicological profile of cannabinoids combined with their lipophilic structure and good penetration across the blood–brain barrier to qualify for the development of drugs for neuroinflammatory diseases.

Another common feature of cannabinoids is their biological pleiotropism, and we show here that both CBG and VCE-003 were able to protect neurons from glutamate and calcium-induced citotoxicity via PPARγ-independent mechanism(s). Thus, VCE-003 seems to retain some of the biological activities mediated by CBG, like rescuing neurons from excitotoxic stimuli. It has been previously demonstrated that several molecules protect neurons from glutamate-induced toxicity by activation of antioxidant response (Li et al. 2009). In addition, it has been shown that activation of an antioxidant response protects primary neuronal cells from ionomycin-induced cell death (Lee et al. 2003). CBG and specially VCE-003 exhibited antioxidant activity in human neuroblastoma cells (data not shown), possibly accounting for their PPARγ-independent neuroprotective effect observed in both HT22 and primary neuronal cells. The mechanism(s) underlying the antioxidant action of VCE-003 is currently under investigation. Moreover, our results suggest that the antiinflammatory and neuroprotective activities of VCE-003 are also mediated through CB_1-independent mechanisms, perhaps involving the CB_2 receptor for which VCE-003 presents certain affinity. Although further experiments are required to determine whether VCE-003 is a CB_2 receptor agonist or antagonist we found that AM630, a CB_2 antagonist, did not affect the neuroprotective activity of VCE-003 in HT22 cells (Supplementary information).

Our observation that VCE-003 restores the motor function impairment in the TMEV murine model of MS suggests that the anti-inflammatory and neuroprotective effects that this molecule exerts in vitro translate into a recovery of TMEV symptomatology in the in vivo model. VCE-003 decreased the activation of microglia in the spinal cord of infected animals, a major step in the development of MS, especially in the progressive form of the disease (Kutzelnigg et al. 2005). The demyelinating disease triggered by TMEV infection shares many similarities with the primary progressive form of MS, particularly the microglia-driven acute inflammatory response (Tsunoda and Fujinami 2009), thus qualifying VCE-003 as a potent anti-inflammatory molecule in vivo.

Leukocyte infiltration is a key step in the establishment of the inflammatory response and the development of the demyelination process (McFarland and Martin 2007). Chemoattractants play a pivotal role in the migration of leukocytes and extravasation to inflammatory sites. Within them, chemokines are of particular interest due to their important role in human inflammatory disorders (Luster 1998). Previous studies showed high-level expression of several chemokines during TMEV-IDD (Murray et al. 2000), and we analysed the potential modulatory role of VCE-003 on chemokine expression. In the spinal cord VCE-003 down-regulated the increased expression of the chemokines Ccl12 and Cxcl11, which are chemoattractant for monocytes and lymphocytes, respectively (Cole et al. 1998; Sarafi et al. 1997), and the chemokine receptors Ccr1 and Cxcr3, which are respectively expressed in activated macrophages and lymphocytes (Gao et al. 1993; Qin et al. 1998). In the brain, VCE-003 showed a slightly different inhibitory profile, since it diminished the expression of Ccl12 and Ccr1, but did not alter the expression of Cxcl11 or Cxcr3. VCE-003 also down-regulated the expression of Ccl7, which interacts with multiple leukocyte receptors (Ben-Baruch et al. 1995), and the expression of the receptor Ccr5, which is expressed on many immune cells, including macrophages, T cells, dendritic cells and microglia (Samson et al. 1996). Thus, VCE-003 effectively reduces the expression of chemokines and might therefore prevent the presence of infiltrated leukocytes in the CNS.

In addition to chemokines, expression of adhesion molecules is also needed to induce leukocyte-endothelial cell adhesion and extravasation of immune cells during pathological processes (Elices et al. 1990). In our hands, TMEV infection resulted in the upregulation of the endothelial adhesion molecules ICAM-1 in the brain and the spinal cord and VCAM-1 in the spinal cord, both effects were prevented in mice treated with VCE-003. These observations are crucial for understanding the beneficial effects of VCE-003 in TMEV-IDD. ICAM-1 is the ligand for lymphocyte function-associated antigen that is mainly expressed on T and B cells (Evans et al. 2009) and VCAM-1 is an endothelial ligand for very late antigen-4 (VLA-4) that is expressed in activated and memory lymphocytes and in dendritic cells (Gonzalez-Amaro et al. 2005). In fact, the block of VLA-4 signalling using specific antibodies (Yednock et al. 1992) is a clinically validated therapeutic strategy in autoimmune and inflammatory disorders, approved for the treatment of MS in 2004 (Miller et al. 2003). In line with our results the administration of the synthetic cannabinoid WIN55212-2 to TMEV-infected mice suppressed ICAM-1 and VCAM-1, interfering with the progression of the demyelinating disease (Mestre et

al. 2009). Moreover, it has also been shown that activation of PPARγ suppresses pro-inflammatory adhesion molecules in human vascular endothelial cells (Wang et al. 2002).

Another important immunomodulatory marker, the transcription factor Foxp3, was not affected in the brain and spinal cord of infected animals but, interestingly, it was increased by VCE-003 in both areas of the CNS. Foxp3 is a specific marker for regulatory T cells (T_{Reg}) (Hori et al. 2003), which have a central role in protecting the organism from autoimmunity (Taams et al. 2002) and may be very important in controlling the inflammatory response in the CNS during human MS (Feger et al. 2007). In fact, depletion of T_{Reg} cells increased susceptibility to EAE in mice, while adoptive transfer of T_{Reg} cells reduced the incidence of the disease (Kohm et al. 2002). Our data, in agreement with recent results in TMEV-IDD (Arevalo-Martin et al. 2012) in which the synthetic CB_1 and CB_2 agonist WIN55212, 2 increases T_{Reg} cells, suggest that somehow VCE-003 may be modulating the pool of T_{Reg} lymphocytes in infected animals, which may be contributing to stopping the progression of the disease. Overall it is possible that VCE-003 may exert its antiinflammatory activity in vivo at two levels, namely an inhibition of proinflammatory cytokines released by activated resident cells (gliosis) and by preventing the infiltration of peripheral immune cells into the brain and the spinal cord.

Fibronectin is an extracellular matrix component synthesized and deposited on active MS lesions (Sobel and Mitchell 1989) that seems to play a critical role in the failure in the remyelination process. Accordingly with recent studies (Haist et al. 2012) fibronectin was upregulated in the spinal cord of TMEV-infected animals and we found that VCE-003 reduced its expression in a highly significant way. In an effort to find remyelinating therapies, differentiation of OPCs near MS plaques have been extensively studied without success (Franklin and Ffrench-Constant 2008). In our study, VCE-003 did not induce the differentiation of primary OPCs cultures, as myelin proteins resulted unmodified after its treatment. It would be possible that VCE-003 by interfering with the fibronectin network may contribute to the alleviation of symptomatology in TMEV-IDD facilitating remyelination, but this point requires further studies. The observation that natural and synthetic PPARγ agonists suppress the production of fibronectin and other components of the extracellular matrix supports that VCE-003 by activating PPARγ receptors can reduce this extracellular matrix protein (Hao et al. 2008).

In summary, we have demonstrated that resorcinol to hydroxyquinone transformation of CBG increased their binding capability to PPARγ receptors and enhanced its transcriptional activity. VCE-003 is a non-cytotoxic compound that inhibited the microglia-driven inflammatory response, protects neurons from toxic insults in vitro, and restores motor function impairment in a murine model of MS. Since phytocannabinoids such as CBD and CBG show excellent pharmacotoxicological profiles we believe that the CBG derivative, VCE-003 may qualify as a novel compound for the treatment of neuroinflammatory and neurodegenerative disorders whose management is currently elusive.

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Conflict of interest MLB, GA and EM have filed a PCT application "Cannabinoid quinone derivatives" (application number PCT-03494). All the other authors declare no conflict of interest.

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