RESEARCH PAPER

Cannabidiol protects an *in vitro* model of the blood–brain barrier from oxygen-glucose deprivation via PPARγ and 5-HT$_{1A}$ receptors

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BACKGROUND AND PURPOSE

In vivo and *in vitro* studies have demonstrated a protective effect of cannabidiol (CBD) in reducing infarct size in stroke models and against epithelial barrier damage in numerous disease models. We aimed to investigate whether CBD also affects blood–brain barrier (BBB) permeability following ischaemia.

EXPERIMENTAL APPROACH

Human brain microvascular endothelial cell (HBMEC) and human astrocyte co-cultures modelled the BBB. Ischaemia was modelled by oxygen–glucose deprivation (OGD) and permeability was measured by transepithelial electrical resistance.

KEY RESULTS

CBD (10 μM) prevented the increase in permeability caused by 4 h OGD. CBD was most effective when administered before the OGD, but protective effects were observed up to 2 h into reperfusion. This protective effect was inhibited by a PPARγ antagonist and partly reduced by a 5-HT$_{1A}$ receptor antagonist, but was unaffected by antagonists of cannabinoid CB$_1$ or CB$_2$ receptors, TRPV1 channels or adenosine A$_{2A}$ receptors. CBD also reduced cell damage, as measured by LDH release and by markers of cellular adhesion, such as the adhesion molecule VCAM-1. In HBMEC monocultures, CBD decreased VCAM-1 and increased VEGF levels, effects which were inhibited by PPARγ antagonism.

CONCLUSIONS AND IMPLICATIONS

These data suggest that preventing permeability changes at the BBB could represent an as yet unrecognized mechanism of CBD-induced neuroprotection in ischaemic stroke, a mechanism mediated by activation of PPARγ and 5-HT$_{1A}$ receptors.

Abbreviations

BBB, blood–brain barrier; CREB, cAMP response element binding protein; CBD, cannabidiol; CBF, cerebral blood flow; HA, human astrocyte; HBMEC, human brain microvascular endothelial cell; OGD, oxygen–glucose deprivation; TEER, transepithelial electrical resistance; VCAM-1, vascular cell adhesion molecule 1

Tables of Links

TARGETS		LIGANDS
Ion channels[a]		8-OH-DPAT
TRPV1 channels		AM251
GPCRs[b]		AM630
5-HT$_{1A}$ receptors		Capsazepine
Adenosine A$_{2A}$ receptors		CCL3
CB$_1$ receptors		CCL4
CB$_2$ receptors		GW9662
Nuclear hormone receptors[c]		Pioglitazone
PPARγ		SCH58261
		WAY100135

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

The blood–brain barrier (BBB) is formed by brain endothelial cells that line the cerebral microvasculature, the capillary basement membranes and the endfeet of astrocytes. Tight junctions restrict the paracellular pathway for diffusion of hydrophilic solutes, allowing the body to control which substances can gain access to the brain (Abbott *et al.*, 2006). Cerebral reperfusion following ischaemia initiates a cascade of events including inflammation, protease activation and oxidative and nitrosative stress, all of which increase BBB permeability (Lo *et al.*, 2003). Increased BBB permeability aggravates haemorrhagic transformation and vasogenic oedema, and uncontrolled cerebral oedema represents the leading cause of patient mortality within the first week following an ischaemic stroke (Hacke *et al.*, 1996).

Cannabidiol (CBD) is the second most abundant plant-derived cannabinoid. CBD has multiple pharmacological targets, behaving as an agonist of TRPV1 channels, PPARγ, the adenosine A$_{2A}$ and 5-HT$_{1A}$ receptors and antagonizing the novel endothelial receptor, GPR55, and μ and δ opioid receptors. CBD is a low-affinity ligand which can modulate cannabinoid CB$_1$ receptor activity (Laprairie *et al.*, 2015; see also Petitet *et al.*, 1998; McPartland *et al.*, 2015) and has weak antagonistic activity toward signalling mediated at CB$_2$ receptors by the cannabinoid ligands, CP 55,940 and WIN 55212-2 (Bisogno *et al.*, 2001; McPartland *et al.*, 2015). A CBD/THC combination (1:1 ratio, Sativex/Nabiximols, GW Pharmaceuticals, UK) is currently licensed internationally in more than 20 countries for the treatment of spasticity in multiple sclerosis, and a product containing only CBD (Epidiolex, GW Pharmaceuticals, UK) has entered an expanded access programme in children with intractable epilepsies. CBD has also received orphan designation status in treating newborn children with neonatal hypoxic-ischaemic encephalopathy.

The protective qualities of CBD in ischaemic stroke using rodent *in vivo* models have been shown in numerous studies. CBD reduces infarct size (when given either before or after middle cerebral artery occlusion) without the development of tolerance, increases cerebral blood flow (CBF), improves motor behaviour and increases survival (Hayakawa *et al.*, 2010). The mechanism of action includes the ability of CBD to ameliorate glutamate neurotoxicity, behave as an antioxidant and anti-inflammatory agent and to attenuate adhesion molecule expression, neutrophils and the transendothelial migration of monocytes. The protective effects of CBD *in vivo* are inhibited by 5-HT$_{1A}$ receptor antagonists, with no role for TRPV1 channels, CB$_1$ or CB$_2$ receptors (Hayakawa *et al.*, 2010). We recently conducted a meta-analysis to examine the effects of exogenous application of endocannabinoids, phytocannabinoids and synthetic cannabinoids on infarct volume, functional outcome and survival. In 21 studies, involving 188 animals, treatment with CBD showed a highly significant reduction in infarct volume ($P < 0.00001$), with a standardized mean difference of −1.20 (England *et al.*, 2015).

At the BBB, *in vivo* studies have shown that CBD decreased BBB permeability in a mouse model of multiple sclerosis (Mecha *et al.*, 2013) and reduced LPS-induced BBB disruption in mice (Fernandez-Ruiz *et al.*, 2013). CBD also modulated the permeability of several other epithelial barriers. For example, blood–retinal barrier permeability is increased in diabetic rats *in vivo* and this was prevented by regular treatment with CBD (El-Remessy *et al.*, 2006). CBD also decreased *in vitro* permeability in human coronary artery endothelial cells exposed to high glucose conditions (Rajesh *et al.*, 2007). In gut epithelial cells, CBD enhanced the speed of recovery of either chemical-induced or cytokine-induced permeability activity via activation of CB$_1$ receptors (Alhamoruni *et al.*, 2010; Alhamoruni *et al.*, 2012). A recent study from our group found that endocannabinoid-like molecules (oleoylethanolamide, palmitoylethanolamide and virodhamine) decreased the oxygen–glucose deprivation (OGD)-induced increase in permeability using a *in vitro* model of the human BBB, showing that endocannabinoids can affect BBB permeability in an ischaemic setting (Hind *et al.*, 2015).

The potential effects that CBD has on BBB permeability are yet to be assessed in the context of ischaemia, and we hypothesized that activity at the BBB could represent an as

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yet unrecognized mechanism of CBD-mediated protection in ischaemic stroke. To address this, we examined the effects of CBD in an *in vitro* BBB model, where the potential mechanisms of action were also probed.

Methods

Cell culture and protocols

Human brain microvascular endothelial cells (HBMECs; ScienCell, USA) and human astrocytes (HAs; ScienCell, USA) were cultured in their specialized media. HAs were seeded on the outside of collagen-coated 0.4 μm pore polytetrafluoroethylene membrane Transwell inserts (12 well type; Corning Costar, USA), and HBMECs were seeded on the inside of the insert. Both cells were grown to confluence to create a contact co-culture model (Hind *et al.*, 2015). Transepithelial electrical resistance (TEER) was measured as a marker of co-culture integrity and as a measure of BBB permeability. The resistance across the membrane was measured using STX2 electrodes linked to an epithelial voltohmmeter (World Precision Instruments, UK).

Ischaemic conditions were simulated using an OGD protocol (Hind *et al.*, 2015). Cell culture media was replaced with glucose-free RPMI medium (Invitrogen, UK) and the plates placed into a GasPak EZ Anaerobe Pouch (Beckton Dickinson, UK) with anaerobic conditions being achieved within 20 min. Inserts were left in OGD conditions for 4 h. After OGD, TEER was read, and the RPMI medium was replaced with the cells' normal medium and returned to the incubator (i.e. the reperfusion period). Throughout the course of the experiments, TEER readings were taken at 0, 4, 6, 8, 10, 12, 24 and 32 h.

The effect of CBD treatment (100 nM, 1 μM and 10 μM) on BBB permeability was assessed when given either before or immediately after the OGD protocol. To test the time window of efficacy, CBD (10 μM) was given at 6 and 8 h (i.e. 2 and 4 h into the reperfusion period). A range of receptor antagonists were used to probe the target sites of action for CBD (at 10 μM) at the BBB; for CB_1 receptors, AM251 (100 nM); for CB_2 receptors, AM630 (100 nM); for TRPV1 channels, capsazepine (1 μM), for PPARγ, GW9662 (100 nM), for adenosine A_{2A} receptors, SCH58261 (100 nM); for 5-HT_{1A} receptors, WAY100135 (300 nM). In these studies, vehicle, the antagonist alone, and a control response to CBD were conducted at the same time. To confirm the role of PPARγ and 5-HT_{1A} receptors, the effects of agonists of these receptors, pioglitazone (10 μM) and 8-OH-DPAT (1 μM) were measured. In some experiments, HBMECs and HAs were grown in monoculture and treated with CBD and/or antagonist to establish which target site(s) of action were activated by CBD in these two individual cell types.

Biochemical analysis

LDH levels at the end of the experiment (32 h) were measured using a commercially available kit (LDH-Cytotoxicity Assay Kit II, Biovision, USA). Medium samples were transferred into an optically clear 96-well plate and Reaction Mix (containing water-soluble tetrazolium-1) was added. After 30 min,

absorbance was measured at 450 nm, subtracting the 650 nm reading to correct for optical imperfections.

DuoSet ELISA kits (R&D Systems, UK) were used to measure the levels of human IL-6, vascular cell adhesion molecule 1 (VCAM-1) and VEGF in medium samples from co-cultures and in medium or cellular samples from monocultures (cellular samples collected in RIPA buffer with phosphatase and protease inhibitors; Sigma-Aldrich, UK). Data were normalized to protein content as determined using a bicinchoninic acid assay (Sigma-Aldrich, UK). The medium from co-culture experiments was analysed for changes in cytokines using a human cytokine/chemokine panel (HCYTOMAG-60 K, Merck Millipore, UK) that measured levels of IFN-γ, IL-1β, IL-2, IL-6, IL-10, chemokines CCL3 and CCL4, TNF-α and VEGF.

Cell signalling analysis

HBMEC monocultures treated with CBD (100 nM, 1 μM and 10 μM) for 28 h were analysed using human Multi-Pathway Magnetic Bead 9-Plex Cell Signalling Multiplex Assay (48-680MAG, Merck Millipore, UK) to screen for potential changes in the following phosphorylated proteins; PKB (Akt), cAMP response element binding protein (CREB), ERK, JNK, NF-κB, STAT3, STAT5A/STAT5B, p38 MAPK (p38), and ribosomal protein S6 kinase β-1.

Materials

Purified CBD was a gift from GW Pharmaceuticals (Cambridge, UK) and was dissolved to 10 mM in ethanol. AM251, AM630, GW9662, SCH58261, (S)-WAY100135 and capsazepine (Tocris UK) were dissolved in dimethyl sulfoxide to stock solutions of 10 mM,). Serial dilutions were made fresh daily.

Data analysis

All data were tested for normality using the D'Agostino and Pearson omnibus normality test, and AUC was calculated using the trapezoidal method. Time course studies (Figures 1–3) were analysed by two-way ANOVA with multiple comparisons at each time point comparing against the vehicle control. Column graphs (Figures 3–6) were compared by one-way ANOVA with Dunnett's *post hoc* test comparing against vehicle controls.

Results

The effects of cannabidiol on BBB permeability after OGD

Exposing the co-cultures to 4 h OGD caused a significant increase in permeability ($P < 0.0001$). In some inserts, we showed that 4 h OGD was also accompanied by a significant increase in Evans Blue cleared volume (normoxia 979 ± 23 compared with 4 h OGD 1127 ± 26, $n = 3$, $P < 0.05$), an alternative method of measuring increases in cellular permeability (Srivastava *et al.*, 2013). Administering CBD immediately before OGD at 100 nM ($P < 0.05$), 1 μM ($P < 0.05$) or 10 μM ($P < 0.01$) reduced the permeability of the BBB when looking at the effect on the time-
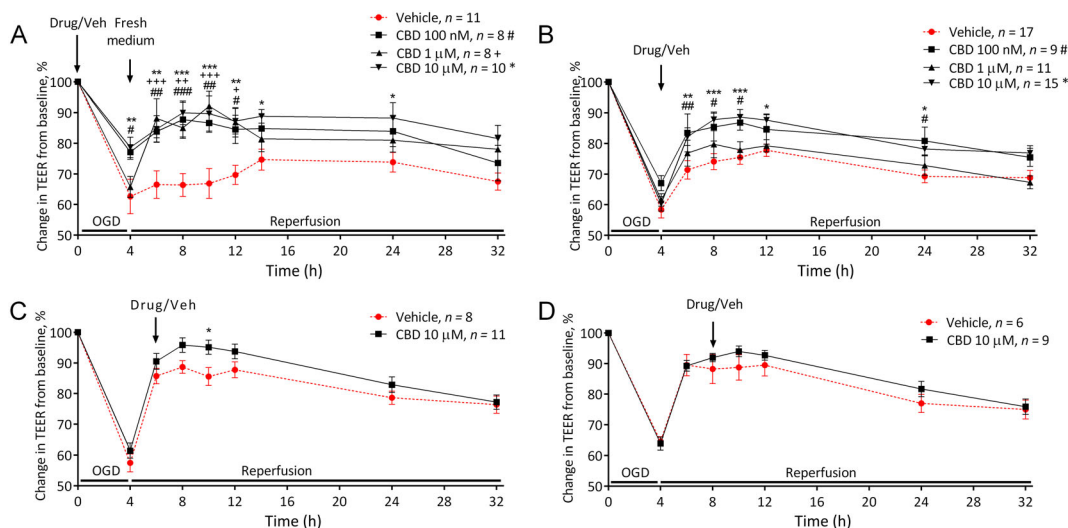
Figure 1

Cannabidiol (CBD) is protective against increased permeability of the BBB associated with oxygen–glucose derivation (OGD). The effects of CBD given before (A), immediately after (B) OGD, or 2 h (C) or 4 h (D) into the reperfusion period on TEER values. Data are given as mean ± SEM (n represents the total number of inserts measured, based on three to four separate experiments). Statistical analysis was conducted by two-way ANOVA with *post hoc* analysis at each time point comparing data against the vehicle control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; 10 μM CBD versus vehicle. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, 1 μM CBD versus vehicle, + $P < 0.05$, ++ $P < 0.01$`, +++ $P <$, 0.001, 100 nM CBD versus vehicle.

course as a whole (0–32 h, AUC, Table 1). All concentrations of CBD reduced permeability at various time points during reperfusion ($P < 0.05$–0.001), but only 100 nM ($P < 0.05$) and 10 μM ($P < 0.01$) attenuated the initial increase in permeability (Figure 1A). Administering CBD immediately after OGD at 100 nM and 10 μM, but not 1 μM, reduced the permeability of the BBB (Figure 1B). Adding 10 μM CBD 2 h into the reperfusion period reduced

permeability at 10 h (Figure 1C). Adding 10 μM CBD 4 h into the reperfusion period did not have any effect on BBB permeability (Figure 1D).

The permeability lowering effects of 10 μM CBD given immediately after OGD were not inhibited by AM251, AM630, capsazepine or SCH58261 (Figure 2 and Table 1). The permeability lowering effects of 10 μM CBD were inhibited in the presence of GW9662 (Figure 3A), and the
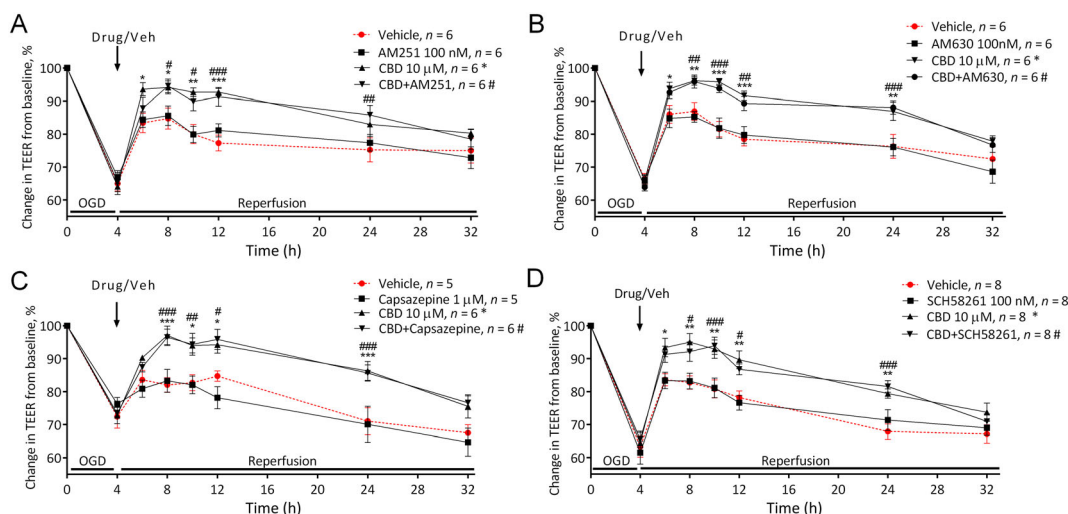

Figure 2

The effects of cannabidiol (CBD) are not mediated by CB$_1$, CB$_2$ receptors or TRPV1 channels. The effect of the receptor antagonists AM251 (A; 100 nM), AM630 (B; 100 nM), capsazepine (C; 1 μM) and SCH58261 (D; 100 nM) on the effects of CBD applied luminally on BBB permeability. Data are given as mean ± SEM (n represents the total number of inserts measured, based on two separate experiments). Statistical analysis was conducted by two-way ANOVA with *post hoc* analysis at each time point comparing data against the vehicle control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, vehicle versus CBD. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, vehicle versus CBD + antagonist.
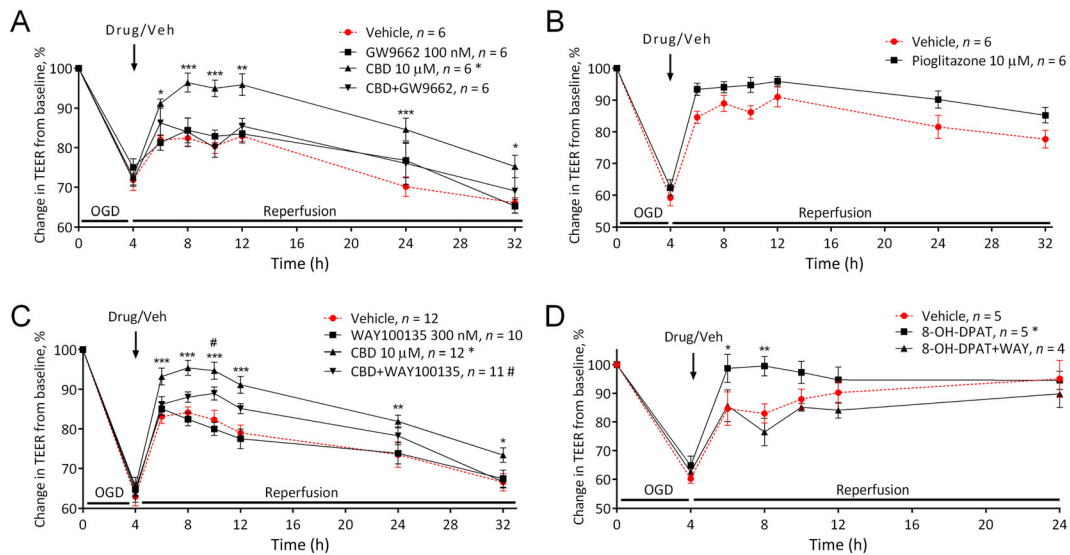
Figure 3

The effects of cannabidiol (CBD) are mediated by PPARγ and 5HT$_{1A}$ receptors. The effect of the receptor antagonists GW9662 (A; 100 nM) and WAY100135 (C; 300 nM) on the effects of CBD on BBB permeability. The effect of the PPARγ agonist pioglitazone (B) and 5HT$_{1A}$ receptor agonist 8-OH-DPAT (D) applied luminally on BBB permeability. Data are given as mean ± SEM (n represents the total number of inserts measured, based on two to four separate experiments). Statistical analysis was conducted by two-way ANOVA with *post hoc* analysis at each time point comparing data against the vehicle control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$,****$P < 0.0001$; vehicle versus CBD. # $P < 0.05$; vehicle versus CBD + antagonist.

PPARγ agonist pioglitazone also reduced BBB permeability after OGD in a similar manner ($P < 0.05$; Table 1 and Figure 3B). In the presence of WAY100135 (a 5-HT$_{1A}$ antagonist), the effects of CBD were no longer significantly different to vehicle except at 10 h (Figure 3C). The 5-HT$_{1A}$ agonist 8-OH-DPAT also reduced BBB permeability after OGD in a similar manner to CBD, which was antagonized by WAY100135 (Figure 3D and Table 1).

Combining all experiments that were conducted with CBD (10 μM) together (n = 45), a highly significant reduction in permeability ($P < 0.0001$) was observed at all time points

following the addition of CBD after OGD (Supporting Information Fig. S1).

Co-culture biochemical assays

In medium from the luminal chamber of co-culture inserts, LDH levels were significantly increased following reperfusion ($P < 0.01$), but LDH levels were not increased in medium from the inserts treated with 10 μM CBD, which had significantly lower LDH levels than vehicle-treated samples ($P < 0.05$; Figure 4A). VCAM-1 was increased in vehicle-treated samples following OGD + reperfusion in medium from luminal
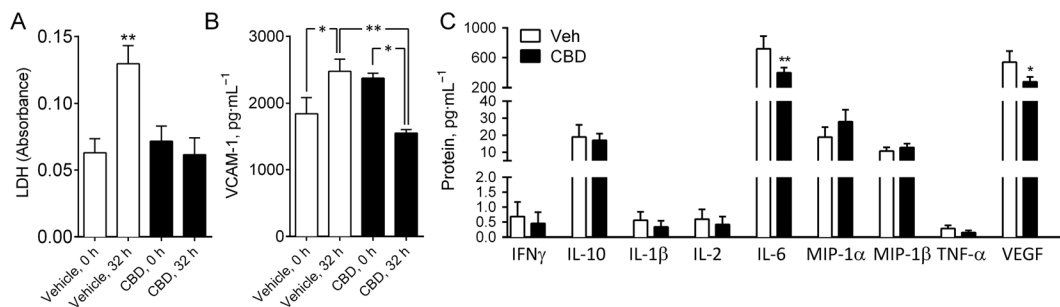

Figure 4

Biochemical analysis of co-culture medium. Levels of LDH (A) and vascular cell adhesion molecule 1 (VCAM-1; B) from the luminal (endothelial) chamber of co-culture inserts at the end of experiment (32 h) (n = 6 inserts). C. Cytokine or chemokine levels from the luminal (endothelial) chamber at 32 h for IFN-γ, L-10, IL-1β, IL-2, IL-6, CCL3, CCL4, TNF-α and VEGF (n = 6 inserts). Data are given as mean ± SEM. Statistical analysis conducted using one-way ANOVA with Sidak's multiple comparisons comparing the vehicle control against CBD responses (n = 6, *$P < 0.05$, **$P < 0.01$).
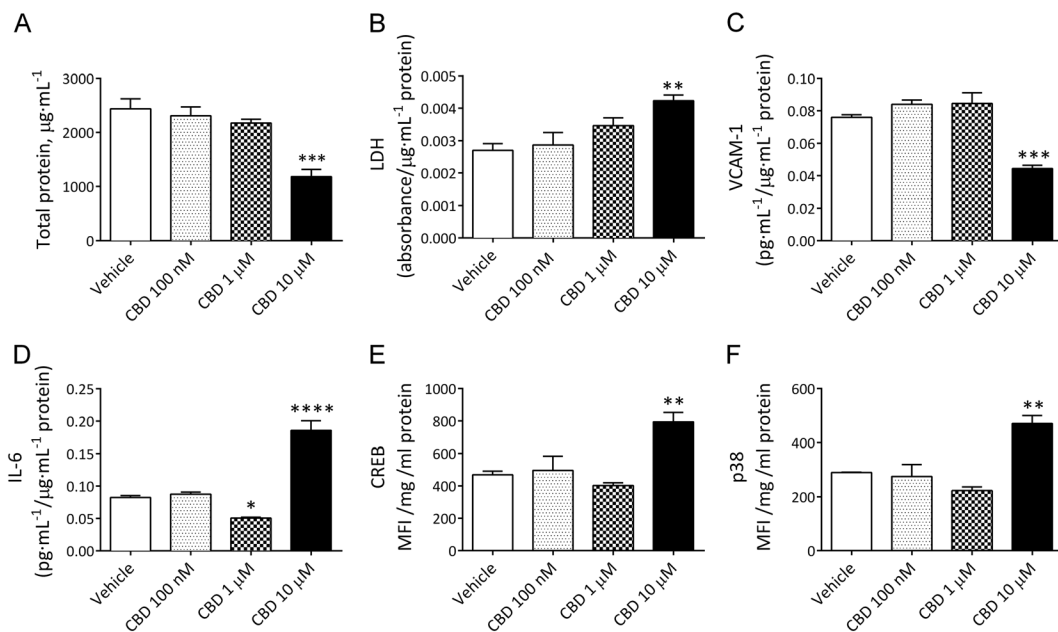
Figure 5

The effects of CBD on HBMECs in monoculture. The total level of protein (A), LDH (B), protein-normalized medium levels of VCAM (C), IL-6 (D), and cellular levels of phosphorylated CREB (E) and p38 (F) in HBMEC treated with 100 nM, 1 μM or 10 μM CBD for 28 h in normal culture conditions. MFI, mean fluorescent intensity. Data are given as mean ± SEM ($n = 3–6$). Statistical analysis conducted using one-way ANOVA with Dunnett's *post hoc* test compared with the vehicle control response, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, **** $P < 0.0001$.
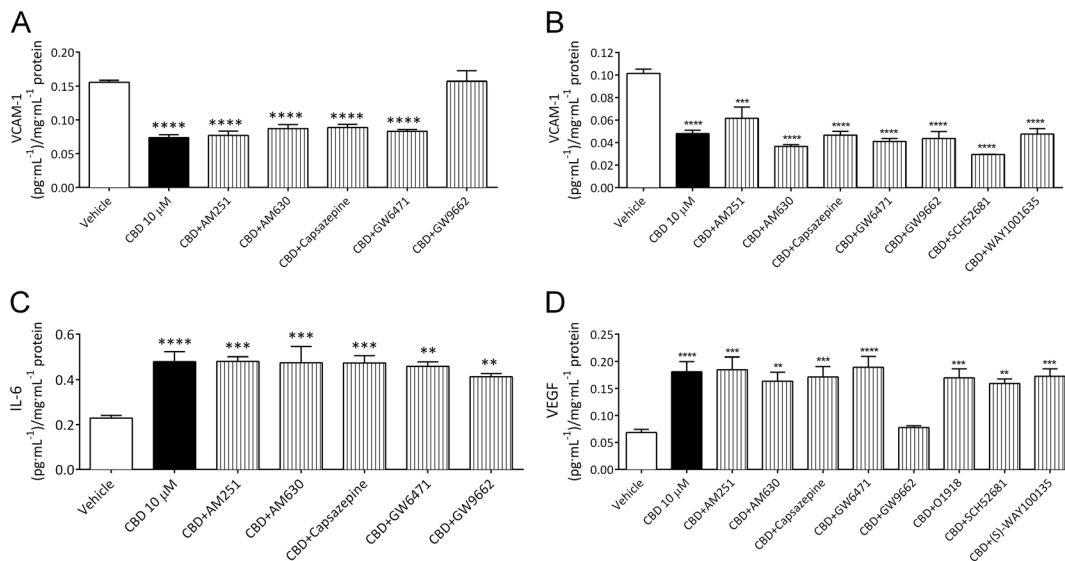

Figure 6

Biochemical analysis of monocultures. The levels of VCAM-1 in cell culture medium from monocultures of either HBMECs (A) or HAs (B) treated for 28 h with CBD alone or in the presence of receptor antagonists in normal conditions. The levels of IL-6 (C) and VEGF (D) in HBMEC medium from monocultures treated for 28 h with CBD alone or in the presence of receptor antagonists. All data normalized to total protein (bicinchoninic acid assay). Data are given as mean ± SEM. Statistical analysis conducted using one-way ANOVA with Dunnett's *post hoc* test ($n = 3–6$), **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

chambers ($P < 0.05$; Figure 4B). Levels of VCAM-1 were significantly reduced after treatment with 10 μM CBD ($P < 0.05$; Figure 4B).

Detectable levels of IL-6, IL-10, CCL3, CCL4, VEGF, INFγ, IL-1β, IL-2 and TNF-α were seen in medium samples at 32 h

from the luminal chambers of the co-culture, (Figure 4C). A similar pattern was observed in the abluminal chamber (Supporting Information Fig. S2). There was a significant reduction in IL-6 ($P < 0.01$) and VEGF ($P < 0.01$) levels in the medium of inserts treated with CBD (Figure 4C).

Table 1

Integrated responses over time (0–32 h) to CBD in combination with an oxygen/glucose deprivation protocol

Pre-hypoxia	AUC
Vehicle ($n = 8$)	1518 ± 85
CBD 100 nM ($n = 6$)	$1144 \pm 104^{*}$
CBD 1 μM ($n = 6$)	$1185 \pm 101^{*}$
CBD 10 μM ($n = 8$)	$1033 \pm 82^{**}$
Immediately after OGD	
Vehicle ($n = 17$)	1151 ± 54
CBD 100 nM ($n = 9$)	930 ± 89
CBD 1 μM ($n = 11$)	1075 ± 68
CBD 10 μM ($n = 15$)	$880 \pm 53^{**}$
2 h post-OGD	
Vehicle ($n = 8$)	931 ± 63
CBD 10 μM ($n = 11$)	788 ± 62
4 h post-OGD	
Vehicle ($n = 6$)	778 ± 84
CBD 10 μM ($n = 9$)	689 ± 39
CB$_1$ receptor studies	
Vehicle ($n = 6$)	798 ± 79
AM251 alone ($n = 6$)	750 ± 60
CBD ($n = 6$)	$531 \pm 49^{*}$
CBD and AM251 ($n = 6$)	$532 \pm 62^{*}$
CB$_2$ receptor studies	
Vehicle ($n = 6$)	831 ± 68
AM630 alone ($n = 6$)	847 ± 58
CBD ($n = 6$)	$551 \pm 37^{**}$
CBD and AM630 ($n = 6$)	$573 \pm 42^{**}$
TRPV1 receptor studies	
Vehicle ($n = 5$)	858 ± 66
Capsazepine alone ($n = 5$)	918 ± 107
CBD ($n = 5$)	$543 \pm 46^{*}$
CBD and capsazepine ($n = 5$)	$535 \pm 32^{*}$
PPARγ receptor studies	
Vehicle ($n = 6$)	889 ± 28
GW9662 alone ($n = 6$)	805 ± 73
CBD ($n = 6$)	$574 \pm 54^{*}$
CBD and GW9662 ($n = 6$)	780 ± 102
Further PPARγ studies	
Vehicle ($n = 6$)	587 ± 71
Pioglitazone ($n = 6$)	$381 \pm 50^{*}$
A$_{2A}$ adenosine receptor studies	
Vehicle ($n = 8$)	976 ± 63
SCH58261 alone ($n = 8$)	949 ± 73
CBD ($n = 8$)	$683 \pm 64^{**}$

(Continues)

Table 1 (Continued)

CBD and SCH58261 ($n = 8$)	$697 \pm 50^{*}$
5-HT$_{1A}$ receptor studies	
Vehicle ($n = 12$)	1033 ± 75
WAY100135 alone ($n = 10$)	1082 ± 56
CBD ($n = 12$)	$768 \pm 67^{*}$
CBD and WAY100135 ($n = 11$)	936.2 ± 44

AUC values are presented as mean ± SEM. Data were compared by one-way ANOVA with Dunnett's *post hoc* test, comparing against the vehicle response in experiments that did not contain antagonist. In experiments that did contain an antagonist, data were compared by one-way ANOVA with Tukey's post hoc test. Variability in the vehicle controls reflects differences in BBB permeability responses from experiment to experiment. In every experiment, vehicle controls and CBD alone positive controls were carried out to account for this. $^{*}P < 0.05$. $^{**}P < 0.01$.

Effects of CBD on HBMECs and HAs in monoculture

The amount of total protein in HBMEC monocultures was lower in samples treated with 10 μM (but not 100 nM or 1 μM) CBD for 28 h in normal conditions ($P < 0.001$; Figure 5A). In medium from HBMEC monocultures, the levels of LDH were significantly higher in samples that were treated with 10 μM CBD (but not 100 nM or 1 μM) for 28 h in normal conditions ($P < 0.01$; Figure 5B). These increases in levels of cell damage were reflected in the morphology of HBMECs (Supporting Information Fig. S3). These observations were in contrast to the situation in co-cultures, where 10 μM CBD inhibited the OGD-induced increase in LDH and visual examination of the cells under the microscope at various time points throughout the 32 h procedure did not reveal any damaging effects of CBD.

In HBMEC monocultures, CBD at 10 μM, but not 100 nM or 1 μM, decreased the levels of VCAM-1 (data normalized to total protein) compared with vehicle in samples that were treated for 28 h in normal conditions ($P < 0.001$; Figure 5C). This effect was inhibited by the PPARγ antagonist GW9662 (Figure 6A). There was a concentration-dependent effect of CBD on IL-6 in normal conditions, where 1 μM decreased ($P < 0.05$), but 10 μM increased ($P < 0.0001$) IL-6 levels (data normalized to total protein; Figure 5D). Receptor involvement in the 10 μM response was probed; however, none of the antagonists tested altered the effect on IL-6 levels (Figure 6C). VEGF levels were increased in HBMEC monocultures treated with 10 μM CBD in normal conditions ($P < 0.001$; Figure 6D) (data normalized to total protein). This effect was inhibited by the PPARγ antagonist GW9662 (Figure 6D).

HBMECs that were treated with 10 μM CBD (but not 100 nM or 1 μM) for 28 h had increased levels of phosphorylated CREB ($P < 0.01$; Figure 5E) and p38 ($P < 0.01$; Figure 5F). The levels of JNK, NF-κB, ERK 1/2, Akt, p70s6K, STAT3 and STAT5 were not altered (Supporting Information Fig. S4).

HA monocultures treated with 10 μM CBD showed similar results to HBMEC monocultures with decreases in protein levels ($P < 0.001$), increases in LDH ($P < 0.001$) and decreases in secreted levels of IL-6 ($P < 0.001$) and VCAM ($P < 0.001$) in

cells exposed to 10 μM CBD (all data normalized to total protein count; Supporting Information Fig. S5). Unlike the endothelial cells, the decrease in VCAM in human astrocytes was not inhibited by GW9662 (Figure 6B) or any other antagonist tested.

Discussion

In this study we have shown that CBD decreases BBB permeability when given either before or after OGD, involving activation of PPARγ and 5-HT$_{1A}$ receptors and associated with reductions in cell damage and VCAM-1, and increases in VEGF. These data increase the case for further investigations into CBD as a future treatment in ischaemic stroke.

Given before OGD, CBD reduced permeability at all concentrations tested. This is consistent with data from *in vitro* and *in vivo* models showing CBD is effective at reducing the permeability of the BBB in multiple sclerosis (Mecha *et al.*, 2013) and sepsis-related encephalitis (Fernandez-Ruiz *et al.*, 2013) and other epithelial barriers in various diseases (El-Remessy *et al.*, 2006; Rajesh *et al.*, 2007; Alhamoruni *et al.*, 2010, 2012). Most strokes occur without warning and a treatment that protects the brain when administered post ictus is more clinically relevant; this makes CBD an attractive candidate as we also showed that 10 μM CBD decreased BBB permeability when given immediately after OGD and also 2 h after reperfusion (i.e. 4 and 6 h from initial onset). Indeed, administration of CBD at 1 or even 3 days (but not 5 days) following middle cerebral artery occlusion increases survival *in vivo* in mice (Hayakawa *et al.*, 2010). CBD afforded protection to the BBB at all concentrations tested when given pre OGD, but only at 10 μM when given post-OGD. The reasons for this may be complex as OGD induces many cellular changes which could influence CBD efficacy such as changes in signalling proteins, Ca^{2+} levels, receptor expression and function, enzymic activities and mRNA translation. CBD decreased the levels of cell damage that were elevated following OGD +reperfusion in co-cultures, contributing to a less disrupted BBB. *In vivo*, CBD decreases the level of cell death in the hippocampus, the liver and the retina following ischaemic stroke in mice (Shiavon *et al.*, 2014), hepatic ischaemia + reperfusion in mice (Fernandez-Ruiz *et al.*, 2013) and in diabetic rats (El-Remessy *et al.*, 2006) respectively. CBD also decreased VCAM-1 after OGD + reperfusion. VCAM-1 is mainly expressed by endothelial cells and selectively binds to the very late antigen-4 expressed on monocytes and lymphocytes (Yusuf-Makagiansar *et al.*, 2002), thus increasing their transmigration across the BBB. VCAM-1 levels are raised during cerebral ischaemia, and it seems likely that they play an important role during the inflammatory response to stroke (Jander *et al.*, 1996). This is consistent with previous studies where CBD reduced VCAM-1 levels using *in vitro* human coronary artery endothelial cells (CB$_{1/2}$ independent; Rajesh *et al.*, 2007) and using *in vivo* mouse models of multiple sclerosis (partly mediated by adenosine A$_{2A}$ receptors; Mecha *et al.*, 2013). In our monoculture studies, CBD reduced VCAM secretion in both HBMECs and HAs. Etymologically, it may seem paradoxical that VCAM-1 is produced by astrocytes, but this finding has also been observed in human astroglial cells (Song *et al.*, 2011).

CBD has a rich pharmacology with known activity at multiple sites. In the present study, we found no role for CB$_1$, CB$_2$ or adenosine A$_{2A}$ receptors or TRPV1 channels. We did however find that permeability reducing properties of CBD were mediated via activity at PPARγ. The concentration of CBD (10 μM) required to affect BBB permeability when given after OGD is in line with CBD's micromolar affinity for PPARγ (O'Sullivan *et al.*, 2009; Fernandez-Ruiz *et al.*, 2013). Furthermore, pioglitazone (a PPARγ agonist) mimicked CBD's permeability-reducing properties. We also found that the CBD-induced decrease in VCAM-1 in HBMEC monocultures was attenuated by PPARγ antagonism, consistent with a previous study showing activation of PPARγ by the CB$_1$/CB$_2$ agonist WIN55,212-2 decreases VCAM-1 levels in mouse brain endothelial cells *in vitro* (Mestre *et al.*, 2009). PPARγ activation by rosiglitazone and pioglitazone decreases BBB permeability induced by inflammation (Ramirez *et al.*, 2008). Our results provide evidence to suggest that activation of PPARγ may play a role in reducing permeability following ischaemia. Other groups have shown that PPARγ activation is protective in rat models of ischaemic stroke; thiazolidinediones (TZDs) such rosiglitazone, as are PPARγ agonists that reduce infarct size and improve functional recovery from ischaemic stroke in rats (Luo *et al.*, 2006). Therefore, PPARγ activity at the BBB in ischaemic stroke could represent an as yet unrecognized mechanism of TZD-mediated protection.

The effects of CBD were also partly inhibited by a 5-HT$_{1A}$ receptor antagonist. We also showed that a 5-HT$_{1A}$ receptor agonist was capable of reducing the increased permeability induced by OGD, and this was inhibited by a 5-HT$_{1A}$ receptor antagonist. Again, the concentration of CBD (10 μM) required to affect BBB permeability is consistent with CBD's micromolar affinity for 5-HT$_{1A}$ receptors (Fernandez-Ruiz *et al.*, 2013). This is the first study to investigate the potential role that 5-HT$_{1A}$ plays in regulating the permeability of the BBB or any epithelial barrier. In mouse models of ischaemic stroke, CBD activity at the 5-HT$_{1A}$ receptor was shown to be responsible, at least in part, for the beneficial effects of reducing infarct volume and increasing CBF (Hayakawa *et al.*, 2010). Therefore, CBD activation of the 5-HT$_{1A}$ receptor at the BBB could potentially contribute to the protective effects reported in the published literature.

The expression of VEGF is potentiated by hypoxia, and it is an important pathogenic factor for induction of vascular leakage in the brain (Schoch *et al.*, 2002), and inhibition of VEGF reduces the permeability of the BBB *in vivo* in the rat (Zhang *et al.*, 2002). In HBMEC monocultures, we found that VEGF levels were increased following treatment with 10 μM CBD, sensitive to PPARγ antagonism. The PPARγ agonist troglitazone increased VEGF levels in OP9 mouse preadipocytes in a GW9662-sensitive manner (Kotake and Hirasawa, 2013). Our findings are in contrast to a study by El-Remessy *et al.* (2006, where they showed that CBD reduced VEGF levels in the blood–retinal barrier following experimental diabetes in the rat. However, it should also be noted that in the co-culture experiments (Figure 4C), a trend for a reduction in VEGF levels was observed with CBD, indicating a difference in the cellular response to CBD between monoculture and co-cultures.

In HA and HBMEC monocultures, we found that CBD-reduced IL-6 levels at 10 and 1 μM, respectively; and there was a trend for reduced levels of IL-6 in medium from the

luminal chamber of our co-cultures that were treated with 10 μM CBD ($P = 0.11$; Figure 4C). CBD decreases IL-6 levels *in vitro* following ischaemic damage to the mouse forebrain (Castillo *et al.*, 2010), which is associated with CBD's ability to reduce infarct volume. IL-6 levels are increased following experimental and human ischaemic stroke (Miao and Liao, 2014), with increased IL-6 levels being shown to interrupt the BBB by disrupting endothelial TJs (Yenari *et al.*, 2006). Indeed, in clinical studies, IL-6 is often suggested as a good predictive marker of stroke severity (Miao and Liao, 2014). However, IL-6-deficient mice do not exhibit improved outcome from stroke (Ceulemans *et al.*, 2010), and production of IL-6 promotes post-stroke angiogenesis, thus promoting long-term histological and functional benefits (Gertz *et al.*, 2012).

To gain a better understanding of the mechanisms through which CBD was having its effects on the HBMEC and HA monocultures, the levels of several well-known signalling proteins involved in cell survival and inflammation were measured, and an increase in the levels of phosphorylated p38 and CREB were observed at 10 μM. p38 is a stress-activated serine/threonine protein kinase, which is a downstream target of proinflammatory cytokines and oxidative stress, and hence causes cellular damage. Increased levels of p38 contribute to transcriptional activation of the IL-6 promoter by modulating the transactivation capacity of the NF-κB subunit (Vanden Berghe *et al.*, 2000). Therefore, the increased levels of p38 may be partly responsible for the increased levels of IL-6 seen in HBMEC monocultures treated with 10 μM CBD.

CBD also increased phosphorylated CREB levels in HBMEC monocultures. Cerebral ischaemia increases glutamate levels, which results in elevated levels of neuronal CREB activation. CREB is known to lead to the expression of certain genes that encode neuroprotective molecules, such as the anti-apoptotic protein Bcl-2, and contributes to neuronal survival following ischaemia (Kitagawa, 2007). However, CREB has also been shown to induce transcription of immune-related genes that possess a cAMP responsive element, such as IL-6, so this could be partially responsible for an increase in IL-6 levels (Wen *et al.*, 2010). The CREB signalling pathway is linked to p38, whereby an increase in p38 raises CREB phosphorylation through interaction with ERK-1/ERK-2 and then mitogen and stress-activated kinases (MSK)-1/MSK-2. CREB then initiates transcription of the anti-inflammatory protein IL-10 and also induces dual specificity protein phosphatase 1, which feeds back to inhibit p38 (Wen *et al.*, 2010).

The effects of CBD on cells in monocultures did not always mirror the data obtained from the co-cultures. For example, in co-culture, CBD decreased LDH levels, but in HBMEC and HA monocultures, CBD (10 μM) increased the levels of cell damage. A potential explanation for why 10 μM CBD was cytotoxic in monocultures but cytoprotective in co-cultures is that in the co-culture, the HBMECs and HAs communicate with each other, and thus is more representative of the BBB *in vivo*. Communication between these two cell types is vital for the formation of tight junctions; furthermore, paracrine signalling occurs between these two cells. Given the large repertoire of astrocyte-released agents, many of which have matching receptors on brain endothelium, a range of

distinct and complex responses of the endothelium can be influenced by astrocyte activity (Abbott *et al.*, 2006). For example, there is an up-regulation of numerous specific transport systems in brain endothelia when they are in contact with glia, an effect that has been observed with GLUT-1, the L-system and A-system amino acid carriers and P-glycoprotein. Several molecules, including glial cell line-derived neurotrophic factor and various steroids, are known to be capable of mimicking aspects of glial-mediated barrier induction of brain endothelium. In the opposite direction, there is also evidence that endothelial cells induce the astrocytic phenotype at the BBB, with leukaemia inhibitory factor, secreted from brain endothelia, being shown to induce astrocytic differentiation (Abbott, 2002). Another potential explanation for the distinct effect of CBD in co-culture and monoculture may be that in co-culture, the cells were grown on a collagen-coated membrane, which may provide a more stable environment for the cells compared with monoculture. Of the two different types of cellular environment employed in this study (i.e. monoculture and co-culture), the co-culture is considered to be the more physiologically relevant environment. Whilst our co-culture reproduced many of the important characteristics of the BBB, regulation of the BBB is highly complex and involves input from an array of different cell types and molecules (Abbott *et al.*, 2006), implying that the actual *in vivo* physiology of the BBB can never be fully reproduced *in vitro*. Our data demonstrate the limitations of performing *in vitro* cell work in a single cell type only, in the absence of other cells that will influence cellular responses.

In conclusion, this study demonstrated that CBD decreased the permeability of the BBB following OGD-reperfusion and has identified a role for PPARγ and 5-HT$_{1A}$ receptors. CBD may achieve this decrease in permeability by attenuating the OGD + reperfusion-associated increases in cell damage and by decreasing VCAM-1. The results presented here (in addition to the existing literature; where CBD has shown neuroprotection, CBF increasing properties and improved motor performance following ischaemic stroke *in vivo* and *in vitro*; Hayakawa *et al.*, 2010) increases the case for further investigations into CBD as a future treatment in ischaemic stroke and potential clinical application. Many neuroprotective drugs have shown preclinical promise, but all those that have reached clinical development failed to demonstrate efficacy or had unacceptable adverse effects (Green and Shuaib, 2006). CBD has an excellent tolerability profile (Bergamaschi *et al.*, 2011) and is currently licensed to treat spasticity in multiple sclerosis (in combination with THC, as the medicine Sativex; GW Pharmaceuticals, Cambridge UK) in over 20 countries. The sequelae of ischaemic stroke are multifaceted; therefore, a drug that targets multiple systems such as CBD may have an increased chance of success compared with compounds aimed at a single-target (O'Collins *et al.*, 2006).

Author contributions

W. H. H. performed the research. T. J. E. and S. O. S. designed the research study. W. H. H. and S. O. S. analysed the data. W. H. H., T. J. E. and S. O. S. wrote the paper.

Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 The effect of 10 μM cannabidiol (CBD) applied after 4 hours OGD on BBB permeability from all experiments (*n* = 45) presented as a time course (A) and as the area under the curve of the whole experiment (B). Data are given as mean ± SEM. Statistical analysis conducted using two-way ANOVA (A) or Student's *t*-test (B). ******* $P<0.001$, ******** $P<0.0001$.

Figure S2 Cytokine/chemokine levels in the abluminal medium of BBB co-culture inserts at 32 hours for IFNγ, IL-10, IL-1β, IL-2, IL-6, macrophage inflammatory protein (MIP)-1α, MIP-1β, TNF-α and VEGF. Data are given as mean ± SEM. Statistical analysis conducted using one-way ANOVA with *post hoc* analysis of selected pairs (vehicle versus CBD).

Figure S3 The effect of 28h treatment with increasing concentrations of cannabidiol (CBD) on HBMEC cell morphology. At the highest concentration, CBD appeared to cause cell damage, which was associated with a significant decrease in protein levels and significant increase in LDH release (see Figure 5).

Figure S4 No differences were observed after CBD treatment in the levels of phosphorylated JNK (A), ERK (B), Akt (c), NFκB (D), p70s6K (E), STAT3 (F) or STAT5 (G) in HBMECs treated for 28 h with CBD. Data are given as mean ± SEM. Statistical analysis conducted using one-way ANOVA with Dunnett's *post hoc* test.

Figure S5 A. Light microscope images of human brain astrocytes treated by vehicle or increasing concentrations of CBD for 28 h in normal conditions. At the highest concentration, CBD appeared to cause cell damage, which was associated with a significant decrease in protein levels (B) and significant increase in LDH release (C). The levels of IL-6 (D) and VCAM (E) in medium from astrocyte monocultures treated for 28 h with CBD. All data was normalised to total protein (BCA assay). Data are given as mean ± SEM. Statistical analysis conducted using one-way ANOVA with Dunnett's *post hoc* test, *n* = 3-6, ****** $P<0.01$, ******* $P<0.001$.