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Phytocannabinoids, Neuroprotection And The Blood-Brain Barrier

Nicole Stone

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Statement of authenticity

I declare that this thesis has been completed by myself under supervision of Professor Saoirse O'Sullivan and Dr Tim England at the Division of Medical Sciences & Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital. This thesis is a record of work that has not been submitted previously for a higher degree.

Nicole Stone December 2020

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COVID-19 impact

During the course of my final year, we were struck with news of a novel coronavirus, known as COVID-19. From March 2020 widespread closure of Universities meant that me and my colleagues were no longer able to continue our laboratory work and were told to start writing up our thesis from home, with no known date to return to the University to complete our work. This heavily impacted my data collection in my final year, which to many is the most productive given you have all methodologies in place and most of kinks ironed out. During the first few months the extra time dedicated to writing was useful, however motivating myself to write continually all day every day quickly became quite challenging. Overall, I believe the impact of the COVID-19 pandemic on my PhD experience was considerable and made my final year very tough. I found that during the pandemic my mental health really suffered and anyone completing a PhD in 2020 has overcome very difficult circumstances to produce a piece of work that is already extremely challenging.

Conferences

Presenting author:

July 2019	'Neuroprotective effects of cannabidiolic acid (CBDA) on cells of the blood brain barrier (BBB) under hypoxic conditions'. **Winner of the pre-Doctoral Presentation Award.** International Cannabinoid Research Society (ICRS), Washington, United States.
November 2018	'Modelling the blood-brain barrier *in vitro* using four primary human cell types'. Oral presentation. 8th UK& Ireland Early Career Blood-Brain Barrier symposium, Oxford, United Kingdom.
July 2018	'Treatment with phytocannabinoid cannabidiolic acid (CBDA) on pericytes under hypoxic conditions *in vitro'*. Poster presentation. International Cannabinoid Research Society, Leiden, The Netherlands.
July 2017	'Phytocannabinoids cannabichromene (CBC) and cannabidivarin (CBDV) modulate mitochondrial complex proteins in primary human astrocytes'. Poster presentation. International Cannabinoid Research Society, Montreal, Canada.

Contributing author:

July 2018	'A review of cannabidiol dosing in patient studies'. Poster presentation. International Cannabinoid Research Society. Leiden, The Netherlands.

Internships

September 2019	International scholarship exchange (PROM) in Department of Neurochemistry, Institute of Pharmacology of the Polish Academy of Sciences. Krakow, Poland.
Feb- March 2019	Professional internship at Blossom Medical Consultancy Ltd as part of the DTP doctoral training programme. Manchester, United Kingdom.

Publications

First author publications directly pertaining to PhD thesis:

Cannabidivarin (CBDV) and Cannabigerol (CBG): effects on Cells of the Blood-Brain Barrier under Ischaemic Conditions. N. Stone, T. England, S. O'Sullivan (2021) *Cannabis and Cannabinoid Research. Online ahead of print.*

A Systematic Review of Minor Phytocannabinoids with Promising Neuroprotective Potential. N. Stone, A. Murphy, T. England, S. O'Sullivan. *British Journal of Pharmacology (2020:177:19).*

A Novel Transwell Blood Brain Barrier Model Using Primary Human Cells. N. Stone, T. England, S. O'Sullivan. *Frontiers in Cellular Neuroscience (2019:13:230).*

An analysis of endocannabinoid concentrations and mood following singing and exercise in healthy volunteers. N. Stone, S. Millar, P. Herrod, D. Barrett, C. Ortori, V. Mellon, and S. O'Sullivan. *Frontiers in Behavioural Neuroscience (2018:12:269).*

Second author publications conducted during the course of PhD studies:

A systematic review of cannabidiol dosing in clinical populations. S. Millar, N. Stone, Z. Bellman, A. Yates, T. England and S. O'Sullivan. *British Journal of Clinical Pharmacology (2019;85:9).*

A systematic review on the pharmacokinetics of cannabidiol in humans. S. Millar, N. Stone, A. Yates and S. O'Sullivan. *Frontiers in Pharmacology (2018;26:9).*

Abstract

The blood brain barrier (BBB) is central to the neurovascular unit (NVU) where it creates a semi-permeable barrier between neuronal tissue and the vascular networks that feed the brain. In neurodegenerative conditions and ischaemic stroke, the BBB becomes compromised and as a result its permeability increases. This not only exacerbates neuronal damage at the site of injury but also causes unwanted extravasation of peripheral immune cells into the brain, fuelling the overactivation of the immune response. Endocannabinoids and phytocannabinoids have both displayed neuroprotective effects, attenuating damage in a range of models including Parkinson's, Huntington's, amyloid lateral sclerosis and ischaemic stroke. The current study aimed to investigate the neuroprotective properties of emerging phytocannabinoids; specifically focusing on the BBB and NVU in the context of ischaemic stroke pathophysiology.

A four-cell blood brain barrier model was constructed consisting of; human brain microvascular endothelial cells (HBMECs), astrocytes, pericytes and neurons. Cells were cultured on collagen coated transwell inserts and permeability was assessed using transepithelial resistance (TEER). A systematic review was conducted to examine work on the neuroprotective properties of minor phytocannabinoids, aside from cannabidiol (CBD) and delta 9-tetrahydrocannabinol (Δ^9-THC). Following on from this, *in vitro* experiments were conducted using minor phytocannabinoids with the most neuroprotective potential; cannabidivarin (CBDV), cannabigerol (CBG) and cannabidiolic acid (CBDA). Inserts or monocultures (four cell model and pericyte, HBMECs and neuronal monolayers) were subjected to either a 4 h oxygen-glucose deprivation (OGD) protocol or an 8 h OGD (astrocyte monocultures), to model ischaemic stroke *in vitro*. Media was analysed for various chemokines and cytokines using enzyme-linked immunoassays or multiplex assays.

From the systematic review, emerging phytocannabinoids cannabidivarin (CBDV) and cannabigerol (CBG) were found to display efficacy in various neurogenerative conditions and of the limited available mechanistic data, were found to mediate some of their effects through peroxisome proliferator-activated receptor gamma (PPARγ). Data showed CBDV (300 nM-10 µM) attenuated MCP-1 levels in HBMEC monolayers, as well as reducing IL-6 (30 nM, 1 µM and 10 µM; $p<0.05$) and VEGF (10 nM- 10 µM; $p<0.01$) levels in astrocyte monocultures post OGD. CBG (10 nM-3 µM; $p<0.0001$) also reduced levels of IL-6 secreted by astrocytes and decreased levels of DNA damage response proteins including Chk1, Chk2, H2A.X and p53 post OGD. Neither CBG, nor CBDV reduced levels of IL-6, VEG or IL-8 in pericytes compared to the vehicle control post OGD. Cannabidiolic acid (CBDA) was also investigated and was found to decrease IL-6 in pericyte monocultures which was mediated, at least in part, by 5-HT_{1A} activation. In a four-cell model of the BBB, CBDA offset increases in permeability vs the vehicle control and offered direct protection to neurons, as shown by a lack of propidium iodide (PI) staining in CBDA treated cells, indicating live cells are present.

Data presented in this thesis show minor phytocannabinoids CBDV, CBG and CBDA provide protection against OGD mediated damage, with CBDA also offering protection against increases in permeability of the BBB post OGD. These novel data warrant further investigation into the neuroprotective properties of phytocannabinoids, particularly in ischaemic stroke.

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Tables and Figures

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1. General Introduction

1.1 Prevalence of Neurological disorders with a focus on ischaemic stroke

Globally, neurological disorders such as ischaemic stroke and dementia accounted for 250.7 million disability adjusted life years in 2015, an increase of 7.45% over the past 25 years (Cao et al., 2007; Feigin et al., 2019). Of these, stroke is the third leading cause of death worldwide; in 2015 6.24 million deaths were reported listing stroke as the cause and this number is predicted to rise to 7.8 million deaths by 2030 (Gorelick, 2019; Lewandowski and Barsan, 2001; Strong et al., 2007). Furthermore, the risk of suffering a stroke doubles every 10 years after the age of 55, with around three-quarters of all strokes occurring in individuals over the age of 65. Similarly, the prevalence of dementia in the over 65s is 0.8%, increasing to 28.5% in those aged ≥90 (Van Der Flier and Scheltens, 2005; Yousufuddin and Young, 2019). As of mid 2019, in the UK there are 18.5% of people over 65 and 2.5% aged over 85, with the proportion of people over 65 expected to grow to 24.6% by 2045 (Park, 2019). These conditions are highly debilitating and place enormous burden on healthcare systems, which is only predicted to worsen as the population continues to age and the number at risk of suffering from these conditions increases (Feigin et al., 2019).

In recent years, there has been a gradual decline in stroke mortality, thought to be as a result of a greater awareness of the risk factors associated with stroke including smoking, cardiac disease, physical inactivity, obesity, diabetes, high blood pressure and stress (Mozaffarian et al., 2016). Despite this, strokes are still a major public health problem exhibiting poor prognosis, high morbidity and mortality, as well as being a key cause of chronic adult disability which directly correlates with long term financial burden (Moretti et al., 2015). Currently, the only licenced, evidence-based treatments for ischaemic stroke are intravenous thrombolysis treatment with tissue-type plasminogen activator (tPA) or endovascular thrombectomy (EVT) (Douglas et al., 2020). Both aim to restore blood flow, mitigate damage and reduce the infarct volume,

however administration is crucially time dependent and not always effective (Bai and Lyden, 2015). Recent evidence, however, has led to an extension in the time for a viable mechanical clot removal, which may be up to 24 h in certain patients (Douglas et al., 2020). Aside from these, no other treatments exist for ischaemic stroke patients and there are no interventions that can help with inevitable tissue damage, which is inversely proportional to functional recovery. Furthermore, there are several risks associated with reperfusion of hypoperfused tissue including post-reperfusion haemorrhage, reperfusion injury and haemorrhagic transformation (Bai and Lyden, 2015). Novel compounds in development for ischaemic stroke have failed to show suitable efficacy and potency at both phase 2 and phase 3 clinical trials, either because they were found to be toxic to humans or no more efficacious than placebo (Schmidt-Pogoda et al., 2020). In light of the above, there is a clear need for novel treatments for ischaemic stroke as well as therapies to better manage post stoke effects.

1.2 Pathophysiology of ischaemic stroke and reperfusion injury

The World Health Organisation (WHO) defines ischaemic stroke as 'rapidly developing signs of focal/global disturbance in cerebral function' (Howard, 2016). The brain is particularly vulnerable to ischaemia due to its high energy dependence and in order to maintain normal levels of cellular metabolism it requires 20% of total oxygen and 25% of total glucose consumption (Howarth et al., 2012). Therefore, due to the high metabolic demand of neuronal tissue, the obstruction of cerebral blood supply causes rapid energy depletion with ATP reserves used within minutes. To compensate for the absence of oxygen anaerobic glycolysis continues to produce ATP together with lactic acid. Early tissue acidosis begins to occur as lactic acid levels rise, causing localised tissue damage and inhibition of further ATP production. As a result, ATP dependent ion pumps quickly become dysregulated causing cytosolic sodium levels to increase and potassium ions to escape to the interstitial space, water then begins to enter cells via osmosis resulting in hydroponic cell swelling and oedema (Krause and Edvinsson, 2002). Ionic imbalances (including high levels of Ca^{2+} and $Na^{2+)}$ and high levels of adenosine diphosphate (ADP) trigger mitochondrial dysfunction and the generation of

harmful reactive oxygen species (ROS), hydroxyl radicals (OH^-), superoxide (O_2^-), as well as the particularly damaging peroxynitrite ($ONOO^-$) (Doyle et al., 2008). Oxidative stress causes passive DNA damage, including nucleotide base modification as well as single and double strand DNA breaks (Chen et al., 1997; Li et al., 2018).

Under physiological conditions the levels of glutamate are carefully regulated, however critical ionic imbalances and uncontrolled elevations in Ca^{2+} as a result of ischaemia-reperfusion results in membrane depolarisation and the reversal of glutamate transporters present in neurones and glial cells (Banati et al., 1993; Nishizawa, 2001). Excess extracellular glutamate overstimulates neurons causing a glutamate efflux along a concentration gradient and its accumulation within the extracellular spaces of neural tissue (Siegal and Sapru, 2011). Increases in Ca^{2+} ions also activate Ca^{2+} dependent enzymes such as caspases and calpain, a non-lysosomal cysteine protease (Reviewed in Momeni, 2011).

Calpains are an abundant family of proteases and early studies have reported the presence of calpain mRNA in glia and in the cell body and dendrites of neurons (Hamakubo et al., 1986; Perlmutter et al., 1990). More recent studies have implicated their activation in post-ischaemic neuronal damage (Bevers and Neumar, 2008; Cao et al., 2007) and recent studies have shown calpain inhibitors may offer protection against ischaemic damage (Hoang et al., 2011; Potz et al., 2017), but the mechanisms in which calpains contribute to ischaemia-reperfusion injury remains to be fully elucidated.

During the acute inflammatory phase of ischaemia-reperfusion, a plethora of pro-inflammatory signals are released in response to oxidative stress, glutamate release and tissue damage. These include pro-inflammatory cytokines such as IL-6, IL-8, TNF-α and IL-1β as well as adhesion molecules such as ICAM-1, VCAM-1 and MIP-1-α, all of which are responsible for initiating acute inflammatory phase responses during the initial onset of ischaemia (Huang, Upadhyay and Tamargo, 2006; Kriz, 2006; Amantea

et al., 2009). TNF-α mediates the early inflammatory response by stimulating the synthesis of IL-6 and IL-1. In addition, IL-6 and IL-1 stimulate the vascular endothelium increasing the expression of ICAM-1, P-selectin and E-selectin (Chiang et al., 1994; Huang et al., 2006). This in turn promotes leukocyte adherence to the endothelium and increases endothelial permeability. Meanwhile, IL-8 acts as a potent chemoattractant of neutrophils and mediates the diapedesis of peripheral leukocytes into the CNS, see Figure 1.1 (Baggiolini et al., 1993; Pieper et al., 2013). Clinically, elevations in these cytokines have been found to correlate with stroke severity and generally a poorer patient prognosis (Pan and Kastin, 2007; Shaafi et al., 2014).

Reperfusion of hypoperfused tissue is necessary to prevent further damage and reinstate normal levels of cellular respiration. However, paradoxically the return of blood flow brings with it the potential for further damage to already fragile neuronal tissue, hindering neuronal repair mechanisms and overall limiting recovery (Del Zoppo and Mabuchi, 2003). The damaging effects of reperfusion injury have been observed in animal models of acute stroke and have been linked to increases in infarct volume as well as early opening of the BBB. Pivotal features of reperfusion injury include, oxidative stress, mitochondrial dysfunction, inflammation and BBB breakdown, which is closely followed by peripheral immune cell infiltration (L and X, 2016). Furthermore, studies have found that acute ischaemic stroke patients exhibit elevations in malondialdehyde (MDA), a marker of oxidative stress as well as reduced antioxidant capacity (Menon et al., 2020; Milanlioglu et al., 2016).

In response to elevated levels of ROS and pro-inflammatory cytokines generated during ischaemia-reperfusion, stored P-selectin is translocated to the cell surface of endothelial cells. Post-reperfusion, levels of P-selectin peak at around 6 h and causes rapid activation of the endothelium promoting the adherence and rolling of peripheral leukocytes. Meanwhile, the activated endothelium synthesises E-selectin which facilitates immune cell infiltration across the BBB. The pathological importance of

integrins in cerebral ischaemia has been highlighted in several studies (Huang et al., 2000).

Once blood flow returns to hypoperfused tissue, oxygen becomes a catalyst for xanthine oxidase to convert hypoxanthine to uric acid producing superoxide, which is subsequently converted to hydrogen peroxide and the hydroxyl radical (Granger, 1988). These additional elevations in ROS, together with those generated during ischaemia, cause lipid and protein peroxidation and the release of fatty acids from cellular membranes generating various eicosanoids such as thromboxanes, prostaglandins and leukotrienes. In particular, thromboxane-A2 contributes to platelet aggregation and blood vessel constriction, exacerbating damage and contributing to the no-flow phenomenon. In support of this, studies have shown that antagonising the thromboxane-A2 receptor led to a reduction in microglia and macrophage activation in mice, as well as the attenuation of pro-inflammatory cytokines (Yan et al., 2016).

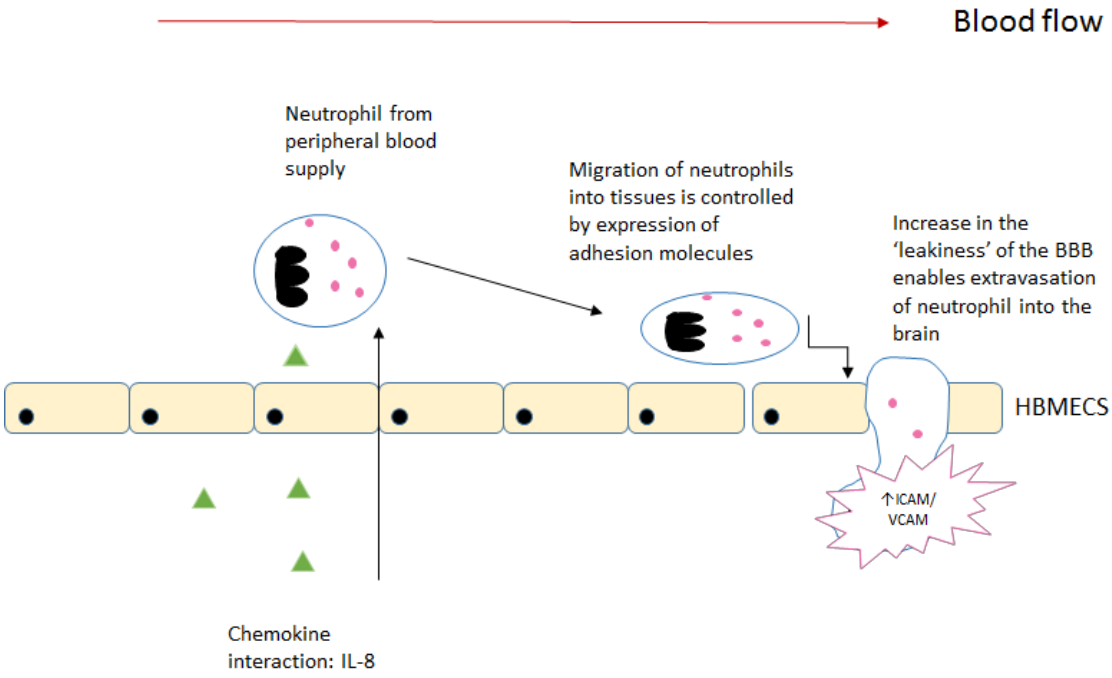

Figure 1.1: Diapedesis of a peripheral neutrophil into the central nervous system (CNS). IL-8 acts as a chemoattractant, whilst increased expression of adhesion molecules by human brain microvascular endothelial cells (HBMECs), such vascular cell adhesion molecule (VCAM)-1 and (intracellular adhesion molecule (ICAM)-1, facilitate the attachment to the endothelium. Created by the author.

1.3 The blood brain barrier (BBB) and neurovascular unit (NVU)

Delicate neuronal tissue is protected by the blood brain barrier (BBB), a specialised structure formed by microvascular endothelial cells, pericytes and astrocytes (Abbott et al., 2006, 2010). These cells, together with microglia, neurons and various matrix components form what is known as the neurovascular unit (NVU)(Blanchette and Daneman, 2015; McConnell et al., 2017). The BBB restricts the movement of blood-borne components into the CNS, maintaining its immune privilege status as well as preventing the entry of xenobiotics.

Endothelial cells form the vascular component of the BBB, they help to maintain angiogenesis and vasodilation, secreting growth factors such as vascular endothelial growth factor (VEGF) and vasodilators such as nitric oxide (NO), ensuring neurons have adequate blood flow to meet metabolic demand. Structurally brain endothelial cells differ from endothelial cells found in the periphery; they lack fenestrations (Fenstermacher et al., 1988), have low pinocytotic activity and a larger mitochondrial content (Oldendorf et al., 1977; Stewart et al., 1994), which is essential to maintain the large number of ATP dependent transporters. Endothelial cells are also connected by specialised tight junctional proteins (TJs) namely, occludins, claudins, zonula occludins (ZO) and junctional adhesion molecule (JAMS), see Figure 1.2 (reviewed in Schoknecht et al., 2015). TJs maintain low paracellular permeability allowing only small polar molecules to cross the barrier unrestricted whilst transporter proteins, namely glucose transporter 1 (GLUT-1) and various solute carrier proteins (SLCs), facilitate the entry of essential larger molecules such as glucose and amino acids into the CNS (Pardridge et al., 1990; Stewart et al., 1994; Zlokovic, 1995 and reviewed in Barar et al., 2016). Additionally, efflux transporter proteins such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), which are present in the luminal side of the endothelium, function to prevent the entry of harmful solutes into the brain (Abbott et al., 2010). Pericytes provide mechanical support to endothelial cells, wrapping around the endothelium via peg and socket style arrangements covering 70-80% of the capillary surface (Allt and Lawrenson, 2001; Armulik et al., 2005; Bell et al., 2010;

Zimmermann, 1923). These cells are part of the vascular smooth muscle lineage and in the brain they secrete vasoactive substances to generate functional responses to changes in the BBB microenvironment (Dore-Duffy, 2008; Von Tell et al., 2006). Specifically, these cells help endothelial cells regulate blood flow, capillary diameter, angiogenesis as well as supporting the stability of the vasculature (Peppiatt et al., 2006).

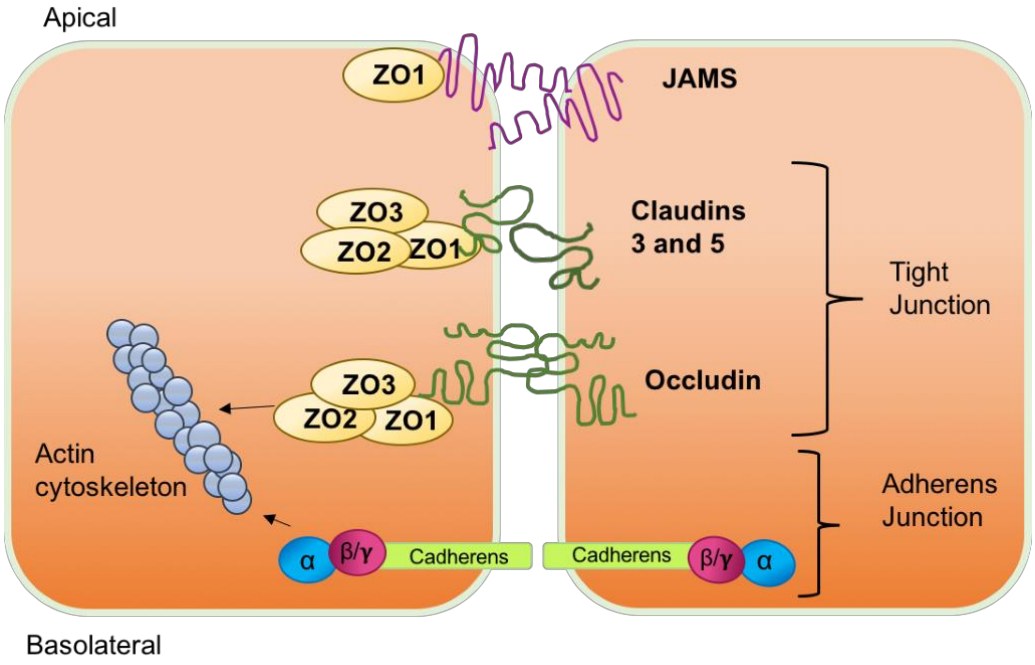

Figure 1.2. A schematic of the tight junctional proteins including zonnula occludins (ZO-1,2,3), junctional adhesion molecules (JAMs), claudins 3,5 and occludin that are present between endothelial cells which form the blood-brain barrier (BBB). Created by the author.

Astrocytes are supportive glial cells and their end feet envelope both pericytes and endothelial cells, enabling crosstalk between the vascular component of the BBB and neurons. They form connections with all neuronal synapses and although they do not propagate impulses themselves, they help to regulate neuronal excitability playing a key role in neurotransmitter homeostasis (Ransom and Ransom, 2012). Astrocytes also provide metabolic support for neurons and express transporters for glutamate, GABA, and glycine to facilitate rapid reuptake of neurotransmitters from the synaptic cleft. Glutamate is a major excitatory neurotransmitter and must be removed rapidly from

the extracellular space to prevent excitotoxicity and astrocytes conduct glutamate reuptake against a concentration gradient using ATP, sodium-dependent glutamate transporters. (Sattler and Rothstein, 2006; Seifert et al., 2006; Sofroniew and Vinters, 2010).

Microglial cells are resident immune cells of the CNS, participating in immune surveillance, neuronal plasticity and CNS homeostasis which is governed by neuronal-glial crosstalk (da Fonseca et al., 2014; Tremblay et al., 2011). Microglia migrate to the brain parenchyma during embryogenesis where they closely associate themselves with the brain microvasculature, participating in BBB development and angiogenesis (Alliot et al., 1999; Nayak et al., 2014). Normally the activation state of microglia is tightly controlled; they are either directed to the ameboid pro-inflammatory M1 phenotype or the anti-inflammatory, ramified M2 phenotype (Kettenmann et al., 2011). The M1 phenotype secrete a variety of proinflammatory mediators such as IL-6 and TNF-α to signal to nearby cells of the presence of tissue injury and recruit immune cells. Conversely, the M2 phenotype governs anti-inflammatory mechanisms, immune surveillance, tissue repair and regeneration, as well as contributing extensively to synaptic plasticity and remodelling (Benarroch, 2013).

1.3.1 Impact of ageing on the BBB and NVU

Since ischaemic stroke occurs predominantly in the elderly, it is important to recognise age-related changes that occur within the NVU. Neuronal loss, cellular senescence, oxidative stress and impaired cellular metabolism occur as part of the natural aging process, but as they accumulate there are consequences to the normal physiology of the brain and NVU. Thus, not only is the prevalence of neurogenerative conditions and ischaemic stroke increased with age, but the capacity of the brain to cope with such diseases is also diminished. Senile endothelial cells exhibit reduced secretion of growth factors which impairs the ability of the brain microvasculature to maintain angiogenesis with age (Lähteenvuo and Rosenzweig, 2012). Pericytes also undergo morphological changes with aging which contribute to BBB impairment and exacerbation of damage by neurodegeneration and ischaemia. In the retina of aging

rats, Hughes et al., (2006) found increases in α-smooth muscle actin as well as alterations in the length and orientation of desmin, features typically found pericyte senescence. Bell and collegues (2010) showed that there is pericyte loss with aging in platelet-derived growth factor receptor (PDGFR)-β deficient mice, which led to microvascular reductions, diminished cerebral blood flow and BBB breakdown. Senile astrocytes exhibit marked hypertrophy, increased expression of glial fibrillary acid protein (GFAP), decreased density of glucose and glutamate-aspartate transporters (GLUT-1 and GLAST), as well as a increases in levels of aquaporin-4 (Berciano et al., 1995; Ferrer, 2017; Nichols et al., 1993; Owasil et al., 2020; Peinado et al., 1998). Therefore, senile astrocytes less equipped to maintain normal physiology and mitigate CNS pathologies and are more vulnerable to damage.

1.3.2 Consequences of ischaemia-reperfusion on BBB physiology

Ischaemia generates large amounts free radicals triggering oxidative stress, lipid peroxidation, protein dysfunction as well as directly downregulating TJs and activating MMPs which degrade extracellular matrix (Haorah et al., 2007; Li et al., 2018; Schreibelt et al., 2007). Moreover, under normal physiological conditions endothelial cells limit immune cell infiltration into the CNS, however post ischaemic BBB breakdown, together with increased expression of chemokines and adhesion molecules, enables uncontrolled infiltration of peripheral leukocytes into the CNS where they secrete matrix metalloproteinases (MMPs) and additional proinflammatory mediators, further exacerbating BBB breakdown (Gidday et al., 2005). Following ischaemia there is also a significant degree of pericyte loss and remaining pericytes have an increased expression of vascular endothelial growth factor (VEGF), which is known to contribute to increases in BBB permeability and correlates to poor prognosis in stroke patients (Bai et al., 2015; Escudero et al., 2020). Both of these pathophysiological responses have been shown to be detrimental to the BBB function as even a modest (20%) loss of pericyte coverage induces vascular changes at the BBB. Pericyte loss is linked to abnormal astrocyte polarization, increased endothelial transcytosis flux and ultimately compromised BBB integrity (Armulik et al., 2010; Bell et al., 2010).

During ischaemia, damaged neurons stimulate the proliferation of astrocytes bordering the lesion, as well as the activation of astrocytes in the ischaemic core (Barreto et al., 2011; Popa-Wagner et al., 2007). This process, known as astrogliosis, triggers alterations in morphology including hyperplasia and hypertrophy, increases in the expression of filament proteins such as GFAP and nestin, as well as stimulating the secretion of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 (Chiang et al., 1994; Herx and Yong, 2001; Li and Chopp, 1999; Liu and Chopp, 2016; Sims and Yew, 2017). Hypertrophic astrocytes exhibit elongated processes which envelope the ischaemic region in an attempt to contain the damaged tissue, preventing its spread to the penumbral region which is potentially salvageable (Kajihara et al., 2001). After a few days post ischaemia, astrocytes begin to form a glial scar which consists of reactive astrocytes and extracellular matrix components such as chrondroitin proteoglycans (CSPGs) and whilst the scar reduces in size over time, interwoven astrocytic end feet and higher levels of GFAP remain present (Sims and Yew, 2017). The development of a glial scar can be both beneficial and counterproductive (reviewed in Ferrer, 2017), whilst it offers protection to the surrounding salvageable and healthy tissue it can also hinder neuronal repair mechanisms. Reactive astrocytes are protective against nitric oxide toxicity post ischaemia (Chen et al., 2001) and astrocyte ablation prolonged infiltration of CD45-positive leukocytes and the failure of the BBB (Bush et al., 1999). In addition, Li and colleagues found that mice lacking in characteristic reactive astrocyte markers, GFAP and vimentin, exhibited larger infarct volumes and a 44% lower uptake of glutamate vs WT controls, highlighting the protective role of reactive astrocytes in ischaemia (Li et al., 2008). On the other hand, prolonged astrocyte activation post stroke can increase neuronal damage via excitotoxic damage, increases in proinflammatory cytokines, as well as contributing to enhanced BBB permeability and preventing axonal sprouting (Li et al., 2014; Silver and Miller, 2004).

Post insult, microglia are rapidly activated by excitotoxic signalling and undergo morphological changes to their M1 phenotype. In the acute phase, they secrete a

plethora of proinflammatory cytokines and nitric oxide which stimulates surrounding cells and recruits other microglia to the site of injury, causing a cycle of uncontrolled activation, which can lead to additional tissue damage (Jolivel et al., 2015; Taylor and Sansing, 2013). Likewise, quantitative evidence has showed that 24 h post insult, the ischaemic core exhibits marked changes in microglia including increased CD11b expression, shorter processes and amoeboid morphology (Morrison and Filosa, 2013). Excessive, microglial activation can disrupt BBB integrity post stroke, downregulating/redistributing tight-junctional proteins and reorganising the actin cytoskeleton (da Fonseca et al., 2014). Despite the clear role of microglia in facilitating damage post ischaemia, studies have shown that the absence of microglia results in a larger infarct size and the deletion of microglia can adversely affect the integrity of the BBB (Dudvarski Stankovic et al., 2016; Szalay et al., 2016). Thus, some degree of microglial activation is necessary to minimise post-stroke injury, but this must be adequately controlled to prevent further tissue damage.

1.4 Endocannabinoids and the endocannabinoid system

The endocannabinoid system consists of the cannabinoid receptors (CB_1 and CB_2), endogenous lipid ligands that activate them and the enzymes involved in their synthesis and degradation. CB_1 was first cloned in 1990, followed by CB_2 in 1993 which shares only 44% homology to CB_1 (Howlett et al., 2002; Munro et al., 1993). Both receptors are G-protein coupled receptors part of the $G_{1/0}$ family and as such their activation inhibits adenylyl cyclase, a key regulatory enzyme responsible for a diverse range of cellular responses, whilst positively activating mitogen-activated protein kinases (Pertwee and Ross, 2002). The distribution of the cannabinoid receptors varies throughout the body, while CB_1 receptors are abundant in the CNS, CB_2 receptors are present but expressed at a significantly lower level (Howlett et al., 2002; Pertwee, 1997). CB_1 receptors are present within the cortex, basal ganglia, hippocampus and cerebellum where they are localised to axon terminals and terminal axon segments (Herkenham et al., 1990). Aside from the CNS, CB_1 receptors are also found in reproductive tissues, the gastrointestinal (GI) tract, heart, lungs and bladder (Pertwee,

1997). On the other hand, CB_2 receptors are expressed by immune cells such as resident microglia where they play a role in orchestrating the immune response, including the release of pro-inflammatory cytokines.

Shortly after CB_1 and CB_2 were discovered, the two most studied endocannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG) were identified from pig brain and canine gut respectively (Devane et al., 1992; Mechoulam et al., 1995). They are synthesised on demand via the enzymatic hydrolysis of precursors derived from phospholipid bilayers in response to cellular stimuli, specifically elevations in intracellular calcium. Synthesis of 2-AG occurs when phospholipase C is converted to diacylglycerol, which is then converted to 2-AG via the enzyme diacylglycerol (DAG) lipase. Conversely, AEA is produced from the phospholipid N-acylphosphadiyl ethanolamide. AEA is also synthesised by the same route as two other major endocannabinoids oleoylthanolamide (OEA) and palmitoylethanolamide (PEA), which in general, are found in higher concentrations than AEA (Di Marzo et al., 2005). Altogether these compounds are known as the N-acylethanolamines (NAGEs) and are known to exert anti-inflammatory effects. The enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAG-lipase) hydrolase AEA (as well as OEA and PEA) and 2-AG respectfully (Reviewed in Di Marzo et al., 2005).

1.4.1 Endocannabinoid system and neuroprotection

Modulation of the endocannabinoid system can illicit neuronal plasticity, neuroprotection and neurotoxicity, indeed endocannabinoids are known to regulate mood, synaptic excitability, hypokinesia and analgesia due to their activity at CB_1 and other receptors such as 5-HT_{1A}, TRPV1 (Cristino et al., 2020). Moderate increases in endocannabinoids post exercise, particularly AEA and 2-AG, may be correlated with improvements in neuronal plasticity (Heyman et al., 2012; Raichlen et al., 2012; Sparling et al., 2003). Heyman et al., (2012) found that increases in AEA in cyclists was positively correlated with increases in brain derived neurotrophic factor (BDNF), and our group showed that singing increased levels of PEA, OEA and AEA and cycling also

increased levels of OEA in healthy volunteers (Stone et al., 2018). Thus, elevations in endocannabinoids post exercise could be linked to the positive effects of physical activity on cognitive function and mood. Endocannabinoids and endocannabinoid receptors are also affected post neuronal injury, for example in a model of stroke CB_1 knockout mice exhibited higher post-ischaemic mortality, greater infarct sizes and worse neurological function and studies in humans have found increased levels of AEA in stroke and PD patients (Naccarato et al., 2010; Parmentier-Batteur et al., 2002; Pisani et al., 2010; Schäbitz et al., 2002).

1.5 Phytocannabinoids

Phytocannabinoids are compounds that are naturally present in the cannabis sativa herb, with cannabidiol (CBD) and delta 9-tetrahydrocannabinol (Δ^9-THC) being the most widely studied. Δ^9-THC was first identified in by Gaoni and Mechoulam, (1964) and later synthesised in 1967 by the same group (Mechoulam et al., 1967). Initial studies on cannabis focused on the analgesic and sedative properties of THC (Paton, 1973) and its psychoactive effects (Pertwee, 1988), which were later found to be mediated by its action at CB_1 receptors. CBD was first identified in 1963 (Mechoulam and Shvo, 1963) and unlike Δ^9-THC it has little affinity for CB_1 receptors, thus displaying no psychotropic activity. Since its discovery, CBD has shown efficacy in a plethora of conditions largely due to its promiscuity at different receptors; acting as an antioxidant, anti-inflammatory and anticancer agent, as well as displaying anxiolytic and anti-convulsant properties (Pisanti et al., 2017; Russo and Marcu, 2017). Other phytocannabinoids were discovered not long after Δ^9-THC and CBD, including cannabigerol (CBG) which was first identified in 1964 (Gaoni and Mechoulam, 1964) cannabichromene (CBC) in 1966 (Gaoni and Mechoulam, 1966), cannabidivarin and THCV in 1970 (Gill, Paton and Pertwee 1970, Vollner, Bienieke and Korte 1969).

1.5.1 Other targets for cannabinoids

Cannabinoids can also modulate a wide range of other receptors aside from the cannabinoid receptors, CB_1 and CB_2. They have also been shown to activate

peroxisome proliferator activated receptors (PPARs), serotonin receptors and transient receptor potential (TRP) channels, as well as orphan GPRs such as GPR55, GPR18 and GPR119 (De Petrocellis and Di Marzo, 2010).

PPARs are ligand activated transcription factors that are part of the nuclear hormone superfamily and are divided into three subtypes; PPARα, PPARδ and PPARγ. They are expressed in almost every cell in the body and are responsible for regulating different aspects of gene expression, primarily those involved in fatty acid metabolism and inflammation (reviewed in Pistis and O'Sullivan, 2017). Upon activation, for example by hormonal or environmental stimuli, PPARs bind to the retinoid X receptor (RXR) forming a heterodimer. This triggers the recruitment of coactivators such as PGC-1α or E1AS binding protein p300 which have histone acetylase activity (Marion-Letellier et al., 2016). The PPAR-RXR heterodimer complex binds along with a cofactor to regions of DNA known as peroxisome proliferator response elements (PPREs) which are present on the promotor regions of target genes (Berger and Moller, 2002; Osumi et al., 1991). It has been deduced that the acetylation of histone proteins alters the organisation of the tightly packed chromatin, thus enabling RNA polymerase II to bind and imitate gene transcription. In the absence of a stimulus, heterodimers are associated with a co-repressor complex which blocks gene transcription (Ziouzenkova and Plutzky, 2004). PPARα is predominantly involved in regulating lipid and lipoprotein metabolism and its expression is relatively high in cardiomyocytes, hepatocytes, the kidney, brown adipose tissue, the GI tract (enterocytes) and immune cell types including monocytes and microglia (Herkenham et al., 1990; Marion-Letellier et al., 2016; Mottillo et al., 2012). PPARδ expression is ubiquitous but varies from tissue to tissue and can be altered in disease states. PPARy is expressed in brown and white adipose tissue, the pancreas, large intestine and various immune cells and its activation mediates inhibition of helper T-cells responses, as well as having a role in glucose metabolism, regulation of fatty acid storage, adipocyte differentiation, insulin sensitivity and cell growth (Tyagi et al., 2011). All PPAR isoforms have a central role in mediating the inflammatory response. In general, PPARs have large, complex ligand

binding domains which enables them to bind a wide range of ligands, including their natural ligands such as fatty acids and eicosanoids (Gervois et al., 2000; Krey et al., 1997). Not surprisingly, as cannabinoids are lipid-based molecules, a number of endocannabinoids and phytocannabinoids have been shown to bind and/or activate different PPAR isoforms (O'Sullivan, 2016). PPARα has been shown to be activated by the endocannabinoid like N-acylethanolamines OEA and PEA at concentrations achieved under normal physiology (Fu et al., 2003). Further, Sun et al., (2007) confirmed that OEA, PEA and AEA bind to the PPARα binding domain. THC was found to increase the activity of PPARγ (O'Sullivan et al., 2005) and the ability of THC to bind to PPARγ was later confirmed by Granja et al., (2012). Emerging evidence has also supported the interaction of PPARγ with other phytocannabinoids such as cannabigerol (CBG) and tetrahydrocannabidiolic acid (THCA) and that interaction with this receptor mediates some of their protective effects (Stone et al., 2020).

Transient receptor potential (TRP) channels are a group of membrane spanning proteins comprised of four subunits and six transmembrane helices (S1-S6). There are six subfamilies of TRP channels; vallinoid type (TRPV), ankyrin type (TRPA), melastatin type (TRPM), polycystin type (TRPP), canonical type (TRPC) and mucolipin (TRPML), which are further divided into different isoforms. TRP channels are predominantly expressed in nociceptive sensory neurons where they mediate the transduction of a number of chemical and physical stimuli, including but not limited to pain, pH and temperature sensation (Reviewed in Zheng, 2013). Following exposure to a stimulus they can gate both mono and divalent cations, including Ca^{2+}, however the full mechanisms associated with TRP activation have yet to be fully understood (Gees et al., 2010). TRP channels, specifically TRPV1, TRPA1, TRPV4, have been implicated in the pathogenesis of several neurodegenerative disorders and ischaemic stroke (Huang et al., 2020; Miyanohara et al., 2015; Pires and Earley, 2018). TRPV4 channels expressed in astrocytes form complexes with AQP4, contributing to osmotic homeostasis, however under ischaemic conductions TRPV4 channels can become overactivated causing osmotic swelling and potentiating injury (Jo et al., 2015). Excessive TRPV1

activation has also been implicated in exacerbating neuronal injury in ischaemic stroke and interestingly, TRPV1 antagonist capsazepine was found to reduce neurological deficits and infarct volume in mice post middle cerebral artery occlusion (MCAO) (Miyanohara et al., 2015).

To date research has found that endocannabinoids, phytocannabinoids and synthetic cannabinoids can activate TRPV1,2,3,4, TRPA1 and TRM8, either as agonists or antagonists, with the majority of research focused on their activation of TRPV1 (Muller et al., 2019). Table I provides a summary of the roles of the different TRP channels that are activated by cannabinoids.

TRP Channel	Location	Role in normal/pathophysiology	Activation by cannabinoids
TRPV1	All major classes of nociceptive neurons, detected in: dorsal root ganglion (DRG), trigeminal ganglion (TG) and nodose ganglion (NG).(Nilius et al., 2008)	• Responsible for strong burning sensations and cutaneous pain. • Analgesia can be evoked if these channels are desensitised • Histaminergic itch. (Shim et al., 2007)	AEA with a binding affinity similar to capsaicin (Zygmunt et al., 1999). OEA (Ahern, 2003). Activated by CBD, CBG and CBC, CBDV can also activate and desensitise (Iannotti et al., 2014; Ligresti et al., 2006).
TRPV2; 50% sequence homology to TRPV1	Medium and large diameter sensory neurons. Heart, GI tract, smooth muscle. Highly expressed in macrophages. (Nilius et al., 2008)	• Insensitive to protons but can be activated by high temperatures (>52 °C) and inflammation. • Chronic pain • Mechano-sensor in vascular smooth muscle cells. (Nilius et al., 2008)	Not activated by endocannabinoids, mainly activated by phytocannabinoids. Activated by CBD (EC_{50} 3.7 µM), THC and CBN (Maréchal and Zou, 2013; Muller et al., 2019)
TRPV3; 43% sequence homology to TRPV1	Present in the DRG, trigmental ganglia. Testis, skin (keratinocytes), tongue.	• Responsible for pain and itch sensations. • Thermosensor between 33-39°C. Becomes sensitized in repeated heat application.	Not activated by endocannabinoids. CBD and THCV were both able to activate TRPV3, CBGV and CBGA were found to desensitise TRPV3 (De Petrocellis et al., 2012)
TRPV4; >40% sequence homology to TRPV1	CNS, epithelial cells, osteoblasts, blood vessels (including cerebral). Astrocytes and microglia (Benfenati et al., 2007)	• Role in vascular function • Osmotic pressure in the brain. • Skin barrier function and nociception • Responds to 25-34 °C (Nilius et al., 2008)	CBDV and THCV evoked a Ca^{2+} response in TRPV4 expressing cells. Desensitised by CBN, CBGV, CBGA, CBG (De Petrocellis et al., 2012)
TRPA1	A subset of peripheral sensory neurons. Dorsal root, vagal and trigeminal ganglion neurons. (Baraldi et al., 2010)	• Activated by compounds in mustard, garlic etc. and by temperatures below 17°C (Bandell et al., 2004; Baraldi et al., 2010) • Important role in neuropathic pain, hyperalgesia, inflammatory pain.	AEA exhibits a high efficacy. CBC (EC_{50} 90 nM), CBD (EC_{50} 110 nM), CBN (EC_{50} 180 nM). (De Petrocellis et al., 2008, 2011, 2012)
TRPM8	Primary afferent neurons (Story et al., 2003).	• Activated below 27°C in response to cooling compounds, menthol eucalyptus. • Involved in androgen receptor positive prostate cancers	Antagonised by AEA and phytocannabinoids including CBD, CBG, THC, and THCA (De Petrocellis et al., 2007, 2008)

Table I: Summary of the different TRP channels activated by endo and phytocannabinoids; TRP channels of vallinoid type (TRPV1,2,3,4), ankyrin type (TRPA1), melastatin type (TRPM8). Endocannabinoids; anandamide (AEA) and oleoylethanolamide (OEA). Phytocannabinoids; cannabidiol (CBD), delta-9 tetrahydrocannabinol (Δ^9-THC), cannabidivarin (CBDV, cannabigerol (CBG), cannabigerolic acid (CBGA), cannabigerovarin (CBGV), cannabichromene (CBC), cannabinol (CBN) and tetrahydrocannabidivarin (THCV).

Serotonin receptors, otherwise known as 5-hydroxytryptamine (5-HT) receptors are involved in the regulation of mood, manifestation of anxiety and depression, as well as immune and cardiovascular regulation. Most 5-HT receptors are metabotropic and are coupled to G-proteins which mediate their signal transduction. Cannabinoids have been shown to modulate the 5-HT_{1A} subtype of these receptors, having effects such as neuroprotection, reducing the stress response and acting as anti-emetics. 5-HT_{1A} receptors are widely distributed in the brain and areas associated with modulating stress and anxiety, such as the raphe nuclei. Resstel et al., (2009) found that CBD (10-20 $mg.kg^{-1}$) decreased stress induced cardiovascular responses which was blocked by WAY100635, a 5-HT_{1A} receptor antagonist. At low doses (5-10 mg.kg-1) CBD is an effective antiemetic in different models of nausea (Kwiatkowska et al., 2004; Rock et al., 2008). Studies have shown that CBDs acidic precursor, cannabidiolic acid (CBDA 0.1/0.5 mg.kg-1, i.p) acts as a more potent anti-emetic than CBD, reducing nausea in three different models in mice, an effect mediated through the 5-HT_{1A} (Bolognini et al., 2013). CBG has been found to act as a 5-HT_{1A} receptor antagonist (Cascio et al., 2010) and recently CBGs neuroprotective effects against rotenone induce neurotoxicity were mediated partly by interaction with 5-HT_{1A} receptors (Echeverry et al., 2020).

GPR55/ GPR18

Despite having a low sequence homology to CB_1 (13.4%) and CB_2 (14.4%), orphan GPCR, GPR55 was postulated to be a third cannabinoid receptor because of its activation by some cannabinoid like compounds. It was first identified in 1999 (Sawzdargo et al., 1999) and is expressed on immune cells, including monocytes, NK and microglial cells and is present in both the CNS and the GI tract (Ryberg et al., 2009). An accumulating body of evidence implicates GPR55 in neuronal hyperexcitability and epilepsy, corroborated by recent findings indicating that GPR55 expression is elevated in the epileptic hippocampus (Gray and Whalley, 2020; Rosenberg et al., 2018). Ryberg et al., (2007) showed that cannabidiol displayed antagonist properties at GPR55 and that anandamide stimulated GTPγS binding with an EC_{50} of 18 nM. CBDs action at GPR55 has been proposed to meditate its

antiepileptic effects; Kaplan and colleagues (2017) found that GPR55 was responsible for CBDs effects on increasing inhibitory neuronal excitability. Despite these promising advances regarding the biological roles of GPR55 and its cannabinoid ligands, the link between the role of GPR55 and other pathologies that affect the CNS, such as neuroinflammation, remains unclear. Saliba et al., (2018) found that activation of GPR55 by antagonists mediated protective effects in microglial cells and Hill et al., (2018) found that GPR55 activation led to an increase in the proliferation rate of human neuronal stem cells *in vitro.*

GPR18, another orphan GPCR, has been found to be activated by Δ^9-THC and abnormal cannabidiol. Early studies showed GPR18 is richly expressed in the testis and spleen (Gantz et al., 1997) and was later found to also be expressed in peripheral leukocytes, microglia and lymph nodes (McHugh, 2012). GPR18 is also involved in the action of N-arachidonoylglycine, the carboxylic metabolite of anandamide, in the modulation of the immune response and the cross talk between microglia and neurons (Burstein et al., 2011).

1.5.2 Phytocannabinoids, ischaemic stroke and neuroprotection

Accumulating evidence supports the neuroprotective effects of phytocannabinoids in various models of neurodegeneration and neurological dysfunction (Cristino et al., 2020; Fernández-Ruiz et al., 2013). Due to their lipophilic nature they readily penetrate the BBB (Deiana et al., 2012) and recently Brzozowska et al., (2016) found that CBD was not a substrate for P-gp or BCRP, thus these transporters do not limit CBD uptake into the brain. Preclinical studies on Δ^9-THC and CBD have been conducted in models of AD, PD, ALS, MS, traumatic brain injury (TBI) and stroke. In an animal model of AD, 3 mg.kg Δ^9-THC + COX-2 inhibitor was shown to reduce amyloid beta plaques (Maroon and Bost, 2018). *In vitro* CBD attenuated the damaging effects of amyloid beta (AB) peptide and in a murine model of AD CBD attenuated reactive gliosis and decreased the production of pro-inflammatory mediators (Esposito et al., 2006, 2007; Iuvone et al., 2004). Similarly, CBD improved signs of EAE in mice due to its capability to suppress

the immune response; including microglial activity and T-cell proliferation (Kozela et al., 2010). In PD patients, CBD decreased PD associated psychosis symptoms and improved patient well-being (Chagas et al., 2014; Zuardi et al., 2009). In ischaemia, Δ^9-THC and CBD have also displayed neuroprotective effects in various *in vitro* and *in vivo* models (Alvarez et al., 2008; Castillo et al., 2010; Louw et al., 2000; Zani et al., 2007). Pre-treatment with CBD (100 and 200 ng/rat) for five days prior to initiating a 60 minute MCAO resulted in a significant decrease in infarct size (Khaksar and Bigdeli, 2017). Ceprián et al., (2017) found that CBD administration ameliorated excitotoxic associated damage and improved neurological outcome post MCAO. Other phytocannabinoids have also exhibited neuroprotective potential (Reviewed in Stone et al., 2020), CBDV and Δ^9-THCV have displayed efficacy an antiepileptics (Amada et al., 2013; Hill et al., 2010, 2013; Ma et al., 2008), CBG as an anti-neuroinflammatory agent (Carrillo-Salinas et al., 2014; Rodríguez-Cueto et al., 2018) and Δ^9-THCA as a neuroprotectant (Nadal et al., 2017).

Clinical studies assessing the therapeutic potential of cannabinoids and neurological disorders have been predominantly in epilepsy, MS and Parkinson's. In a human trial with MS patients, Δ^9-THC ameliorated urinary incontinence, spasticity and tremors in MS, but no effect on disease progression was observed (Baker et al., 2000; Freeman et al., 2006; Zajicek et al., 2003). So far, CBD alone (Epidiolex, GW Pharmaceuticals, Cambridge, UK) and as a 1:1 formulation with Δ^9-THC (Sativex, GW Pharmaceuticals, Cambridge, UK) are the only licenced cannabis-based medicines and are prescribed to treat Dravet syndrome and Lennox-Gastaut syndrome, both rare forms of childhood epilepsy. There are, however phase 3 trials in the pipeline to assess whether CBD oil is effective in ALS (Urbi et al., 2019), as well as phase 2 trials to assess the efficacy of Sativex in spasticity associated with motor neuron disease and multiple sclerosis (MS) (Markovà et al., 2019; Riva et al., 2019). The aforementioned findings emphasise the neuroprotective potential of cannabinoids, the role of the endocannabinoid system in neurological disease and the clinical translatability of these compounds.

1.6 Summary

Most therapies targeting stroke and other neurodegenerative conditions have only targeted neurons in order to preserve functionality. However, from the high number of failed neuroprotective agents, it is clear that protecting neurons alone is insufficient to improve neurological outcome in these conditions. Various compounds have displayed efficacy in animal models of ischaemic stroke, however there has been little success when these compounds reach clinical development (Schmidt-Pogoda et al., 2020). O'Collins et al., (2006) highlighted the importance of only taking worthy drugs to clinical trial and only if the animal study data is robust, valid and relevant to the clinical cause. In order to be a successful treatment in conditions such as ischaemic stroke, multiple facets of the disease need to be targeted. The unique pharmacology of cannabinoids makes them particularly desirable as novel therapies as they can potentially target multiple areas of dysfunction, as well as modulate different cells that constitute the NVU.

1.7 Thesis aims

1.7.1 Rationale

In vitro models to simulate stroke are useful tools to screen neuroprotective agents and determine which compounds look the most promising to be taken to *in vivo* studies (Antonic et al., 2012). We aimed to develop a more clinically relevant four-cell NVU model consisting of primary human cells to model ischaemic stroke *in vitro* using a previously described oxygen-glucose deprivation (OGD) protocol (Hind et al., 2015, 2016). Using OGD to model stroke exhibits the majority of effects associated with *in vivo* ischaemia-reperfusion injury and is therefore a relevant methodology to simulate stroke *in vitro* (Holloway and Gavins, 2016). Secondly, we aimed to gain insight into the neuroprotective properties of CBG, CBDV and CBDA or lack thereof and to establish whether these compounds may be useful as potential treatments for ischaemic stroke. CBG and CBDV effects in ischaemia are largely unknown but were found to be protective in several pre-clinical models of other neurodegenerative diseases (based

on the results of our systematic review; Chapter 4). In light of these data, we hypothesised that CBDV and CBG would also prove to be protective in a model of ischaemic stroke. CBDA has been found to exhibit similar biological and pharmacological properties to CBD, but is significantly overlooked compared to its neutral derivative (Reviewed in Formato et al., 2020). Given that previous work from our group found CBD prevented increases in BBB permeability in an OGD model (Hind et al., 2016), we hypothesised that CBDA may also be protective against BBB permeability, perhaps by a similar mechanism to CBD.

1.7.2 Objectives

Therefore, the aim of this thesis was to assess the neuroprotective properties of minor phytocannabinoids, CBG, CBDV and CBDA, on cells of the NVU and to assess whether CBDA, like CBD, was able to modulate BBB permeability in an *in vitro* model of ischaemic stroke. To achieve this, we set out to test CBG, CBDV and CBDA in monocultures of cells of the NVU and to test CBDA in a BBB model of our own design. To simulate ischaemic stroke *in vitro* we used an oxygen-glucose deprivation (OGD) protocol.

Specifically, our objectives were as follows:

1. To develop a more clinically relevant in *vitro* BBB model comprised of primary human cells to study permeability.

Based on evidence from previous studies on CBD, results from our systematic review and the wider literature:

2. To investigate the neuroprotective properties of minor phytocannabinoids, CBG, CBDV and CBDA in cells of the NVU using an OGD protocol.
3. To investigate whether CBDA modulates BBB permeability when administered before an OGD protocol.
4. To explore the potential mechanisms of action by which CBDV, CBG and CBDA mediate their protective properties following outcomes from aims 2 and 3.

In addition to my central thesis aims, during my PhD studies we also investigated the levels of endocannabinoids on volunteers who underwent exercise activities and singing. This provided additional lab training, academic publication writing and clinical study design experience. Specific aims of this project were:

1. To assess whether different activities influenced mood as measured by visual analogue score (VAS) questionnaire.
2. To establish whether changes in mood were correlated with levels of circulating endocannabinoids, namely anandamide, 2-AG, OEA and PEA.

2. General Methods

Cell culture

All cell culture work was performed under sterile conditions within a biological class II cabinet (HEPA filtered). Personal protective equipment (PPE) was worn during cell culture and aseptic technique was used throughout. Surfaces were disinfected with 1% distel, followed by 70% ethanol. Each item was disinfected with 70% ethanol before placing in the safety cabinet. Cell cultures were maintained in an incubator; 37°C with 5% CO_2 and 95% air.

Cell types

Primary cell cultures included human astrocytes (HA; SC-1800), human pericytes (HP; SC-1200), human brain microvascular endothelial cells (HBMECs; SC-1000) and human neurons (HN; SC-1520). All cells were purchased from Caltagmedsystems (UK), originally sourced from Sciencell (USA) in vials of 1x10^6 cells per mL. In order to prevent de-differentiation, cells were not used beyond passage 6, as per manufacturer's recommendations.

Specialised medium

Cells were maintained in their respective recommended specialised medium purchased from Caltagmedsystems (UK), originally sourced from Sciencell (USA). Media was supplied as a basal phenol red formula containing essential nutrients along with a bullet pack consisting of foetal bovine serum (FBS), 10,000 units/mL penicillin and streptomycin (pen-strep, 1% final volume). Endothelial cell medium was supplemented with 5% FBS, pericyte and astrocyte medium were supplemented with 1% FBS. Neuronal medium was not supplemented with FBS. A growth supplement mix specific to each cell type was added to each of the different media (Caltagmedsystems (UK). These were originally formulated and sourced from Sciencell (USA).

Revival, Sub-culturing and cryopreservation

To initiate cell cultures vials of cells in freezing medium containing 5% dimethylsulfoxide (DMSO) were removed from liquid nitrogen storage and gently

thawed in a water bath at 37°C. Once vial contents were approximately 50-75% thawed they were pipetted into T75 cell culture flask (75cm^2) containing 13 mL of the appropriate pre-warmed medium. The flask was then placed in an incubator (37°C, 5% CO_2) and the cells were allowed to adhere to the flask surface for a minimum of 6 h before replacing the medium. Upon reaching 80-90% confluency cells were passaged depending on each cell types doubling number and experimental demand. Typically, astrocytes and HBMECs were passaged in a 1:3 ratio of cells to fresh medium and pericytes in a 1:5 ratio cells to fresh medium. Cells were either passaged into new flasks (to increase cell populations), into cell culture plates or inserts, or cryopreserved for future use.

Cells were passaged as follows: medium was aspirated and discarded; 8 mL of pre-warmed phosphate buffered saline (PBS, Gibco) was used to rinse the cell monolayer to remove any excess medium containing FBS. This was then aspirated and discarded and 2 mL of trypsin-EDTA (0.25% in 1x PBS) was pipetted into the T75 flask. For HBMECs 2 mL StemPro™ accutase (Gibco, ThermoFisher Scientific) was used instead of trypsin. The flask was gently rocked from side to side to ensure full coverage of the cells with Trypsin-EDTA, then placed into the cell culture incubator (37°C, 5% CO_2) for 1-2 minutes. The flasks were removed from the incubator and firmly tapped. The flasks were observed under a light microscope to ensure cells were detached. If cells were still adhering to the flask, cells were placed back in the incubator for 1-2 minutes and the process repeated until cells were detached. Once cell detachment was confirmed, 5-7 mL of cell culture medium was added to neutralise the trypsin. Media was rinsed over any remaining cells left on the bottom of the flask and repeated to ensure the maximum number of cells were re-suspended in the media. Then medium was aliquoted into a falcon tube. Cells were centrifuged at 1000 rpm (320 xg) for 5 minutes to form a cell pellet. The supernatant was carefully aspirated and discarded. The cell pellet was gently re-suspended in 1 mL of medium. The cell suspension was subsequently diluted further to the desired split ratio (i.e 3 mL for a 1:3 ratio, 1 mL into

each T75 flask), to be seeded into cell culture plates (i.e 12 mL, 500 µL each well for a 24 well plate), or to be cryopreserved for later use.

To cryopreserve cells, cells were pelleted in the procedure described above, then resuspended in their recommended medium containing 5% DMSO. DMSO acts as a cryopreservative, preventing unwanted crystals forming in the freezing process and preserves cell viability. Then the cell suspension was aliquoted into 1 or 2 mL cryovials and placed on ice for 10 minutes before being transferred to a Mr Freeze cryocontainer to be stored at -80°C overnight, cooling at approximately 1 °C /minute. The following day cryovials were transferred to liquid nitrogen for long term storage until required.

Oxygen-glucose derivation (OGD) protocol

To simulate ischaemia *in vitro*, an oxygen-glucose deprivation (OGD) protocol was followed (Hind et al., 2015, 2016). Normal cell culture medium (specific to each cell type, detailed previously) was removed and replaced with glucose-free RPMI medium (ThermoFisher, UK). Cell culture plate were placed in an anaerobe BD GasPack (UK) for 4 h for HBMECs, pericytes and neuron or 8 h for astrocytes and placed back into the cell culture incubator (37°C, 5% CO_2). Following this, plates were removed from the anaerobic bags and glucose-free medium was aspirated and replaced with normal cell culture medium. Cell culture plates were then returned to the cell culture incubator for either a 20 h or 16 h reperfusion period.

Sample collection; media and lysates

Post OGD experiments, medium samples were collected from cell culture plates into 1 mL Eppendorf tubes and analysed immediately or stored at -80°C until subsequent analysis. During analysis all samples were kept on ice (approximately 4°C) to minimise sample degradation. Once cell medium had been removed, cell monolayers were gently washed with 1X PBS. Ice cold RIPA buffer (Sigma-Aldrich R0278) containing pierce protease and phosphatase inhibitors (inhibitor cocktail Thermo Fisher: A32959)

was added to each well. Cell culture plates were then placed on a plate shaker in the cold room (4°C) for 30-60 minutes to lyse the cells. The cell lysis mixture was then either immediately analysed, or frozen at -80°C and stored in liquid nitrogen.

Phytocannabinoids

Cannabidiol (CBD) was purchased from Tocris, UK. Cannabidiolic acid (CBDA) was purchased from Sigma, UK. Cannabigerol (CBG) and cannabidivarin (CBDV) were gifts from STI pharmaceuticals, UK. CBD, CBG and CBDV were dissolved in 100% ethanol at stock concentrations of 10 mM and CBDA was dissolved in 100% acetonitrile at a stock of 10 mM. All stocks were stored at -20°C. Fresh dilutions were prepared daily as required.

Antagonists

All antagonists were purchased from Tocris, UK and were used at the following concentrations AM251 (100 nM), AM630 (100 nM), GW6471 (100 nM), GW9962 (100 nM), (S)-WAY 100135 (300 nM), capsazepine (1 µM), O1918 (1 µM), CID16020046 (1 µM), SB366791 (1 µM) (Hind et al., 2016). All antagonists except for (S)-WAY 100135 were dissolved in dimethyl sulfoxide (DMSO), (S)-WAY 100135 was dissolved in deionised water. All antagonists were prepared as stock solutions of 10 mM and stored at -20°C. Dilutions were prepared fresh as required.

Microplate spectrophotometer

A microplate spectrophotometer (Thermo Scientific, UK) with SkanIt Software was used to colorimetrically measure optical density to determine protein concentration. illuminating samples and measure the light intensity returned relative to its specific wavelength. A standard curve was created using samples at known protein concentrations. Samples of unknown protein concentrations were determined using the equation of the line created by the standard curve, unknowns were interpolated from the standard curve using Prism software (GraphPad, USA).

Bicinchoninic acid (BCA) assay

A bicinchoninic acid (BCA) assay was performed on cell lysates to quantify the amount of protein present. This was to enable normalisation of data from multiplex and ELISA assays to compensate for any cell variation between wells. An 8-point standard curve was constructed using samples of known concentrations of BSA protein (0-2 mg/mL). 10 µL of sample or standard was pipetted into a clear, flat bottomed 96 well plate (Griener, UK). A solution of Cu^{2+} and BCA was prepared at a 1:50 ratio and 100 µL added to respective wells. This was incubated for 20 minutes at 37°C and for a further 10 mins at room temperature before reading absorbance at 562 nm using Varioskan™ LUX platereader, (Thermofisher, UK) using SkanIt Software. Unknowns were interpolated from the 8-point standard curve in µg/mL using Prism Software (GraphPad, USA).

Enzyme-linked immunoassays (ELISA)

Human DUOset sandwich enzyme-linked immunosorbent assays (ELISAs) were used to measure the presence and concentration of secreted cellular proteins and used as per the manufacturer's instructions. The following kits were used over the course of the project: vascular cell adhesion molecule-1 (DY809), human intracellular adhesion molecule-1 (DY720), interleukin-8 (DY208), monocyte chemoattractant protein-1 (DY279) and interleukin-6 (DY206). To assay: clear, flat-bottomed, high-binding 96-well microplates (Greiner Bio-One, 655061) were incubated with capture antibody (see specific kit for details on concentrations used) diluted in 1xPBS overnight at room temperature. The next day plates were washed 3x with wash buffer (0.05% TWEEN in PBS) and blotted on clean absorbent towels to remove excess liquid. Next, 300 µL of blocking solution (1% BSA in PBS) was added to the wells for 1 h at room temperature to prevent any non-specific binding of protein to the plate wells. The supplied standard was reconstituted and diluted, then an 8-point standard curve was constructed by serial dilution. Quality controls were prepared at low and high concentrations based on the standard curve. The plates were washed again, 3x with wash buffer and after the

third wash plates were blotted on absorbent towels. 100 µL of standard, QC or samples was pipetted into the plate and incubated for 2 h at room temperature to allow the protein of interest to bind to the capture antibody coated on the plate bottom. After this incubation step plates were washed 3x again with wash buffer and blotted on absorbent paper, then a biotinylated secondary antibody solution (1% BSA in PBS, see specific kit for concentration details) was added to the wells and incubated for 1 h at room temperature. Plates were washed with wash buffer and blotted, and horseradish peroxidase (HRP; prepared in 1% BSA in 1X PBS) was added to the wells and plates were incubated at room temperature for 20 minutes. For this step and the last step plates were wrapped in foil as these steps are light sensitive. Following a final wash with wash buffer and blotting of the plates, a colour forming solution (which consisted of mixing equal amounts of colour reagent A (H_2O_2) and reagent B (3,3',5,5'-tetramethylbenzidine, TMB) was added to the plates and incubated at room temperature for a further 20 minutes. Stop solution consisting of 2N sulphuric acid (H_2SO_4) was added to each well to end the reaction. Optical density was determined using a spectrophotometer at the wavelengths 450 nm and 570 nm. Readings were determined by subtracting values at 570 nm from 450 nm. Sample concentrations were determined by extrapolating unknowns from the 8-point standard curve comprised of known concentrations. A plate wash was conducted between each step to prevent any non-specific binding of excess protein and ensure consistency and robust data each time the kit was performed. Fresh reagents were prepared every three months or as required. In every plate, QCs were included to ensure inter and intra batch consistency and were deemed acceptable if their interpolated values were within a variance of <15% of the desired concentration.

Multiplex immunoassays

Multiplex immunoassays enable the simultaneous detection of more than one analyte within an individual sample where fifty distinct coloured beads can be created from the combination of two fluorescent dyes which are then pre-coated with capture antibody. Luminex xMAP® technology and a MAGPIX™ analysing system from Merck

Millipore were used alongside a MILLIPLEX MAP DNA Damage/Genotoxicity Magnetic Bead Panel kit (48-621MAG) to detect DNA damage response (DDR) proteins: ataxia-telangiectasia mutated (ATR-Total), checkpoint kinases 1, 2 (Chk,1 Ser345 and Chk2, Thr68), histone family member X (H2AX, Ser139), mouse double minute 2 homolog (MDM2, total), cyclin-dependent kinase inhibitor 1 (p21, total), tumour protein (p53, Ser15). Internally labelled fluorescent dyes with magnetite encapsulated functional polymer outer coating with a surface of carboxyl groups to enable covalent coupling of ligands. Samples and the beads were pipetted into the supplied black, clear bottomed 96 well plates. Plates were incubated overnight on a plate shaker at a speed in which beads were in a constant motion but avoiding splashing (as per manufacturer's recommendations) and in the absence of light to enable antibody-analyte binding. The next day a mixture of specific biotinylated secondary antibodies were added. The biotinylated secondary antibodies bind to the analytes of interest, forming an antibody-antigen sandwich. Following this, phycoerythrin (PE)-conjugated streptavidin was added to bind the biotinylated detection antibodies. Samples were either re-suspended in sheath fluid or assay buffer depending on manufacturer's instructions for individual kits before reading on the MAGPIX^{TM} analysing system. The system uses two distinct light emitting diodes (LEDs): one to classify the bead based on its fluorescence and determine the analyte being detected and one to determine level of analyte bound by the extent of the PE signal. Depending on the specifications for individual kits, mean fluorescent intensity (MFI) data were exported into PRISM software (GraphPad, USA) and if required, interpolated from a standard curve of known concentrations to determine concentration of unknowns (cytokine panel kits). All data were normalised to total protein determined by a BCA assay.

Cell Viability assay

A resazurin reduction assay was performed to determine astrocyte viability. 10 µL of resesazurin was added directly to cells cultured in 96 well plates and incubated for 4 h in the cell culture incubator (37°C, 5% CO_2). Viable cells (i.e those that are

metabolically active) are able to convert resazurin substrate, which is deep blue in colour, to resorufin product which is both pink in colour and fluorescent, see figure 2.1.

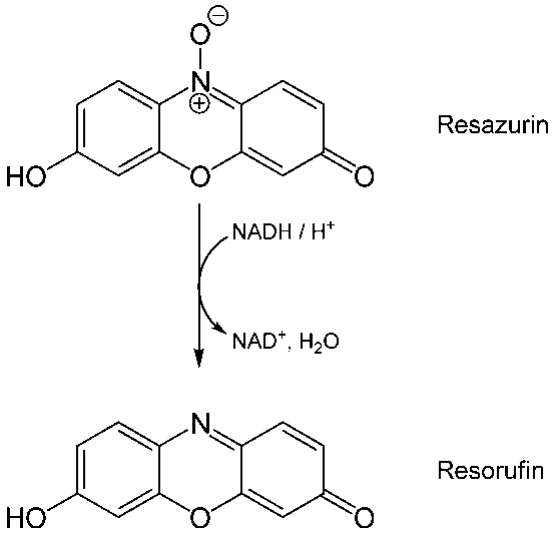

Figure 2.1: Conversion of resazurin to resorufin, this process occurs in mitochondria of metabolically active cells and correlates to cell viability.

After a 4 h incubation period, the resorufin product was measured using a microplate fluorometer (Varioskan™ LUX, Thermofisher, UK) with filters set to 560 nm excitation and 590 nm emission. The accumulation of product is proportional to the number of viable cells in each well.

LDH Assay

A colorimetric lactate dehydrogenase (LDH) assay was used to evaluate cytotoxicity (Abcam, UK). LDH is a stable cytosolic enzyme present in all cell types and if cell membrane integrity is compromised (i.e. as a result of damage), LDH is rapidly excreted into the extracellular space. Therefore, the LDH quantification is a reliable measure of cellular apoptosis and necrosis in cultured cells. Cell culture medium or standard was pipetted into a 96 well plate (Griener, UK) and WST substrate mix was added, as per manufacturer's instructions. If LDH is present in the sample NAD is reduced to NADH, which then reacts with the WST substrate mix. Optical density of the

sample was read at 450 nm using a plate reader (Varioskan™ LUX, Thermofisher, UK) every 15 minutes up to 1 h. The yellow colour generated is proportional to the number of damaged (lysed) cells and LDH activity was determined by interpolating unknown values from a standard curve of known NADH concentrations.

Transepithelial resistance (TEER)

Transepithelial resistance (TEER) is a useful, relatively non-invasive technique and is used to evaluate barrier integrity of cells grown in a four-cell culture model (REF). The tips of STX-3 chopstick electrodes were firstly placed in 70% ethanol for 5 minutes, then placed into endothelial cell culture medium to equilibrate for 15 minutes. When cells reached full confluency, the four-cell model cell culture plates were removed from the cell culture incubator, sterilised with 70% ethanol and placed inside the biological safety cabinet. Plate lids were removed, and the electrodes were placed either side of the cell layer, ensuring the shorter electrode was placed in the upper compartment (apical) and the longer electrode was placed in the lower compartment (basolateral). The ohmic resistance of the inserts was determined by applying a current to STX-3 chopstick electrodes (which have silver chloride pellets for passing current)(Srinivasan et al., 2015).The resistance of the monolayer was then measured and calculated according to Ohm's law using an Epithelial Voltohmmeter (EVOM™, World Precision Instruments). A background 'blank' well containing medium but no cells was used as a control and measured. Each experimental well was measured twice and an average of the two readings was calculated. The blank well was subtracted from the average reading and multiplied by the surface area (SA) of the insert (1.12). An equation summarising this is shown below:

$$\text{TEER reported in Ohms } (\Omega.cm^2) = ((R_1+R_2*)/2) \times SA**(cm^2)$$

*Where * is the measured reading in Ohms (Ω) and **SA is specific to the model of insert used in cm^2.*

In order to ensure reproducible TEER measurements, the electrodes were placed in the same location in each well for every measurement. Particular care was also taken when placing electrodes into each well as to not disturb the cell monolayer on the apical surface of the insert, or the coverslip containing neurons on the plate bottom. For details on full BBB methodology, refer to Chapter 3 (Stone et al., 2019).

Propidium Iodide (PI) Staining

A life/death assay utilising propidium iodide (PI) staining was conducted to determine the fraction of dead neurons post OGD. At 72 h post OGD, neurons were washed with 1X PBS and incubated with a 100 µL/mL solution of PI prepared in PBS for 1-2 minutes at room temperature in the absence of light. As PI is a membrane impermeant dye, it is excluded from live cells but can bind to double stranded DNA of dead or dying cells by intercalating between base pairs. Images were obtained immediately using a Nikon DS-Fi1 digital camera linked to an upright fluorescence microscope (Nikon Eclipse 50i) with a 20x objective. Images were visually assessed to determine proportion of dead cells.

Statistical Analysis

Statistical analysis was conducted using GraphPad prism software (USA), versions 7-8. Data were tested for normality using the D'Agostino & Pearson omnibus normality test to determine whether a non-parametric (non-normal data) or parametric test (normal data) were to be applied. In general, when data compared three or more groups, e.g vehicle vs various drug concentrations, a one-way analysis of variance (ANOVA) test was used. Multiple comparisons were adjusted using Dunnett's or Sidak's statistical tests. If a data set was looking a response affected by two independent factors, e.g TEER measurement and time, then a two-way ANOVA was used and multiple comparisons adjusted for using Turkey's statistical test. Typically, data are presented as mean ± standard error of the mean (SEM) of two or three experimental repeats, unless stated otherwise. Data were considered significant if p-values were $p<0.05$.

1. A Novel Transwell Blood Brain Barrier Model Using Primary Human Cells

The following chapter is presented in its final manuscript format and is published in Frontiers in Cellular Neuroscience (Stone et al., 2019).

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A Novel Transwell Blood Brain Barrier Model Using Primary Human Cells

Nicole L. Stone, Timothy J. England and Saoirse E. O'Sullivan*

Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Nottingham, United Kingdom

Structural alterations and breakdown of the blood brain barrier (BBB) is often a primary or secondary consequence of disease, resulting in brain oedema and the transport of unwanted substances into the brain. It is critical that effective *in vitro* models are developed to model the *in vivo* environment to aid in clinically relevant research, especially regarding drug screening and permeability studies. Our novel model uses only primary human cells and includes four of the key cells of the BBB: astrocytes, pericytes, brain microvascular endothelial cells (HBMEC) and neurons. We show that using a larger membrane pore size (3.0 μM) there is an improved connection between the endothelial cells, astrocytes and pericytes. Compared to a two and three cell model, we show that when neurons are added to HBMECs, astrocytes and pericytes, BBB integrity was more sensitive to oxygen-glucose deprivation evidenced by increased permeability and markers of cell damage. Our data also show that a four cell model responds faster to the barrier tightening effects of glucocorticoid dexamethasone, when compared to a two cell and three cell model. These data highlight the important role that neurons play in response to ischaemia, particularly how they contribute to BBB maintenance and breakdown. We consider that this model is more representative of the interactions at the neurovascular unit than other transwell models and is a useful method to study BBB physiology.

Keywords: blood-brain barrier, transwell, *in vitro*, BBB model, BBB permeability, primary human cells, stroke

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Förster Carola,
Universitätsklinikum Würzburg, Germany
Babette Weksler,
Cornell University, United States

****Correspondence:***
Nicole L. Stone
mzxns2@nottingham.ac.uk;
nicole.stone@nottingham.ac.uk

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INTRODUCTION

The blood brain barrier (BBB) is a unique interface that separates the peripheral blood supply and neuronal tissue. Structurally, the BBB is comprised of specialized brain microvascular endothelial cells (HBMECs), perivascular cells (pericytes) and astrocytes (Abbott et al., 2006, 2010). Neurons and microglia also contribute to the maintenance of the BBB and form what is known as the neurovascular unit (NVU) (Abbott et al., 2006). Pericytes contribute 22–32% of the cerebral vasculature and together with vascular smooth muscle cells and endothelial cells they maintain vascular function (Martini and Bartholomew, 2017). In the CNS, pericytes are present at a higher ratio to HBMECs in the brain compared to the periphery and recent studies have shown the extensive role of pericytes in BBB development and maintenance (Kacem et al., 1998; Armulik et al., 2011; Sweeney et al., 2016). As well as offering mechanical support, they also regulate vessel contractility, endothelial proliferation, blood flow and angiogenesis (Bergers and Song, 2005;

Dore-Duffy, 2008). Pericytes have also been shown to secrete angiogenic factors such as vascular endothelial growth factor (VEGF), that support endothelial cell survival and proliferation (Darland et al., 2003). In pathologies such as ischaemic stroke, large gaps can develop between adjacent pericytes, increasing barrier permeability and vessel leakage. These alterations in pericyte morphology, coupled with an upregulation in the expression of adhesion molecules and leukocyte integrin ligands, contribute to the extravasation of peripheral leukocytes into the brain following ischaemic insult (Pieper et al., 2013). Thus, pericytes play a large role in cerebral vascular function under normal physiological conditions as well as vascular dysfunction in hypoxia.

Several studies have also highlighted the roles of neurons and glia in BBB development and maintenance. Neural progenitor cells present in the ventricular neuroepithelium have been shown to aid endothelial cell recruitment during early BBB development, which is largely governed by the Wnt signaling pathway (Risau et al., 1986). Specifically, Wnt signaling in early CNS development is responsible for vascular stabilization and angiogenesis (Liebner et al., 2008). Further to this, early neuronal signaling has been shown to be essential for the maturation of the BBB, specifically, tight junction (TJ) organization. A study carried out using rat microvascular endothelial cells and neuronal progenitor cells, showed that in the presence of neural progenitor cells, endothelial cells established regular TJ formation including: claudin 5, zonula occludens (ZO-1) and occludin (Weidenfeller et al., 2007). After maturation, maintenance of the BBB and preservation of brain homeostasis is largely dependent on adequate perfusion to neuronal tissue and neuronal signaling to cerebral vessels, a process known as hyperaemia (Attwell et al., 2010). Studies have shown that neuronal-astrocyte crosstalk is important for appropriate vessel contractility and blood flow, depending on metabolic demand (Zonta et al., 2003; Attwell et al., 2010; Macvicar and Newman, 2015). In cerebral ischaemia, astrocytes sense elevations in Ca^{2+} ions and increases in extracellular glutamate released by neurons and respond accordingly, secreting a range of vasoactive substances to help mitigate the effects of the blood vessel occlusion (Macvicar and Newman, 2015). Altogether, interactions between both neural and vascular cells within the NVU is considered to be paramount in BBB functionality because together they induce and strengthen barrier properties; helping to maintain its key features including low paracellular permeability and functional tightness (Abbott et al., 2010). Breakdown of the BBB in conditions such as ischaemic stroke can lead to severe consequences to brain homeostasis, therefore, modeling these interactions is necessary to understand the complex signaling networks between these cell types and how they are influenced in disease states.

To date, a number of BBB models have been developed ranging from HBMEC monolayers to more sophisticated spheroid and chip style models, see **Table 1**. After the successful isolation of brain endothelial cells, the first, most simplistic BBB models were developed utilizing HBMECs as a single monolayer in the abluminal side of transwell inserts, see **Table 1** (Borges et al., 1994; Hartz et al., 2010). Later addition of other BBB cell types (namely astrocytes and pericytes), led to the development of co-culture transwell systems which exhibited greater barrier strength, exhibited by higher transepithelial resistance (TEER) and lower permeability than single HBMEC models, see **Figure 1**. More recent transwell systems typically use three cell types originating from either bovine, porcine or rodent origin, see **Table 1** (Gaillard et al., 2000; Nakagawa et al., 2009; Thomsen et al., 2015).

Whilst modeling using non-human cells is cheaper and easier to obtain, they are not comparable to human cells, with many studies showing key differences in morphology and function, particularly their sensitivity to glutamate and expression of efflux transporter proteins (Oberheim et al., 2009; Warren et al., 2009; Zhang et al., 2016). More complex BBB models are also available, such as spheroid or microfluidic models and offer a closer representation of the *in vivo* environment. However, these set ups are difficult and expensive to assemble (Ruck et al., 2015). Therefore, there is a need to develop a multicellular transwell model that incorporates multiple NVU cell types to study their interactions, particularly the role of neurons and their influence on barrier strength in both physiological and disease states. Transwell systems still offer a distinct advantage in that they are relatively easy to setup and control, as well as offering a range of endpoints to study. Measuring TEER in these types of models is commonplace as it provides a reliable, non-invasive quantitative measure of barrier integrity, enabling repeated measurements to be taken over the desired time period with minimal damage to cells (Srinivasan et al., 2015). Further to this, transwell models enable access to both the apical and basolateral (basal) compartments for drug application and medium sampling as well as being able to visualize cells over the course of the experiment.

Our aim was therefore to create a novel four cell human BBB model to study changes in permeability post oxygen-glucose deprivation (OGD) and for use in *in vitro* pharmacology. We initially focused on model development, refining a protocol first outlined by Hind (2014) by optimizing the inserts themselves, insert coating, cell seeding densities and cell culture timelines. Finally, we incorporated a method of seeding neurons on plastic coverslips which were placed on the bottom of 12 well cell culture plates. Thus, our model maintains the ease of the transwell setup but utilizes four primary human cells, making it a closer representation of the human *in vivo* environment.

MATERIALS AND METHODS

Primary cells (astrocytes, pericytes, HBMECs, and neurons) and specialized cell culture medium (astrocyte medium, pericyte medium, endothelial cell medium, and neuronal medium) were obtained from ScienCell, United States supplied by Caltag Medsystems, United Kingdom. Poly-L-lysine and porcine fibronectin were also obtained from ScienCell, United States supplied by Caltag Medsystems, United Kingdom. Collagen coated inserts, 3.0 μm, 12 mm were obtained from Corning, United Kingdom. Plastic coverslips (Thermanox® 13 mm diameter), Accutase dissociation reagent and glucose free RPMI medium were obtained from Thermo Fisher Scientific, United Kingdom.

TABLE 1 | Different models of the blood brain barrier; their features, advantages, and disadvantages.

Model type	Typical components	Advantages	Limitations	Representative of BBB phenotype	References
Single-cell transwell systems (non-co-culture)	A monolayer of HBMECs cultured in the apical compartment of the transwell insert.	Very easy to set up. Minimal cost. Low labor intensity. Useful if wanting to study endothelial cells alone.	TEER is typically low.	Cobblestone appearance of HBMECs, barrier formation. Little information on the impact of additional cell types.	Borges et al., 1994; Hartz et al., 2010
Co-culture /multicellular transwell systems	HBMECs cultured on the apical side of the transwell insert and astrocytes and/or pericytes cultured on the underside of the transwell insert.	Time and cost effective. Higher TEER. Greater barrier stability.	Some models are not fully in contact.	Closer representation of the BBB with the addition of important cell types. Able to study interactions between cell types and how they influence BBB phenotype.	Hind, 2014; Wang et al., 2015; Appelt-Menzel et al., 2017
Spheroid	3D organization of cells typically using matrigel. Typically consists of HBMECs and astrocytes and/or pericytes with some models containing neuronal cell types.	3D Cell model. No scaffold. Reduced de-differentiation.	Cannot measure permeability with this model. Expensive and greater skill required.	Microvessels wrap around endothelial cells and provide structural support. Helps to induce tight junction proteins. Closely represents the *in vivo* set up with cells in direct contact with each other. Applications include: cancer drug and neurotoxicity screening.	Cho et al., 2017; Nzou et al., 2018
Microfluidic systems/3D chip-style models	3D organization of cells with the added benefit of a "flow" system to mimic cerebral blood flow. Typically consists of HBMECs and astrocytes and/or pericytes with some models containing neuronal cell types.	Advantage of mimicking sheer stress which is essential for HBMECs optimum phenotype.	Difficult to set up and maintain adequate flow unless linked to a computer system.	Useful to assess the impact of blood flow on cell development and optimum phenotype. Also useful in studying cell migration and metastatic progression.	Yeon et al., 2012; Wang et al., 2017

HBMECs = human brain microvascular endothelial cells, TGFβ = transforming growth factor beta, TEER = transepithelial resistance, BBB = blood brain barrier.

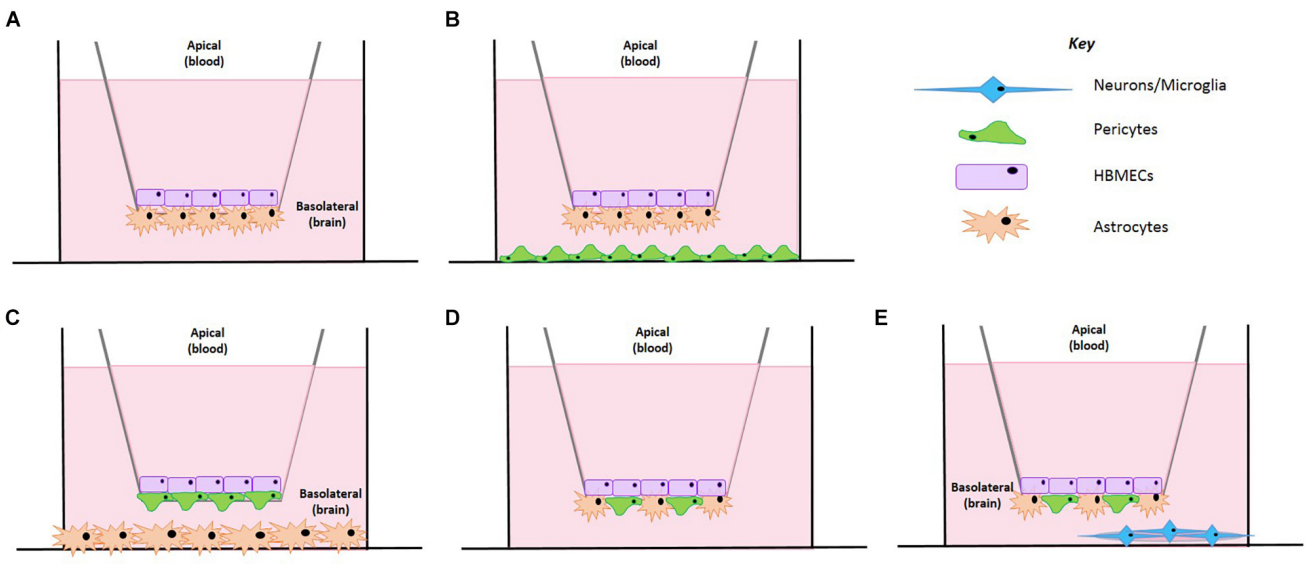

FIGURE 1 | Schematic representation of the BBB model development. **(A)** A co-culture cell model containing HBMECs and astrocytes. **(B)** HBMECs seeded on the apical side, astrocytes seeded on the underside of the insert and pericytes seeded on the plate bottom. **(C)** HBMECs seeded on the apical side of the insert, pericytes seeded on the underside of the insert and astrocytes seeded on the plate bottom. **(D)** HBMECs seeded on the apical side of the insert with mixed culture of astrocytes and pericytes on the underside of the insert. **(E)** HBMECs seeded on the apical side of the insert with mixed culture of astrocytes and pericytes on the underside of the insert and neurons seeded on a poly-L-lysine coated coverslip on the plate bottom.

Cells were maintained in a humidified incubator (37°C, 5% CO_2). Astrocytes and pericytes were cultured and used between passages 4 and 6. Human brain microvascular endothelial cells (HBMECs) were used between passages 3 and 5 and neurons were used at passage 1. During subculture, flasks containing HBMECs were coated with 2 $\mu g \cdot cm^2$ of fibronectin before reviving or splitting cells as per manufacturers recommendations. Cells were passaged at 80–90% confluency. Inserts contained 1.2 mL of medium in the basolateral compartment and 800 μL in the apical compartment.

STX-3 probes and Ohms meter were obtained from World Precision Instruments, United Kingdom. Dexamethasone was obtained from Sigma, United Kingdom and dissolved in DMSO at a stock concentration of 10 mM and subsequently diluted in cell culture medium. GasPak^{TM} EZ anaerobe container systems were obtained from BD, United Kingdom.

Model Validation

Our model was based on an initial co-culture set up established by Hind (2014) and previous models by Allen and Bayraktutan (2009). Our model was modified and developed in a number of preliminary experiments including comparison of insert pore sizes, insert coating, cell organization and addition of multiple cell types.

Pore Size, Insert Size and Coating

Initially, pore sizes of Corning, United Kingdom inserts were compared (0.4 μm vs. 3.0 μm) as well as cell culture plates (12 well vs. 24 well). This was to determine the best initial setup that provided the highest and most stable barrier resistance, as well as giving the best cell contact. During protocol development, we found addition of pericytes in the smaller 24 well plates yielded poor results and insufficient TEER, suggesting inadequate barrier formation. Possibly as a result of inadequate cellular growth in such a small surface area and environment. Therefore, 24 well plates were switched back to 12 well plates, which resulted in substantially higher TEER readings. Following work carried out by Niego and Medcalf (2013), we also found that inserts with a 3.0 μm pore size had higher TEER values than 0.4 μm inserts, suggesting that increased contact between the cells in the apical and basolateral sides of the insert resulted in greater barrier strength, see **Figure 2A**.

Addition of Multiple Cell Types and Cell Positioning

Despite these improvements on the co-culture model, the need for additional cell types was critical to create a closer representation of the *in vivo* BBB. We established three different set ups as shown in **Figure 1**. In one, astrocytes were seeded on the basolateral side of the inserts and pericytes on the bottom of the culture dish (**Figure 1B**), in another pericytes were seeded on the basolateral of the inserts whilst astrocytes were seeded on the bottom of the culture dish (**Figure 1C**) and finally the last set up involved a mixed culture of astrocytes and pericytes seeded on the basolateral side of the insert (**Figure 1D**). In all models tested, HBMECs were seeded in the apical side of the transwell insert.

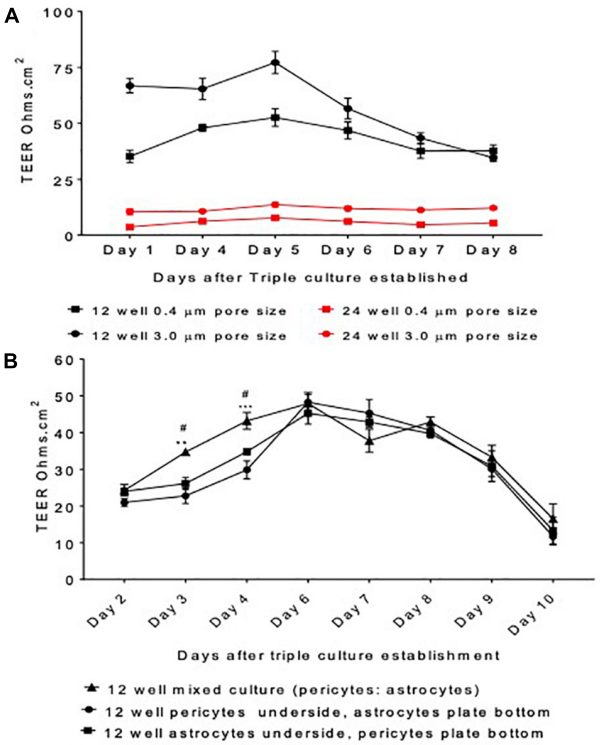

FIGURE 2 | Model protocol development **(A)** measured transepithelial resistance (TEER) as a marker of barrier tightness comparing a 12 well plate transwell set up vs. a 24 well plate transwell set up and insert pore size 3.0 μm vs. 0.4 μm. HBMECs seeded on the apical side of the insert, astrocytes underneath and pericytes on the plate bottom. **(B)** The organization of cells was optimized by comparing the TEER generated by a mixed culture of astrocytes and pericytes, pericytes or astrocytes alone on the underside of transwell inserts and astrocytes or pericytes on the cell culture plate bottom. HBMECs were seeded on the apical side of the insert. Data given as mean ± SEM, n = 4–6 from two experimental repeats. Statistical analysis conducted using 2-way ANOVA with Turkey's multiple comparisons test, $^{**}P < 0.01$ and $^{***}P < 0.001$ mixed culture astrocytes and pericytes vs. pericytes underside the insert and astrocytes on plate bottom. $^{\#}P < 0.05$ mixed culture astrocytes and pericytes vs. astrocytes underside the insert and pericytes on plate bottom.

The final set up offered a closer replication of the organization held at the *in vivo* BBB, as cells would be in direct contact allowing them to exchange vital growth factors required for cellular growth and development. We found that mixed culture of pericytes and astrocytes exhibited significantly higher TEER values when compared to the set-up with pericytes seeded on the plate bottom and astrocytes underneath the insert or astrocytes on the plate bottom and pericytes underneath the insert on days 3 and 4, $P < 0.05$ and $P < 0.01$, respectively (**Figure 2B**). Furthermore, this set up was also considered the most stable, as shown by steadier TEER readings and was altogether more physiologically relevant. This set up was therefore taken forward in subsequent four cell protocol development.

To test the viability of adding neurons to the model, we originally seeded neurons on the bottom of the 12 well plate in which the inserts were hung. This, however, was

not feasible as the TEER probes touched the bottom of the plate causing unwanted damage to the cells. Therefore, we decided to utilize coverslips that could be positioned on the plate bottom, but not take up the entirety of the well, allowing the probe to sit where the cells were not present. After testing both poly-L-lysine coated glass and plastic coverslips, we found that plastic coverslips coated were the most effective in neuronal adhesion and this method was used in the final model.

Four Cell Method Overview

After optimization, our four cell BBB model consisted of four major NVU cell types arranged in a transwell permeability set-up (see **Figure 1E**). The assembly of this involves seeding different cell types at different times on the apical and basolateral sides of the transwell insert. During this time, neurons are seeded on plastic coverslips placed on the bottom of a separate 12 well plate to develop neurite before putting both parts of the model together on the final day of model establishment. Cell culture medium in both compartments was replaced every other day and the final set up was left to equilibrate for 2 days before commencing experiments. Greater than 85% of inserts are feasible for use in experiments and the model remained viable for up to 5 days.

Insert Coating and Astrocyte Seeding

On day one, the basolateral side of transwell inserts were coated with poly-L-Lysine and astrocytes were seeded on the basolateral side of the inserts, see **Figure 3**. Briefly, 3.0 μm, 12 mm collagen coated inserts (Corning, United Kingdom) were carefully removed from outer packaging and placed into 12 well cell culture plates using sterile forceps. A solution of poly-L-Lysine (2 $\mu g/cm^2$) was prepared in sterile water, homogenously mixed and carefully pipetted using a Pasteur pipette to just cover the basolateral of the insert, see **Figures 4A,i**. Plates containing inserts were then returned to the incubator, 37°C, 5% CO_2 for 1 h as per supplier recommendations. After 1 h, plates were removed from the incubator and washed twice with sterile water to remove any residual poly-L-lysine. All remaining liquid was removed by careful aspiration. Transwell inserts were then flipped inside the plate and the lid removed (**Figure 4B**). On the newly coated inserts, 100 μL of astrocyte cell suspension in astrocyte medium (3.13×10^5 cells) was pipetted quickly onto the basolateral side of the transwell and the lid carefully replaced (see **Figures 4C,ii**). Plates were returned to the cell culture incubator for 2–3 h for the cells to adhere. After this time, transwell inserts were reverted and any excess medium was removed by aspiration. Medium was topped up in the apical and basolateral compartments, see **Figure 4iii**. Again, plates were returned to the incubator.

Pericyte Seeding

On day 2, plates were removed from the incubator and the astrocyte medium was removed with care so as to not disturb the layer of cells on the basolateral side of the insert. Inserts were then inverted again and 100 μL of 6.25×10^4 pericyte cell suspension was added to the astrocyte cell layer on the basolateral side of the transwell inserts, giving an approximate ratio of 5:1 astrocytes to pericytes (Pardridge, 1999). Plate lids were quickly replaced and returned to the incubator for 2–3 h. After this time, transwell inserts were reverted and any excess medium was removed by aspiration and a mixture of astrocyte and pericyte medium (1:1) was added to the apical and basolateral compartments.

HBMEC Seeding

Once astrocytes and pericytes reached 90% confluency (approximately day 4 from model initiation, see **Figure 3**), the astrocyte:pericyte (1:1) medium in the apical compartment was removed and 100 μL of HBMEC cell suspension (7.5×10^4) in HBMEC medium was added to the apical compartment of transwell inserts and cells were left to adhere for a minimum of 5 h, then medium was topped up to 700 μL with endothelial cell medium and plates returned to the incubator.

Neuronal Seeding

On the same day as HBMEC seeding, plastic coverslips (13 mm diameter) were coated with poly-L-lysine and placed in the cell culture incubator for a minimum of 1 h, as per supplier recommendations. Plates containing coverslips were carefully removed from the incubator and coverslips were washed twice with sterile water and left to air dry in the cell culture hood. Following this, cryopreserved neurons were revived into 3 mL of neuronal medium (to give a total cell suspension of 4 mL) and 100 μL of cell suspension was added to each coverslip (thus seeded at a density of approximately 2.5×10^4 cells per cm^2 within the optimum range according to the manufacturer's

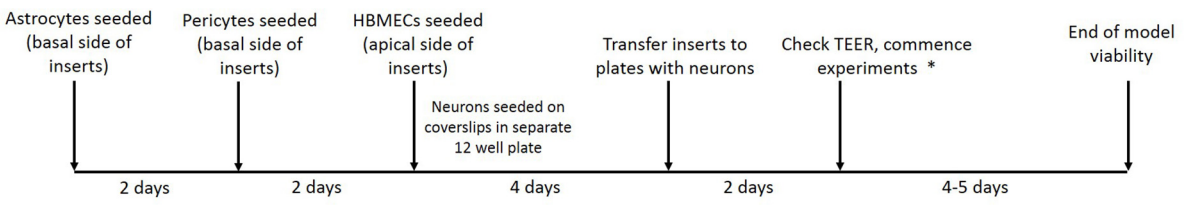

FIGURE 3 | Timeline showing stages of model establishment. On day 1, inserts were coated and astrocyte seeded on the basolateral side of transwell inserts and on day 3 pericytes were seeded on the basolateral side of inserts to form a mixed culture. On day 6 HBMECs were seeded on the apical side of inserts and neurons were seeded on coated plastic coverslips in a separate 12 well plate. On day 10/11, inserts are carefully lifted out of their current plate and placed into the second 12 well plate containing the neurons seeded on coverslips. After 2 days, TEER measurements are taken to ensure adequate barrier formation. *In our lab OGD experiments were commenced at this point and were viable for 4–5 days.

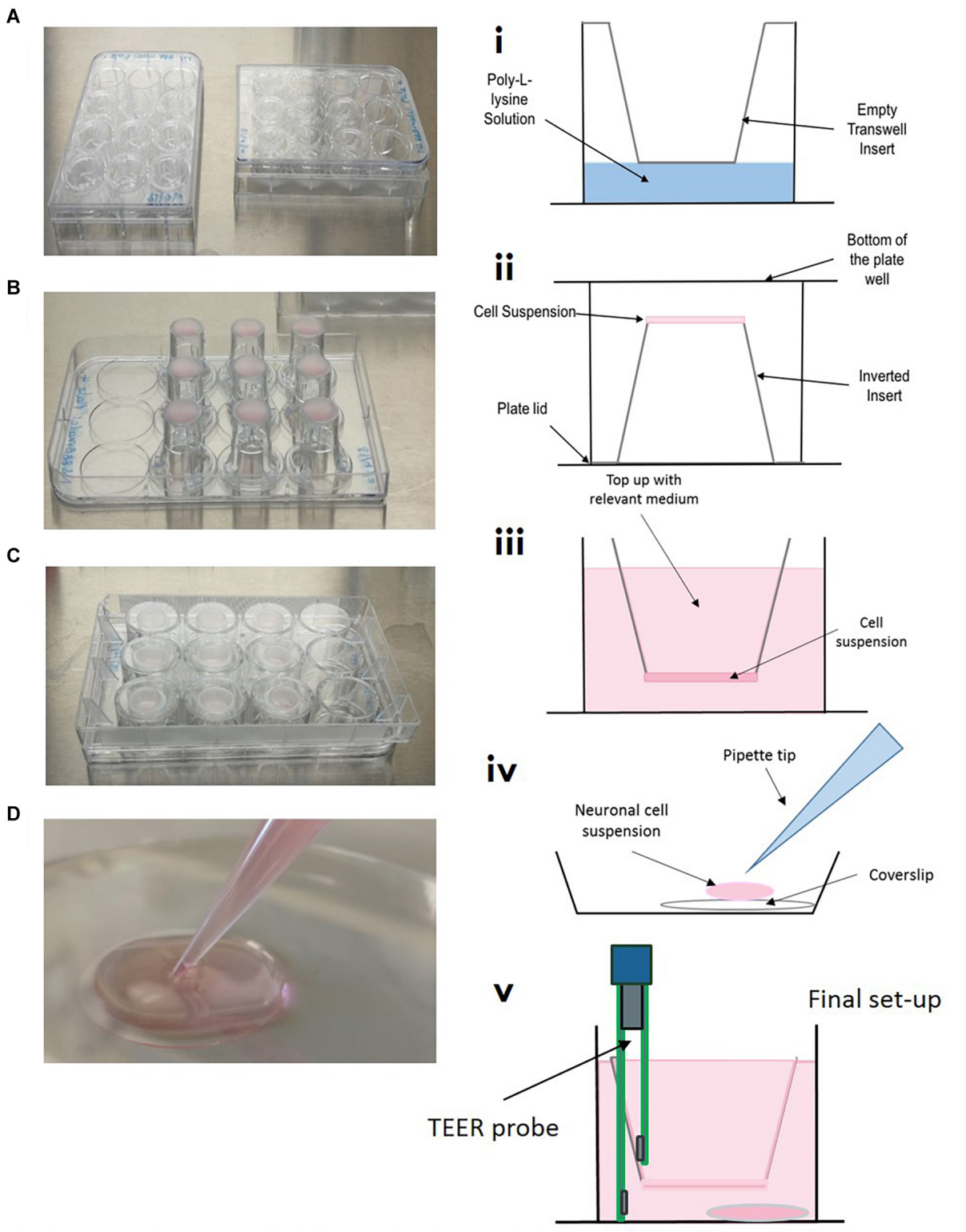

FIGURE 4 | Model setup **(A–D)** and **(i–v)**. **(A/i)** Inserts are placed into 12 well plate, coated with poly-L-lysine and washed, ensuring all of the liquid is removed. **(B/ii)** Inserts are carefully flipped inside the plate and the plate removed. 100 μL of relevant cell suspension is carefully placed on the underside of the insert. **(C/ii)** The bottom of the cell culture plate acts as a "lid" and is replaced as quickly as possible, plates are then returned to the incubator for the cells to adhere for 3–4 h. **(iv)** In a separate 12 well plate, coverslips are placed in the bottom of the culture dish, coated with poly-L-lysine and seeded with neuronal cell suspension. **(v)** Once all cells have been seeded on transwells, inserts are carefully transferred to plates containing neurons on coverslips.

recommendations) (**Figures 4D,iv**). Medium was topped up after 2 h and half of the medium replaced every 2–3 days. After light microscope observation, neurons began showing extensive neurite growth at approximately day 5. At this point HBMECs will have almost formed a confluent monolayer above the astrocytes and pericytes. Transwells were then carefully lifted out of their current 12 well plate using sterile forceps and placed into the 12 well plate containing the neuronal coverslips. Fresh HBMEC medium was applied to the apical compartment and a mix of pericyte, astrocyte and neuronal medium (1:1:2, respectively) was added to the basolateral compartment. This was to maintain a low concentration of fetal bovine serum optimum for neuronal maintenance, whilst also preserving growth of astrocytes and pericytes. As all cells were confluent and the barrier was adequately formed, conditions were able to be maintained in the different compartments.

Oxygen-Glucose Deprivation (OGD) Protocol

An oxygen-glucose deprivation (OGD) protocol was used to increase barrier permeability, simulating the effects of ischaemic stroke *in vitro* (Hind et al., 2015, 2016). Normal cell culture medium was removed from transwell inserts and replaced with glucose free RPMI medium (Thermo Fisher Scientific, United Kingdom) and placed in a 0% O_2 environment (GasPak^{TM} anaerobe pouch Beckton Dickinson, Oxford, United Kingdom) for 20 min to ensure anaerobic conditions for a further 4 h. There was no initial pre-conditioning period. Reperfusion was initiated by removing plates from the anaerobe pouch and returning cells to their normal medium (HBMEC medium in the apical compartment and in the basolateral compartment a mix of pericyte, astrocyte and neuronal medium, 1:1:2, respectively). TEER was measured at baseline (0 h), immediately post OGD (4 h), 24, 48, and 72 h.

Evaluation of Barrier Integrity

Transepithelial resistance (TEER) was measured prior to commencing OGD experiments to ensure model barrier integrity; inserts should exhibit a TEER value of $\geq$45 Ω/cm^2 (**Figure 4v**). Light microscope observation was also carried out to ensure cell confluency and successful neurite formation. To ensure consistency, TEER measures should always be read at least 24 h after a medium change. Briefly, STX3 electrodes were sterilized by placing the tips of the probe in 70% ethanol, and then equilibrated for 15 min in endothelial cell culture medium at room temperature. The STX probe was then connected to an EVOM^2 meter (Both World Precision Instruments, United Kingdom) and inserted into the transwell insert. The electrode has two parts that are uneven in length, the longer part of the electrode was placed so it gently touched the bottom of the cell culture plate, whilst the shorter electrode rested slightly above the insert dish, not quite making contact the HBMEC cell layer. Care should be taken to avoid disrupting the neurons on the bottom of the cell plate, see technical comments and limitations. As TEER values are very susceptible to change, it is important to keep the electrode upright and avoid tilting as this can cause fluctuation in the TEER values. A background reading for an insert with just cell culture medium was taken and subtracted from each reading (readings were repeated twice to ensure reproducibility), this was then multiplied by 1.12 to address the cell culture insert area (cm^2) (Hind, 2014).

Dexamethasone Protocol

Dexamethasone is a synthetic glucocorticoid and several groups have shown that is able to artificially improve barrier strength (Shi and Zheng, 2005; Pyrgos et al., 2010; Hind, 2014). Therefore, we used dexamethasone as a positive control to investigate any potential difference in the response of the three versus four cell model to a drug application. Baseline TEER readings were recorded and medium replaced, then dexamethasone was added to the apical compartment of the transwell insert, giving a final concentration of 1 μM. TEER was measured at 2, 4, and 24 h.

Data Analysis

Data analysis was carried out using GraphPad prism software (La Jolla, CA, United States). Data are presented as mean ± SEM and analyzed using two-way ANOVA, followed by Sidak's or Turkey's multiple comparisons test. $^*P < 0.05$ was considered significant.

Technical Comments and Limitations

A critical step for setting up the four cellular model is timing and the revival and seeding of human neurons. Addition of the cells at incorrect timings will result in the model not working as effectively and TEER values will be lower than anticipated. We have therefore outlined a timeline for setting the model up (**Figure 2**), steps 4 and 5 can vary depending on the time taken for barrier formation to take place and for neurons to form neurite. Improper technique when seeding neurons on the coverslips will result in a lack of uniformity and inadequate neurite formation. Ensure coverslips are adequately air dried and neuronal cell suspension is carefully but adequately mixed during the revival and seeding process. Avoid removing neurons from the incubator for long periods.

When taking TEER values, ensure that the larger part of the STX probe does not touch the neurons cultured on the coverslip. This is especially important if multiple readings are being made (recommended). Utilization of neurons after primary experiments have been completed is also possible. Staining can be done on the coverslips using a variety of techniques including propidium iodide (PI) and DAPI staining, neurons can be lysed and intracellular assays can be performed.

RESULTS

Protocol Development

During BBB model development various set ups were compared including; insert pore size, plate size, and cell organization. **Figure 2** highlights stages in protocol development and their respective TEER values, prior to the addition of neurons into the model. **Figure 2A** shows that a larger pore size (3.0 μm) exhibited greater barrier integrity (as shown by greater TEER readings) than the smaller pore size (0.4 μm). Furthermore, the

12 well inserts displayed considerably greater TEER readings than the 24 well inserts. Continuing model development using 12 3.0 μm inserts, **Figure 2B** compares three different cell culture set-ups days after model establishment. On days three and four, the inserts containing a mixed culture of astrocytes and pericytes displayed significantly higher TEER readings than set-ups containing astrocytes or pericytes seeded on the underside of the inserts or the cell culture plate bottom, $P < 0.05$ and $P < 0.01$, respectively.

OGD Model Simulation

To assess the effect of having different cells present, changes in TEER from models D and E shown in **Figure 1**, were compared following an OGD protocol. **Figure 5A** highlights the different responses of a three cell and four cell model in response to a 4 h OGD protocol, followed by a reperfusion period. The three-cell mode exhibited approximately a 30% drop in TEER from baseline after 4 h OGD. This contrasts to the four-cell model which exhibited a 50% drop in TEER post OGD and was significantly different to the three-cell model $P < 0.05$. After OGD, when reperfusion was initiated, TEER was able to return to baseline in the three-cell model, however, BBB permeability only marginally recovered by 20% in the four-cell model. This was significantly different at 24 h ($P < 0.01$) but not 48 or 72 h. Images 5B and C show light microscope images of neurons in the four-cell model before and immediately after the OGD protocol, respectively. In **Figure 5C** neuronal clumping is clearly visible along with apparent neurite fragmentation compared to **Figure 5B** showing healthy neurons prior to OGD.

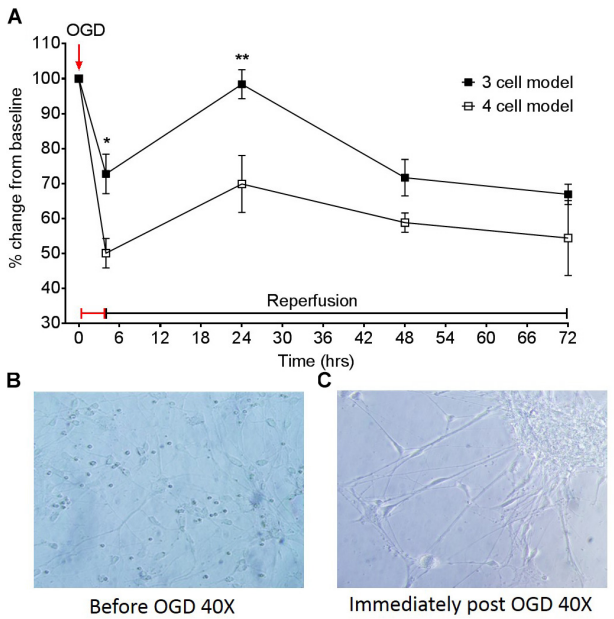

FIGURE 5 | (A) Effect of a 4 h oxygen-glucose deprivation (OGD) protocol on transepithelial resistance (TEER) as a marker of barrier tightness in a three cell model (HBMECs, astrocytes, and pericytes) and a four cell model (HBMECs, astrocytes, pericytes, and neurons). Neuronal images taken from the four cell model **(B)** before OGD 40× and **(C)** neuronal images immediately post OGD 40×. Data given as mean ± SEM, n = 3–6 from two experimental repeats, calculated as a % change from baseline TEER readings. Statistical analysis was conducted using 2-way ANOVA with Sidak's multiple comparisons test, $^{*}P < 0.05$ and $^{**}P < 0.01$ was considered significant.

Dexamethasone Application

Dexamethasone increased barrier tightness in all three models, as shown by increases in TEER and exhibiting overall significance as a result of drug interaction in the three cell and four cell model, $P < 0.05$. The two-cell model was the most unstable out of the three models, as shown by greater fluctuations and variability in TEER measurements (**Figure 6A**). The three-cell model was considerably more stable but differences in TEER between dexamethasone treated and control were only observed after 2 h (**Figure 6B**). The four-cell model was the fastest to exhibit an increase in barrier tightness (i.e., increased TEER) as a result of dexamethasone application (**Figure 6C**) and this reached significance compared to the vehicle control at 2 and 24 h ($P < 0.05$).

DISCUSSION

The BBB can be compromised in a range of different conditions, including but not limited to ischaemic stroke, Alzheimer's disease, cancer, and multiple sclerosis (MS). Research into these disorders that affect the BBB is plagued by translational difficulty, resulting in many potential compounds and/or therapies failing to surpass phase I/II clinical trials. This is at least partly due to a lack of suitable *in vitro* models that can predict drug effectiveness pre-clinically. Most, if not all, current BBB models exhibit "pitfalls" whether that be cost, time or resources. Models that offer the closest representation of the BBB are often complex and expensive to replicate, adding to the cost of the drug screening process. To help improve the translatability of *in vitro* data, we developed a transwell style model that incorporates four primary human cell types, representing the NVU more than other BBB models currently available. We found that our novel four-cell model was superior in modeling ischaemic stroke and drug application *in vitro* compared to a three-cell and a two-cell model as shown through changes in TEER as a measure of barrier integrity and dexamethasone application.

Implications for Drug Testing

The effect of dexamethasone was assessed in three transwell models; a two cell, three cell and four cell model. Greater instability in barrier strength and a slower response was exhibited by the two-cell model after dexamethasone application. This could also suggest that models containing just two cell types, in this case astrocytes and HBMECs, would also react differently to other drug applications and are therefore are not sufficient to truly model drug interactions at the BBB. Whilst the three-cell model shared the same trend in increasing barrier strength, it exhibited more stable TEER values compared to the two-cell model and dexamethasone

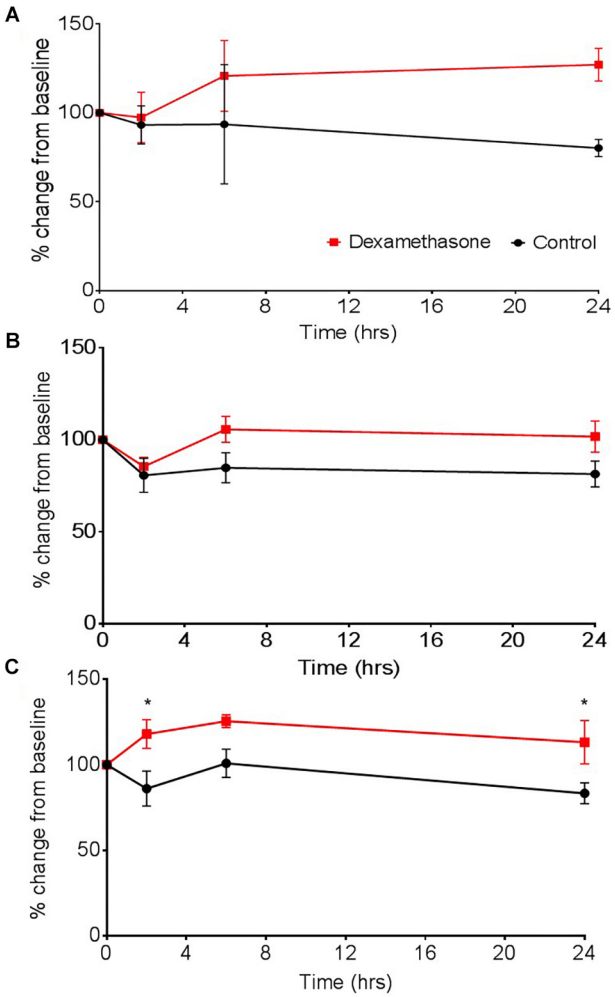

FIGURE 6 | The effect of dexamethasone on transepithelial resistance (TEER) as a measure of barrier tightness in **(A)** a two cell (astrocytes and HBMECs), **(B)** three cell (astrocytes, HBMECs, and pericytes), and **(C)** four cell model (astrocytes, HBMECs, pericytes, and neurons). Dexamethasone (1 μM) was added to the luminal side and used as positive control that is known to decrease permeability, thus increase TEER. Data represented as mean ± SEM, $n = 4$ from two experimental repeats. Statistical analysis was conducted using 2-way ANOVA with Sidak's multiple comparisons test, $^*P < 0.05$ was considered significant.

treated wells were overall significantly different to the vehicle control. Also, by introducing pericytes (generating a three cell model) there was a large increase in baseline TEER from 30 to 40 Ω, again highlighting the role of pericytes in strengthening vascular stability at the BBB and the need for their presence in BBB models (Bergers and Song, 2005; Dohgu et al., 2005; Nakagawa et al., 2007; Ferland-McCollough et al., 2017) Interestingly, the four-cell model exhibited a significant increase in barrier tightness (as shown by an increase in TEER) compared to the vehicle control at just 2 h after dexamethasone application. Although neurons in this model do not directly interact with the BBB, neurons have been shown to secrete a number of vasoactive substances, including VEGF, which influence barrier forming properties and early angiogenesis (Engelhardt, 2003; Eichmann and Thomas, 2013). These comparison data highlight the variations in data obtained from models containing different cell types and the impact this can have on drug screening. This stresses the importance of having a more representative BBB model containing additional cells present at the NVU.

Implications for Protocol Testing

Currently there are a wide range of *in vitro* BBB models available, but despite promising developments in modeling the BBB, there are still gaps in model design, primarily the inability to include all cell types present in the NVU. Whilst most transwell systems incorporate astrocytes and HBMECs, only more recent studies have introduced pericytes or neurons into these model designs. To gain a better understanding of how these cells contribute to the breakdown of the BBB in ischaemic conditions, we subjected our three cell and four cell models to an OGD protocol and measured TEER overtime to assess changes in barrier integrity. Interestingly, we found that with the presence of neurons our model exhibited a larger decrease in TEER compared to the three-cell model, which only contained astrocytes, pericytes and HBMECs. Similarly, whilst the three-cell model was able to recover 24 h post OGD the four-cell model only marginally recovered by approximately 20%, highlighting the role of and sensitivity of neurons in the level of damage ensued by the OGD protocol. Altogether, we have shown that with the addition of neurons our model became more vulnerable to damage; exhibiting a greater loss of barrier strength shown by a decrease in TEER, supporting previous work which showed that ischaemic neurons disrupt the endothelial barrier through increasing VEGF secretion (Li et al., 2014). Thus, omitting neurons from a BBB modeling stroke would underestimate the damage caused and contribution of neurons to the breakdown of the BBB post ischaemia.

Limitations and Future Development

Although our model now includes four cell present in the NVU, our model does not incorporate flow which is an important feature to maintain the BBB phenotype *in vitro*. Studies have shown that sheer stress is critical to increase cell longevity and influence cell phenotype, regulate BBB transport, preventing de-differentiation (Desai et al., 2002; Chiu et al., 2005; Partyka et al., 2017). Culturing HBMECs under physiological shear stress, is particularly important in a ischaemic stroke setting because there is an interruption in blood flow. Microfluidic systems that mimic physiological flow have the advantage in that they can simulate continuous flow improving translation to the environment (Partyka et al., 2017; Wang et al., 2017).

Equally, there is increasing evidence of the role of microglial cells in BBB breakdown. These resident brain immune cells have been shown to release pro-inflammatory mediators that increase barrier permeability and reduce levels of certain TJs, thus playing a key role in BBB breakdown in pathological states

(da Fonseca et al., 2014; Shigemoto-Mogami et al., 2018) Therefore, future work should assess whether additional cells and shear stress can be incorporated into transwell style models.

CONCLUSION

The overall function of the NVU is the perfusion of brain tissue to supply neurons with essential nutrients and the ability of neurons to regulate this blood flow. Glia, namely astrocytes, act as mediators between the vascular and neural compartments (Lo et al., 2015). Pericytes provide an extra level of communication between the endothelia and astrocytes as well as serving a prominent immune function (Darland et al., 2003; Armulik et al., 2011; Kovac et al., 2011). During cerebral ischaemia, these complex interactions are disrupted, and homeostasis is lost as a consequence of functional, morphological and metabolic changes within the NVU (Lo et al., 2015). It is important to model how these cells interact in both normoxic and ischaemic conditions to study the pathophysiology of ischaemic stroke. Finally, transwell systems offer noticeable advantages over the more complex models as they maintain the ease simpler cell culture set up and often use minimal resources. We believe our model offers a closer representation of the BBB, whilst maintaining the ease of a transwell setup.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

NS performed the research. TE and SO'S designed the research study. NS and SO'S analyzed the data. NS, TE, and SO'S wrote the manuscript.

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REFERENCES

Abbott, N. J., Patabendige, A. A. K., Dolman, D. E. M., Yusof, S. R., and Begley, D. J. (2010). Structure and function of the blood-brain barrier. *Neurobiol. Dis.* 37, 13–25. doi: 10.1016/j.nbd.2009.07.030

Abbott, N. J., Rönnbäck, L., and Hansson, E. (2006). Astrocyte–endothelial interactions at the blood–brain barrier. *Nat. Rev. Neurosci.* 7, 41–53. doi: 10.1038/nrn1824

Allen, C. L., and Bayraktutan, U. (2009). Antioxidants attenuate hyperglycaemia-mediated brain endothelial cell dysfunction and blood-brain barrier hyperpermeability. *Diabetes Obes. Metab.* 11, 480–490. doi: 10.1111/j.1463-1326.2008.00987.x

Appelt-Menzel, A., Cubukova, A., Günther, K., Edenhofer, F., Piontek, J., Krause, G., et al. (2017). Establishment of a human blood-brain barrier co-culture model mimicking the neurovascular unit using induced pluri- and multipotent stem cells. *Stem Cell Rep.* 8, 894–906. doi: 10.1016/j.stemcr.2017.02.021

Armulik, A., Genové, G., and Betsholtz, C. (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* 21, 193–215. doi: 10.1016/j.devcel.2011.07.001

Attwell, D., Buchan, A. M., Charpak, S., Lauritzen, M., MacVicar, B. A., and Newman, E. A. (2010). Glial and neuronal control of brain blood flow. *Nature* 468, 232–243. doi: 10.1038/nature09613

Bergers, G., and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro. Oncol.* 7, 452–464. doi: 10.1215/S1152851705000232

Borges, N., Shi, F., Azevedo, I., and Audus, K. L. (1994). Changes in brain microvessel endothelial cell monolayer permeability induced by adrenergic drugs. *Eur. J. Pharmacol. Mol. Pharmacol.* 269, 243–248. doi: 10.1016/0922-4106(94)90092-2

Chiu, J. J., Chen, L. J., Chang, S. F., Lee, P. L., Lee, C. I., Tsai, M. C., et al. (2005). Shear stress inhibits smooth muscle cell-induced inflammatory gene expression in endothelial cells: Role of NF-κB. *Arterioscler. Thromb. Vasc. Biol.* 25, 963–969. doi: 10.1161/01.ATV.0000159703.43374.19

Cho, C. F., Wolfe, J. M., Fadzen, C. M., Calligaris, D., Hornburg, K., Chiocca, E. A., et al. (2017). Blood-brain-barrier spheroids as an in vitro screening platform for brain-penetrating agents. *Nat. Commun.* 8:15623. doi: 10.1038/ncomms15623

da Fonseca, A. C. C., Matias, D., Garcia, C., Amaral, R., Geraldo, L. H., Freitas, C., et al. (2014). The impact of microglial activation on blood-brain barrier in brain diseases. *Front. Cell. Neurosci.* 8:362. doi: 10.3389/fncel.2014.00362

Darland, D. C., Massingham, L. J., Smith, S. R., Piek, E., Saint-Geniez, M., and D'Amore, P. A. (2003). Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival. *Dev. Biol.* 264, 275–288. doi: 10.1016/j.ydbio.2003.08.015

Desai, S. Y., Marroni, M., Cucullo, L., Krizanac-Bengez, L., Mayberg, M. R., Hossain, M. T., et al. (2002). Mechanisms of endothelial survival under shear stress. *Endothel. J. Endothel. Cell Res.* 9, 89–102. doi: 10.1080/10623320212004

Dohgu, S., Takata, F., Yamauchi, A., Nakagawa, S., Egawa, T., Naito, M., et al. (2005). Brain pericytes contribute to the induction and up-regulation of blood-brain barrier functions through transforming growth factor-β production. *Brain Res.* 1038, 208–215. doi: 10.1016/j.brainres.2005.01.027

Dore-Duffy, P. (2008). Pericytes: pluripotent cells of the blood brain barrier. *Curr. Pharm. Des.* 14, 1581–1593. doi: 10.2174/138161208784705469

Eichmann, A., and Thomas, J. L. (2013). Molecular parallels between neural and vascular development. *Cold Spring Harb. Perspect. Med.* 3:a006551. doi: 10.1101/cshperspect.a006551

Engelhardt, B. (2003). Development of the blood-brain barrier. *Cell Tissue Res.* 314, 119–129. doi: 10.1007/s00441-003-0751-z

Ferland-McCollough, D., Slater, S., Richard, J., Reni, C., and Mangialardi, G. (2017). Pericytes, an overlooked player in vascular pathobiology. *Pharmacol. Ther.* 171, 30–42. doi: 10.1016/j.pharmthera.2016.11.008

Gaillard, P. J., Voorwinden, L. H., Nielsen, J. L., Ivanov, A., Atsumi, R., Engman, H., et al. (2000). Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur. J. Pharm. Sci.* 12, 215–222. doi: 10.1016/S0928-0987(00)00123-8

Hartz, A. M. S., Miller, D. S., and Bauer, B. (2010). Restoring blood-brain barrier P-glycoprotein reduces brain amyloid- in a mouse model of alzheimer's disease. *Mol. Pharmacol.* 77, 715–723. doi: 10.1124/mol.109.061754

Hind, W. H. (2014). *The Effects of Cannabinoids on Blood-Brain Barrier Permeability in vitro*. Ph.D. thesis, University of Nottingham, Nottingham.

Hind, W. H., England, T. J., and O'Sullivan, S. E. (2016). Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARγ and 5-HT1A receptors. *Br. J. Pharmacol.* 173, 815–825. doi: 10.1111/bph.13368

Hind, W. H., Tufarelli, C., Neophytou, M., Anderson, S. I., England, T. J., and O'Sullivan, S. E. (2015). Endocannabinoids modulate human blood-brain barrier permeability in vitro. *Br. J. Pharmacol.* 172, 3015–3027. doi: 10.1111/bph.13106

Kacem, K., Lacombe, P., Seylaz, J., and Bonvento, G. (1998). Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: a confocal microscopy study. *Glia* 23, 1–10.

Kovac, A., Erickson, M. A., Banks, W. A., Neuwelt, E., Abbott, N., Abrey, L., et al. (2011). Brain microvascular pericytes are immunoactive in culture: cytokine, chemokine, nitric oxide, and LRP-1 expression in response to lipopolysaccharide. *J. Neuroinflam.* 8:139. doi: 10.1186/1742-2094-8-139

Li, Y. N., Pan, R., Qin, X. J., Yang, W. L., Qi, Z., Liu, W., et al. (2014). Ischemic neurons activate astrocytes to disrupt endothelial barrier via increasing VEGF expression. *J. Neurochem.* 129, 120–129. doi: 10.1111/jnc.12611

Liebner, S., Corada, M., Bangsow, T., Babbage, J., Taddei, A., Czupalla, C. J., et al. (2008). Wnt/β-catenin signaling controls development of the blood–brain barrier. *J. Cell. Biol.* 183, 409–417. doi: 10.1083/jcb.200806024

Lo, E., Mendelow, A., Sacco, R., and Wong, L. (2015). "7. The neurovascular unit and responses to ischaemia," in *Stroke*, eds J. Grotta, G. Albers, J. Broderickm, S. Kasner, E. Lo, R. Sacco, et al. (Amsterdam: Elsevier).

Macvicar, B. A., and Newman, E. A. (2015). Astrocyte regulation of blood flow in the brain. *Cold Spring Harb. Perspect. Biol.* 7, 1–15. doi: 10.1101/cshperspect.a020388

Martini, F., and Bartholomew, E. F. (2017). *Essentials of Anatomy & Physiology*. Amsterdam: Elsevier.

Nakagawa, S., Deli, M. A., Kawaguchi, H., Shimizudani, T., Shimono, T., Kittel, Á, et al. (2009). A new blood-brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes. *Neurochem. Int.* 54, 253–263. doi: 10.1016/j.neuint.2008.12.002

Nakagawa, S., Deli, M. A., Nakao, S., Honda, M., Hayashi, K., Nakaoke, R., et al. (2007). Pericytes from brain microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. *Cell. Mol. Neurobiol.* 27:687. doi: 10.1007/s10571-007-9195-4

Niego, B., and Medcalf, R. L. (2013). Improved method for the preparation of a human cell-based, contact model of the blood-brain barrier*. *J. Vis. Exp.* 81:e50934. doi: 10.3791/50934

Nzou, G., Wicks, R. T., Wicks, E. E., Seale, S. A., Sane, C. H., Chen, A., et al. (2018). Human cortex spheroid with a functional blood brain barrier for high-throughput neurotoxicity screening and disease modeling. *Sci. Rep.* 8:7413. doi: 10.1038/s41598-018-25603-5

Oberheim, N. A., Takano, T., Han, X., He, W., Lin, J. H. C., Wang, F., et al. (2009). Uniquely hominid features of adult human astrocytes. *J. Neurosci.* 29, 3276–3287. doi: 10.1523/JNEUROSCI.4707-08.2009

Pardridge, W. M. (1999). Blood-brain barrier biology and methodology. *J. Neurovirol.* 5, 556–569. doi: 10.3109/13550289909021285

Partyka, P. P., Godsey, G. A., Galie, J. R., Kosciuk, M. C., Acharya, N. K., Nagele, R. G., et al. (2017). Mechanical stress regulates transport in a compliant 3D model of the blood-brain barrier. *Biomaterials* 115, 30–39. doi: 10.1016/j.biomaterials.2016.11.012

Pieper, C., Pieloch, P., and Galla, H. J. (2013). Pericytes support neutrophil transmigration via interleukin-8 across a porcine co-culture model of the blood-brain barrier. *Brain Res.* 1524, 1–11. doi: 10.1016/j.brainres.2013.05.047

Pyrgos, V., Mickiene, D., Sein, T., Cotton, M., Fransesconi, A., Mizrahi, I., et al. (2010). Effects of immunomodulatory and organism-associated molecules on the permeability of an in vitro blood-brain barrier model to amphotericin B and fluconazole. *Antimicrob. Agents Chemother.* 54, 1305–1310. doi: 10.1128/AAC.01263-09

Risau, W., Hallmann, R., and Albrecht, U. (1986). Differentiation-dependent expression of proteins in brain endothelium during development of the blood-brain barrier. *Dev. Biol.* 117, 537–545. doi: 10.1016/0012-1606(86)90321-0

Ruck, T., Bittner, S., and Meuth, S. G. (2015). Blood-brain barrier modeling: challenges and perspectives. *Neural Regen. Res.* 10, 889–891. doi: 10.4103/1673-5374.158342

Shi, L. Z., and Zheng, W. (2005). Establishment of an in vitro brain barrier epithelial transport system for pharmacological and toxicological study. *Brain Res.* 1057, 37–48. doi: 10.1016/j.brainres.2005.07.046

Shigemoto-Mogami, Y., Hoshikawa, K., and Sato, K. (2018). Activated microglia disrupt the blood-brain barrier and induce chemokines and cytokines in a rat in vitro model. *Front. Cell. Neurosci.* 12:494. doi: 10.3389/fncel.2018.00494

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., and Hickman, J. J. (2015). TEER measurement techniques for in vitro barrier model systems. *J. Lab. Autom.* 20, 107–126. doi: 10.1177/2211068214561025

Sweeney, M. D., Ayyadurai, S., and Zlokovic, B. V. (2016). Pericytes of the neurovascular unit: key functions and signaling pathways. *Nat. Neurosci.* 19, 771–783. doi: 10.1038/nn.4288

Thomsen, L. B., Burkhart, A., and Moos, T. (2015). A triple culture model of the blood-brain barrier using porcine brain endothelial cells, astrocytes and pericytes. *PLoS One* 10:e0134765. doi: 10.1371/journal.pone.0134765

Wang, Y., Wang, N., Cai, B., Wang, G. Y., Li, J., and Piao, X. X. (2015). In vitro model of the blood-brain barrier established by co-culture of primary cerebral microvascular endothelial and astrocyte cells. *Neural Regen. Res.* 10, 2011–2017. doi: 10.4103/1673-5374.172320

Wang, Y. I., Abaci, H. E., and Shuler, M. L. (2017). Microfluidic blood–brain barrier model provides in vivo-like barrier properties for drug permeability screening. *Biotechnol. Bioeng.* 114, 184–194. doi: 10.1002/bit.26045

Warren, M. S., Zerangue, N., Woodford, K., Roberts, L. M., Tate, E. H., Feng, B., et al. (2009). Comparative gene expression profiles of ABC transporters in brain microvessel endothelial cells and brain in five species including human. *Pharmacol. Res.* 59, 404–413. doi: 10.1016/j.phrs.2009.02.007

Weidenfeller, C., Svendsen, C. N., and Shusta, E. V. (2007). Differentiating embryonic neural progenitor cells induce blood-brain barrier properties. *J. Neurochem.* 101, 555–565. doi: 10.1111/j.1471-4159.2006.04394.x

Yeon, J. H., Na, D., Choi, K., Ryu, S. W., Choi, C., and Park, J. K. (2012). Reliable permeability assay system in a microfluidic device mimicking cerebral vasculatures. *Biomed. Microdevices.* 14, 1141–1148. doi: 10.1007/s10544-012-9680-5

Zhang, Y., Sloan, S. A., Clarke, L. E., Caneda, C., Plaza, C. A., Blumenthal, P. D., et al. (2016). Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron* 89, 37–53. doi: 10.1016/j.neuron.2015.11.013

Zonta, M., Angulo, M. C., Gobbo, S., Rosengarten, B., Hossmann, K. A., Pozzan, T., et al. (2003). Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. *Nat. Neurosci.* 6, 43–50. doi: 10.1038/nn980

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.1 Four-cell model method development and supplemental information

During model development there were several challenges that required many troubleshooting experiments and method validation. Cells were required to be passaged on specific days to seed cells onto the inserts and the model itself could only be used for a maximum of five days. This posed several logistical issues, and some cultures were lost during the process of finding optimal days to passage for model set-up. Overall, the model development was very challenging, and it is important to appreciate it has limited applications given the expense of using primary human neurons, complex assembly, and the finite lifespan of the model in culture. The model is aimed for use only after preliminary screening of compounds has been completed and lead compounds have been identified.

Cell morphology was difficult to assess due to the material and coating of the transwell inserts and staining of individual cell types on the inserts was not possible due to the porous nature of the inserts. Whilst the increase in TEER is suggestive of barrier development, interactions between individual cell types were not assessed and could merely have been endothelial cells blocking the transwell pores. *In vitro* OGD protocols to model stroke have been adapted and used by numerous research groups because it recapitulates many of the cellular pathways associated with ischaemia-reperfusion injury (Holloway and Gavins, 2016). Specifically, the OGD protocol used in this study was based on previous work conducted by Hind and colleagues (Hind et al., 2015, 2016) and was found to successfully increase barrier permeability, as well as increase levels of damage and inflammatory markers. It is worth noting that the OGD protocol oxygen conditions are classed as ‘anoxia’ not ‘hypoxia’, effectively modelling a ‘worst-case’ scenario. Therefore, it would be useful for future studies to perform experiments with varying levels of hypoxia, for example between 0.5 and 2% oxygen, to model the *in vivo* environment more closely and to ascertain whether different levels of hypoxia affect the degree of BBB permeability. Despite these caveats, the model enabled

assessment barrier permeability under both physiological and pathophysiological conditions and could be used for a range of applications in drug discovery.

2. A Systematic Review of Minor Phytocannabinoids with Promising Neuroprotective Potential

The following chapter is presented in its final manuscript format and is published in The British Journal of Pharmacology (Stone et al., 2020).

Statement of author contributions: NS contributed significantly to the concept and design of the systematic review with help from SOS. NS performed the primary search and data extraction; XM conducted the secondary search and reviewed the extracted data. NS prepared and formatted figures and drafted the manuscript with input by all authors.

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REVIEW ARTICLE

A systematic review of minor phytocannabinoids with promising neuroprotective potential

Nicole L. Stone | Alexandra J. Murphy | Timothy J. England | Saoirse E. O'Sullivan

Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital, Derby, UK

Correspondence
Nicole L. Stone, Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital, Derby DE22 3DT, UK.
Email: nicolestone@hotmail.co.uk

Embase and PubMed were systematically searched for articles addressing the neuroprotective properties of phytocannabinoids, apart from cannabidiol and Δ^9-tetrahydrocannabinol, including Δ^9-tetrahydrocannabinolic acid, Δ^9-tetrahydrocannabivarin, cannabidiolic acid, cannabidivarin, cannabichromene, cannabichromenic acid, cannabichromevarin, cannabigerol, cannabigerolic acid, cannabigerivarin, cannabigerovarinic acid, cannabichromevarinic acid, cannabidivarinic acid, and cannabinol. Out of 2,341 studies, 31 articles met inclusion criteria. Cannabigerol (range 5 to 20 mg·kg^{-1}) and cannabidivarin (range 0.2 to 400 mg·kg^{-1}) displayed efficacy in models of Huntington's disease and epilepsy. Cannabichromene (10–75 mg·kg^{-1}), Δ^9-tetrahydrocannabinolic acid (20 mg·kg^{-1}), and tetrahydrocannabivarin (range 0.025–2.5 mg·kg^{-1}) showed promise in models of seizure and hypomobility, Huntington's and Parkinson's disease. Limited mechanistic data showed cannabigerol, its derivatives VCE.003 and VCE.003.2, and Δ^9-tetrahydrocannabinolic acid mediated some of their effects through PPAR-γ, but no other receptors were probed. Further studies with these phytocannabinoids, and their combinations, are warranted across a range of neurodegenerative disorders.

KEYWORDS
Alzheimer's, epilepsy, Huntington's, neurodegeneration, neuroprotection, phytocannabinoids

1 | INTRODUCTION

According to the World Health Organization (WHO), neurodegenerative diseases will be the second most prevalent cause of death by 2040 (Gammon, 2014). The cellular mechanisms of these diseases typically overlap with neuronal dysfunction and a common thread is neuronal cell death, regardless of definitive clinical presentations. Typically, neurodegenerative diseases are categorized as amyloidoses, which includes Alzheimer's disease and British familial dementia; synucleinopathies, which includes Lewy body disorders such as Parkinson's; and proteinopathies, which includes amyotrophic lateral sclerosis and tauopathies (Kovac, 2018). Other common neurological disorders include epilepsy and stroke, characterized by recurring, unprovoked seizures and vascular pathology, respectively. Recently, stroke was reclassified as a neurological disease by the International Classification of Disease (ICD) 11, highlighting that while strokes predominantly have a vascular origin, the neurological consequences are often severe (Shakir, 2018).

Current treatments for neurodegenerative and neurological conditions are often limited and usually rely on managing symptoms

Abbreviations: 3-NP, 3-nitropropionic acid; 6-OHDA, 6-hydroxydopamine; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; Δ^9-THCV, Δ^9-tetrahydrocannabivarin; BBB, blood–brain barrier; CBC, cannabichromene; CBCA, cannabichromenic acid; CBCV, cannabichromevarin; CBDA, cannabidiolic acid; CBDV, cannabidivarin; CBDVA, cannabidivarinic acid; CBG, cannabigerol; CBGA, cannabigerolic acid; CBGV, cannabigerivarin; CBGVA, cannabigerovarinic acid; CBM, cannabis-based medicine; CBN, cannabinol; CNCVA, cannabichromevarinic acid; MS, multiple sclerosis; RTT, Rett syndrome; TBZ, tetrabenazine; VMAT, vesicular monoamine transporter.

rather than having a significant effect on delaying disease progression (Kiaei, 2013). For example, **Huntington disease** is managed with **tetrabenazine (TBZ)** 75–200 mg per day to alleviate chorea (involuntary movement), but because it acts as a **vesicular monoamine transporter (VMAT)** inhibitor, interfering with both **5-HT** and **dopamine** degradation, patients can develop neuropsychiatric symptoms along with other side effects (Hayden, Leavitt, Yasothan, & Kirkpatrick, 2009; Kaur, Kumar, Jamwal, Deshmukh, & Gauttam, 2016; Wyant, Ridder, & Dayalu, 2017). Other first-line treatments, for example, **L-Dopa** in **Parkinson's disease**, often cause side effects and do not delay disease progression. Finally, **cholinesterase** inhibitors such as **donepezil** are only minimally effective in improving cognition for the treatment of **Alzheimer's disease**. In light of this, there is clearly an urgent need to develop new therapies with more tolerable side effect profiles to combat these debilitating conditions and increase the quality of life of the ageing population.

Over 120 different phytocannabinoids have been isolated from *Cannabis sativa* (ElSohly & Gul, 2015). Of these, **Δ^9-tetrahydrocannabinol (Δ^9-THC)** and **cannabidiol (CBD)** are the most abundant and widely studied. Δ^9-THC is responsible for the psychoactive effects of cannabis, which are mediated through the **cannabinoid CB_1 receptor (**Pertwee, 2008). Δ^9-THC also interacts with other targets including transient receptor potential (TRP) channels, the orphan G-protein receptor, **GPR55**, and peroxisome proliferator-activated receptors (PPARs; Pertwee & Cascio, 2015). CBD has also been shown to modulate a wide range of pharmacological targets including **5-HT_{1A}** receptors, **PPARγ** and **TRPV1** channels, but has no psychotropic effects because it does not activate central CB_1 receptors (see Ibeas Bih et al., 2015, and Russo & Marcu, 2017). Interaction with these targets has given CBD status as a neuroprotectant, anti-inflammatory agent and antioxidant (Fernandez-Ruiz et al., 2013; Maroon & Bost, 2018). These features, along with its favourable safety profile in humans (Millar et al., 2019; World Health Organization, 2017) has made CBD, in many respects, a more desirable drug candidate than Δ^9-THC. CBD has shown promise in several animal models of neurodegeneration as well as clinical trials for Parkinson's, Alzheimer's and **amyotrophic lateral sclerosis (**Iuvone, Esposito, de Filippis, Scuderi, & Steardo, 2009). Furthermore, a fixed combination of CBD and Δ^9-THC (1:1) is currently licenced by GW Pharmaceuticals under the brand name Sativex® to treat pain and spasticity associated with multiple sclerosis (MS), and Epidiolex® (pure CBD) is licensed to treat Lennox–Gastaut syndrome and Dravet syndrome, which are severe forms of childhood epilepsy. Other cannabis-based medicines (CBMs) are also under development. GW Pharmaceuticals has four compounds (structures are not disclosed) in the pipeline for neurological conditions including glioblastoma, schizophrenia and neonatal hypoxic-ischaemic encephalopathy (GW Pharmaceuticals, 2019).

Phytocannabinoids are highly unique compounds, they are promiscuous in action, modulating a range of pharmacological targets as well as exhibiting high antioxidant capability due to their phenolic structures and the presence of hydroxyl groups (Borges et al., 2013; Hampson, Grimaldi, Axelrod, & Wink, 1998; Yamaori, Ebisawa, Okushima, Yamamoto, & Watanabe, 2011). These features, along with their lipophilicity and ability to act as anti-inflammatory agents, makes them desirable therapeutic candidates for the treatment of CNS disorders, as they can effectively cross the blood–brain barrier (BBB), modulate the immune response, and target the many aspects of neurodegeneration (Deiana et al., 2012). These characteristics have been well established for Δ^9-THC and CBD but are less well known for some of the minor constituents of the plant. Thus, in order to understand the full therapeutic potential of *Cannabis sativa*, the pharmacology of the lesser-known components of the plant should be elucidated (Turner, Williams, Iversen, & Whalley, 2017). Given the wide-ranging neuroprotective effects of Δ^9-THC and CBD already established, it is not unreasonable to suggest other phytocannabinoids may exhibit similar or more potent neuroprotective properties. Therefore, the aim of this systematic review was to collate all available data on the neuroprotective effects of **Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA)**, **Δ^9-tetrahydrocannabivarin (Δ^9-THCV)**, **cannabidiolic acid (CBDA)**, **cannabidivarin (CBDV)**, **cannabichromene (CBC)**, cannabichromenic acid (CBCA), cannabichromevarin (CBCV), **cannabigerol (CBG)**, cannabigerolic acid (CBGA), cannabigerivarin (CBGV), cannabigerovarinic acid (CBGVA), cannabichromevarinic acid (CBCVA), cannabidivarinic acid (CBDVA), and **cannabinol (CBN)**. These phytocannabinoids were selected based on their abundance in the plant, ease of synthesis, efficacy in other fields (e.g., as anticancer agents or treatments for inflammatory bowel disease), and similarities in their structure to CBD and Δ^9-THC (which have already shown promise as a neuroprotectants and displayed safety in humans) and are therefore more likely to have neuroprotective potential and exhibit human translatability.

2 | METHODS

2.1 | Data sources and search strategy

An electronic search was conducted using the search engines PubMed and Embase from its inception to June 2019. This was carried out in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Moher, Liberati, & Tetzlaff, 2009; Shamseer et al., 2015; Tóth, Schumacher, Castro, & Perkins, 2010) Search terms included Δ^9-tetrahydrocannabinolic acid, Δ^9-tetrahydrocannabivarin, cannabidiolic acid, cannabidivarin, cannabichromene, cannabichromenic acid, cannabichromevarin, cannabigerol, cannabigerolic acid, cannabigerivarin, cannabigerovarinic acid, cannabichromevarinic acid, cannabidivarinic acid and cannabinol (and their corresponding abbreviations), phytocannabinoids, neurovascular unit, pericytes, neurons, astrocytes, human brain microvascular endothelial cells, brain, neuroinflammation, hyperexcitability, neurodegeneration, Huntington's, Alzheimer's, Parkinson's, epilepsy, and stroke. Two independent reviewers carried out the searches by November 2019, and the reference lists of the final papers were hand searched for any additional studies.

2.2 | Eligibility and exclusion criteria

Conference abstracts and review articles were excluded. No restrictions were applied to type of study, publication year, or language. Inclusion criteria were as follows: an original, peer reviewed article that involved the application of emerging phytocannabinoids in an in vivo or in vitro model of neurodegeneration or neuronal damage. Studies that looked at two derivatives of CBG, known as VCE-003 or VCE-002.3 were also included because current research is focused on these compounds, based on their increased affinity for PPARγ. Studies that assessed CBD, Δ^9-THC, Δ^9-THC:CBD 1:1 (Sativex®), or similar combinations of phytocannabinoids (i.e., different ratios of phytocannabinoids) were excluded from this review. After duplicates and irrelevant articles were removed, the full text was obtained for the remaining articles, and studies were examined for data regarding Δ^9-THCA, Δ^9-THCV, CBDA, CBDV, CBC, CBCA, CBCV, CBG, CBGA, CBGV, CBGVA, CBCVA, CBDVA, and CBN application in an in vitro and/or in vivo model of neuroprotection or neuronal damage. Dose and route of administration were extracted from in vivo studies and where possible range and average were calculated. If studies reported mechanistic data, this was also described in Section 3.

3 | RESULTS

The preliminary search retrieved 2,341 studies, which after duplicates were removed left 1,851. A total of 107 cannabinoid studies were retrieved; once exclusion criteria were applied, 26 articles were considered to be potentially relevant and their full texts obtained. After additional screening (including reviewing reference lists for any potential studies), 28 studies were included in this review; see Figure 1. Table 1 summarizes the *in vitro* data included in this review, and Table 2 summarizes the *in vivo* data.

Within the 28 studies, the neuroprotective models were epilepsy (*n* = 7), Huntington's disease (*n* = 6), Parkinson's (*n* = 4), amyotrophic lateral sclerosis (*n* = 3), neuroprotection (not disease specific, *n* = 2), multiple sclerosis (MS; *n* = 1), Rett syndrome (*n* = 2), neuroinflammation (*n* = 1), Alzheimer's (*n* = 1), and oxidative stress (*n* = 1). Fifteen papers studied CBG or its derivatives, five studies used CBN, eight studies used CBDV, and four studies used CBC. Only two studies used Δ^9-THCV, and three used Δ^9-THCA. CBDA was only included in one study. No data on the neuroprotective effects of CBGA, CBGV, CBCA, CBCV, CBCVA, CBGVA, or CBDVA were identified. Figure 2 shows some of the minor phytocannabinoids structures with CBD and Δ^9-THC for reference, and Table 3 summarises the neurological conditions for which emerging cannabinoids have shown therapeutic potential.

3.1 | Cannabigerol (CBG) and its derivatives

Nine studies included *in vitro* data, and eight included *in vivo* data on CBG and its derivatives that are formed by the oxidation of CBG

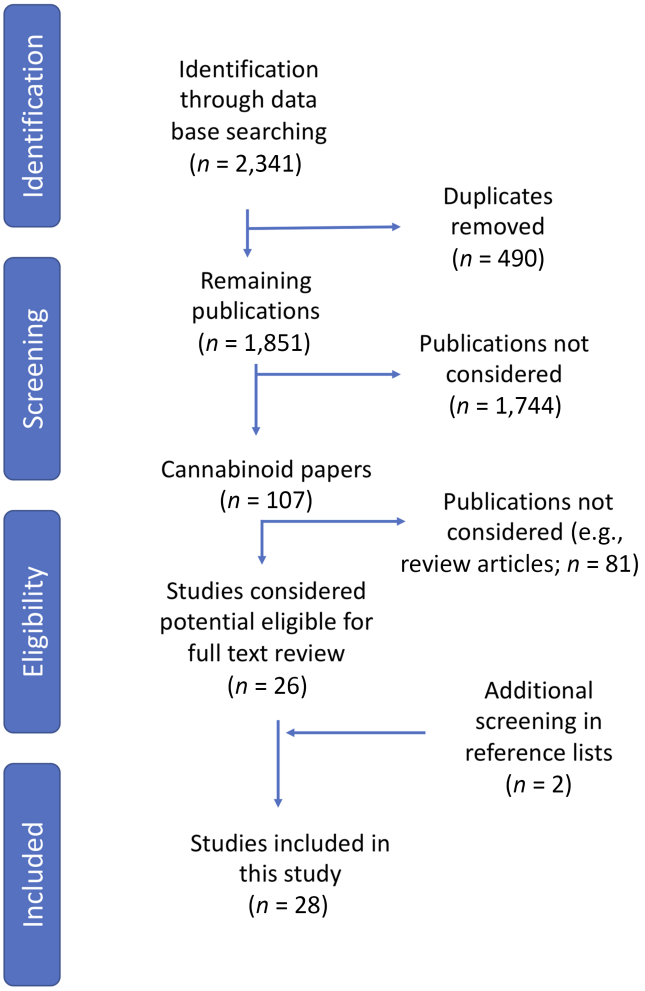

FIGURE 1 Overview of methodology used in the search process, identification, screening, eligibility, and inclusion

(Carrillo-Salinas et al., 2014; Díaz-Alonso et al., 2016; García et al., 2018). VCE-003 and VCE-003.2 have displayed increased affinity for PPARγ, thus maintaining their anti-inflammatory properties while having little affinity for CB_1 and **CB_2** receptors (VCE-003 K_i > 40 μM for CB_1 and K_i > 1.76 μM CB_2, Granja et al., 2012, and VCE-003.2 K_i > 40 μM for both CB_1 and CB_2, García et al., 2018). All studies except one reported a positive effect of CBG, VCE-003, or VCE-002.3, compared with control in the disease model being studied. In an *in vivo* model, using 3-nitropropionic acid (3-NP) to induce Huntington's disease, CBG (10 $mg \cdot kg^{-1}$ per day i.p.) significantly attenuated the up-regulation of **COX-2**, **iNOS**, **IL-6**, and **TNF-α** (Valdeolivas et al., 2015). CBG treatment also prevented 3-NP-induced neuronal loss, recovered catalase, SOD and GSH, compared with control, and down-regulating genes that were directly associated with Huntington's disease including sgkl, Cd44, and normalized levels of huntingtin-associated protein-1. Aggregation of mutant **Huntingtin protein** was diminished, and motor deficits such as hindlimb clasping and dystonia and general locomotor activity were also improved (Valdeolivas et al., 2015). Hill et al. (2014) assessed the anti-convulsant potential of CBG (50–200 $mg \cdot kg^{-1}$ i.p.) when

TABLE 1 Summary of included *in vitro* studies

Phytocannabinoid	Compound	Concentration/Incubation period	Neuro model	Cells used	*n* number	Results	Study
Cannabigerol (CBG)	Cannabigerol derivative VCE-003.2	500 nM for 21 days	Huntington's disease	Mouse embryonic stem cells (R1 line)/P19 neurospheres	$n = 3$	VCE-003.2 increased CTIP-2 positive cells, promoted neuronal like-differentiation and significantly larger P19 neurospheres versus vehicle treated cells ($P < 0.01$)	Aguareles et al. (2019)
	Cannabigerol derivative VCE-003	1, 5, 10 μM (human T-cells). 1 and 2.5 μM (RAW 264.7 cells) for 3 days post stimulation	Autoimmune Encephalomyelitis to model multiple sclerosis (MS)	Jurkat, BV2 RAW 264.7 cells. Human peripheral T-cells	$n = 3$[a]	1 μM reduced expression of iNOS in BV2 microglial cells. Antagonists AM630 (CB_2) and GW9662 (PPARγ) blocked these effects. Prevented T cell division at 1 and 5 μM and inhibition of the release of all soluble mediators (T-cells)	Carrillo-Salinas et al. (2014)
	Cannabigerol derivatives: VCE-003 and VCE-003.2	1–50 μM (N2a) for 24 h 50 nM–50 μM (HiB5) 30, 10, and 3 μM for 6 h	Huntington's disease	(N2a cells/HiB5 cells) Immortalized striatal neuroblasts expressing huntingtin/mutant repeats	$n = 3$[a]	VCE-003.2 improved cell viability (10 and 25 μM) and prevented excitotoxicity in N2a cells. VCE-003.2. Reduced the number of cells with aggregates (neuroblasts) and improved neuronal viability post serum deprivation	Diaz-Alonso et al. (2016)
	VCE-003 cannabigerol quinone derivative	0.1-, 1-, 10-, and 25-μM CBG/VCE-003 (HTT cells, 24 h) (microglia, 18 h; hippocampal cells; mice treated 15 days 5 $mg \cdot kg^{-1}$ i.p. VCE-003[b])	Multiple sclerosis	HEK293 cells and primary microglial cells. HT22 mouse hippocampal cells	$n = 3$[a]	VCE-003 protected neuronal cells from excitotoxity. Reduction in IL-1β, IL-6, TNF-α, PGE_2, and MIP-1-α in microglia (1, 10, and 25 μM) VCE-003 ameliorated MS symptoms induced by TMEV	Granja et al. (2012)
	VCE-003.2 cannabigerol derivative	BV2 cells 5 μM VCE-003.2 for 21 h. VCE-003.2 (M-213 cells) Vehicle (0.1% DMSO)	Parkinson's disease model induced by LPS (conditioned medium from BV2	Mouse microglial BV2 cells. M-213 (striatal cell line) neuronal cells	BV2 cells: $n = 14$, 7 repeats	In BV2 cells, VCE-003.2 significantly decreased TNF-α COX-2 and iNOS mRNA. Attenuated	Garcia et al. (2018)

(Continues)

TABLE 1 (Continued)

Phytocannabinoid	Compound	Concentration/Incubation period	Neuro model	Cells used	*n* number	Results	Study
		versus 0.1, 0.5, and 1 μM for 40 h	cells added to M-213 cells)			TNF-α and IL-1β secreted in medium of BV2 cells (5 μM)	
	Cannabigerol	MTT assay: 1, 2.5, 5, 7.5, 10, 12.5, 15, and 20 μM pretreated 24 h. NSC-34: pretreated with 7.5 μM	Neuroinflammation—medium from LPS stimulated macrophages	NSC-34 motor neurons	*n* = 3 repeats	CBG at 2.5 and 7.5 μM increased cell viability approximately 20% compared to control. CBG pretreatment inhibited apoptosis and reduced; IL-1β, TNF-α, INF-Y (NSC-34 motor neurons). CBG restored decreased Nrf2 levels	Gugliandolo et al. (2018)
	Cannabidiol* and cannabigerol	Electrophysiology: 1/10 μM 20 min. hNAv cells: 1 nM–200 μM for 100 s	PTZ seizures	Transverse hippocampal slices, SH-SY5Y, hNAv cell lines	SH-SY5Y—*n* = 6 mouse cortical neurons *n* = 8 hNAv *n* = 3	10-μM CBG significantly reduced peak Nav current in SH-SY5Y cells and mouse cortical neurons. CBG was also effective as a low affinity Nav channel blocker.	Hill et al. (2014)
	Cannabigerol derivative VCE-003.2	0.1, 0.5, 1, and 5 μM added 1 h prior to LPS, for 24 h	Amyotrophic lateral sclerosis	Astroglial cells (mutant SOD1 mice)	*n* = 4, 6 samples per group	VCE.003.2 at 1 and 5 μM attenuated levels of TNF-α and IL-1β, elevated due to LPS stimulation	Rodrígueuz-Cueto et al. (2018)
	Cannabigerol	6 h—supplementary information cannot be accessed	Huntington's disease	Immortalized striatal progenitor cells: STHdh$^{Q7/Q7}$ and STHdh $^{Q111/Q111}$ cells	*n* = 3 repeats	CBG dose-dependently activated PPARγ	Valdeolivas et al. (2015)
	Cannabigerol	1-μM 24-h ATP assay/viability and differentiation for 2 days	Neuroprotection	Adult neural stem cells/progenitor cells (NSPC)	*n* = 6	CBG had no significant effect on any of the endpoints measured.	Shinjyo & Di Marzo (2013)
Cannabidivarin (CBDV)	Cannabidivarin	1, 10, and 100 μM 30 min after epileptiform activity for 30 min	Epilepsy-spontaneous local field potentials (LFPs)	Transverse hippocampal slices male/female Kyoto rats	*n* > 5 slices from *n* > 5 animals	CBDV decreased amplitude and duration of LFPs and increased Mg2 + free induced LFPs frequency (>10 μM).	Hill et al. (2012)
	Cannabidivarin (+CBD)	3, 10, 30 μM 30–40 min after control readings for 1 min	Epilepsy	HEK cells (HEK293) transfected with	*n* = 4	CBDV was anticonvulsant, and TRPV1 antagonist	Iannotti et al. (2014)

TABLE 1 (Continued)

Phytocannabinoid	Compound	Concentration/Incubation period	Neuro model	Cells used	*n* number	Results	Study
				TRPV1, TRPV2, and TRPA1.		capsazepine blocked this effect. 10 μM CBDV tended to increase phosphorylation at the S800 site of TRPV1.	
Cannabichromene (CBC)	Cannabichromene	1-μM 24-h ATP assay/viability and differentiation for 2 days	Neuroprotection	Adult neural stem cells/ progenitor cells (NSPC)	*n* = 6	CBC raised viability in B27 medium. CBC had no significant effect on proliferation. In B27 medium, CBC up-regulated nestin, but reduced GFAP.	Shinjyo & Di Marzo (2013)
Cannabinol (CBN)	Cannabinol/Δ^8 THC	100, 20, 4, 0.8, 0.16, or 0 μM for 48 h	Huntington's disease	PC12 cells expressing polynucleotide repeats (103 glutamines)	*n* = 2 repeats, average 3– 4 wells	Cannabinol reduced LDH activity in medium at 20 and 100 μM. At 100 μM, CBN decreased LDH release by 84%. Protective EC_{50} of CBN was determined to be 30 μM in this model.	Aiken, Tobin, & Schweitzer, 2004
	Cannabinol (+THC and CBD)	0.1, 1, 2.5, 5, and 10 μM for 24 h	Oxidative stress and neuroprotection	Primary cerebral granule cells (rats/mice), CB1 expressing cell lines. PC12 and HT22 cell lines	*n* = 3	Cannabinol was shown to be a potent antioxidant.	Marsicano, Moosmann, Hermann, Lutz, and Behl (2002)
Tetrahydrocannabidivarin (Δ^9-THCV)	Δ^9-THCV	0, 5, 10, 20, 40, and 50 μM applied directly after epileptiform activity. 20-min pretreatment at 10 μM	In vitro electrophysiology (epileptiform bursting)	Brain slices obtained from male and female outbred rats	*n* = 5	Δ^9-THCV (20–50 μM) decreased burst incidence, PDS amplitude and frequency. The most significant effect was at 50 μM. Δ^9-THCV also decreased epileptiform burst speed (40 μM). Δ^9-THCV was found to act as a CB_1 ligand in receptor binding assays.	Hill et al. (2010)
Tetrahydrocannabinolic acid (Δ^9-THCA)	Δ^9-THCA	0.01, 0.1, 1, and 10 μM for 48 h	Parkinson's disease	Dopaminergic neuronal cell culture	*n* = 3–4 wells/ treatment	Δ^9-THCA had no effect on the survival of dopaminergic	Moldzio et al. (2012)

(Continues)

TABLE 1 (Continued)

Phytocannabinoid	Compound	Concentration/Incubation period	Neuro model	Cells used	*n* number	Results	Study
						neurons, but at 10 μM led to an increased cell count (123%) and morphology was ameliorated versus control cultures.	
Mixed	Δ^9-Tetrahydrocannabinolic acid (Δ^9-THCA) and cannabidiolic acid (CBDA), cannabigerol (CBG)	0, 0.5, and 1 μM (Δ^9-THCA) N2a cells—48 h. 0 and 0.1–15 μM (Δ^9-THCA, CBDA, and CBGA in HEK-293 T cells)—6 h. 1–10 μM Δ^9-THCA STHdh$^{Q7/Q7}$ cells—1 h/CB	Huntington's disease/ neurodegeneration	HEK-293 T Neuro-2a STHdh$^{Q7/Q7}$ And STHdh $^{Q111/Q111}$ cells	*n* = 5 repeats	Δ^9-THCA increased neuronal cell viability post serum deprivation and increased mitochondrial mass. This effect was blocked by a PPARγ antagonist GW9662. All cannabinoid acids induced PPARγ transcriptional activity in HEK293 cells.	Nadal et al. (2017)
	Cannabichromene, cannabidiol, cannabidivarin, cannabigerol, cannabinol, Δ^9-tetrahydrocannabinol, Δ^9-tetrahydrocannabinolic acid	0, 0.1, 1, and 10 μM for 48 h	Neuroprotection	N18TG2 cells (neuroblastoma cell line)	In triplicate with 2–5 repeats	Emerging phytocannabinoids did not affect the number of dopaminergic neurons. CBG and CBC decreased GSH levels (0.1 and 1 μM and 1 and 10 μM). 0.1 μM CBDV reduced GSH levels by 9.6%; THC, THCA, and CBN have no effect. CBDV and CBN decreased resazurin reduction at 10 μM (32.9 and 38.9%) and affected PI uptake at all concentrations. CBG also affected PI uptake at 0.1 and 10 μM.	Rosenthaler et al. (2014)
	Cannabigerol, cannabichromene, cannabidivarin, and cannabinol (as well as THC,	250 nM–10 μM Oxytosis assay, 30 min. Energy loss assay: 22 h.	Alzheimer's disease	MC65 cells (human nerve cell line), Ht22 cells (mouse hippocampal cell	*n* = 6 (twice in triplicate)	CBG, CBDV, CBC, CBN, and THCA prevented oxytosis.	Schubert et al. (2019)

TABLE 1 (Continued)

Phytocannabinoid	Compound	Concentration/Incubation period	Neuro model	Cells used	*n* number	Results	Study
	CBD, and CBD derivative DMCBD*)	Trophic factor withdrawal, 48 h		line), and BV2 microglial cell line		CBG, CBDV, CBC, and CBN preserved trophic factors. THCA was toxic to MC65 cells at 1 μM; however, CBDV, CBC, CBN, and CBDA prevented amyloid toxicity at ≤100 nM. CBDV, CBG, CBC, and CBN (100 nM) prevented MC65 neurons from accumulating amyloid β (Aβ).	

[a]Results from 3 independent experiments.
[b]For in vivo data see Table 2.

TABLE 2 Summary of included *in vivo* studies

Phytocannabinoid	Compound	Dose/route/time	Neuro model	Animals[a]	*n* number	Results	Study
Cannabigerol (CBG)	Cannabigerol derivatives VCE-003 and VCE-003.2	10 mg·kg^{-1} of body weight intraperitoneally per day until kill	Two models of Huntington's disease	**M** CD1 mice (12 weeks)	*n* = 7 each group	QA model: VCE-003.2 RotaRod performance, prevented neuronal loss, microglial activation and reduced astrogliosis. 3NP model: VCE-003.2 improved motor deficits, reduced all pro-inflammatory mediator release, and prevented neuronal loss.	Díaz-Alonso et al. (2016)
	Cannabigerol derivative VCE-003.2	10 mg·kg^{-1} oral once daily for 3 days before kill	Huntington's disease	**M** C57/6 N mice (10 weeks)	*n* = 3–6 mice/condition	VCE-003.2 promoted neurogenesis, increased GFAP-positive cells, and reduced microglial activation. Mice performed better on the RotorRod test drug treated versus vehicle.	Aguareles et al. (2019)
	Cannabigerol derivative VCE-003.2	Oral 10 mg·kg^{-1}, 20 mg·kg^{-1}, 16 h after LPS for 28 days daily	LPS-induced Parkinson's disease	C57BL/6 **F** mice, 7–11 months old	*n* = 6 mice per group	20 mg·kg^{-1} partly corrected altered cylinder rearing test but poor activity in rotarod and CAA tests. VCE—003.2 attenuated TNF-α, IL-1β (greatest effect at 20 mg·kg^{-1}) and recovered TH nigrostriatal neurons.	Burgaz, García, Gómez-Cañas, Muñoz, and Fernández-Ruiz (2019)
	Cannabigerol derivative VCE-003	Daily 5 mg·kg^{-1} i.p. for 21 days	Autoimmune encephalomyelitis (EAE) to model MS	**F** C57BL/6 mice	*n* = 6 animals per group	5 mg·kg^{-1} of VCE-003 decreased EAE symptoms. VCE-003 decreased microglial/macrophage activation, reduced demyelination, maintained myelin structure, and reduced axonal damage lesions. Significant decrease in all measured inflammatory mediators.	Carrillo-Salinas et al. (2014)
	VCE-003 cannabigerol quinone derivative	15 days 5 mg·kg^{-1} i.p. VCE-003 treated 60 days after infection	Multiple sclerosis (MS) induced by TMEV	SJL/J mice	*n* = 12	Clinical score (0–5) was significantly improved with VCE-003 treatment. VCE-003 completely recovered motor activities to normal levels.	Granja et al. (2012)

TABLE 2 (Continued)

Phytocannabinoid	Compound	Dose/route/time	Neuro model	Animals[a]	*n* number	Results	Study
	VCE-003.2 cannabigerol derivative	10 mg·kg^{-1} i.p. 16 h post LPS and then daily for 21 days	Parkinson's disease model—LPS induced	**M** C57BL/6 mice	*n* = 4–6 subjects per group	VC-003.2 prevented nigrostriatal neuronal loss and reduced microgliosis. Elevation in iNOS was decreased by VC-003.2 versus control.	García et al. (2018)
	Cannabigerol	50–200 mg·kg^{-1} i.p. 1 h before PTZ seizures	PTZ seizure model (85 mg·kg^{-1} i.p.)	**M** Wistar Kyoto rats	*n* = 72	CBG had no effect on seizure severity, incidence, or timing and did not alter animal mortality. CBG displayed no anti-convulsant effects.	Hill et al. (2014)
	Cannabigerol derivative VCE-003	10 mg·kg^{-1} i.p. animals 60 days old up to age 18 weeks	Amyotrophic lateral sclerosis	**M** B6SJL-Tg (SOD1*G93A) 1Gur/J versus WT	*n* = 5–6 animals per group	In SOD1 mice, VCE-003.2 delayed disease progression and reduced a number of neuropathological signs. Weight loss was reduced, as were anomalies in clinical score.	Rodríguez-Cueto et al. (2018)
	Cannabigerol (CBG)	4 intraperitoneal injections every 24 h at a dose of 10 mg·kg^{-1} for 6 weeks (4 weeks after birth to 10 weeks)	Huntington's disease induced by 3NP/R6/2 variant mice	16-week-old **M** C57BL/6 mice/4- to 10-week-old R6/2 mice	*n* = 6–8 animals/experiment	CBG improved motor activities, prevented neuronal loss, increased GFAP staining, and decreased Iba-1 staining. CBG down-regulated Huntington associated genes and decreased inflammatory mediators.	Valdeolivas et al. (2015)
Cannabidivarin (CBDV)	Cannabidivarin (CBDV)	Pretreatment vehicle versus 400 mg·kg^{-1} CBDV oral for 3.5 h	Seizures induced by PTZ 95 mg·kg^{-1}	Wistar-Kyoto rats (3/4 weeks old).	*n* = 51	400 mg·kg^{-1} CBDV significantly decreased seizure severity and increased latency to first signs of seizure. CBDV did not significantly affect gene expression changes induced by PTZ.	Amada et al. (2013)
	Cannabidivarin (CBDV)	50, 100, and 200 mg·kg^{-1} i.p. injection 1 h/30 min before induced seizures. 400 mg·kg^{-1} oral gavage 13.5/3.5 h before intraperitoneal PTZ	Epilepsy (mES seizures; 30 mA, 100 Hz for 200 ms, or generalized seizures 85 mg·kg^{-1} PTZ injected intraperitoneally	**F/M** adult Wistar Kyoto rats. Non-Agouti (DBA/) mice 3–4 weeks, ICR (CD-1) mice 5 weeks old	*n* = 80 (10/group). 640 Wistar rats 3–4 weeks old (*n* = 15/group)	200 mg·kg^{-1} CBDV—90% of mice remained seizure free. In rats, CBDV significantly decreased PTZ seizure severity and rodent mortality (200 mg·kg^{-1}) and delayed seizure onset. On co-administration	Hill et al. (2012)

(Continues)

TABLE 2 (Continued)

Phytocannabinoid	Compound	Dose/route/time	Neuro model	Animals[a]	*n* number	Results	Study
						experiments, 2.9% of rats (*n* = 7) exhibited a fatal reaction to CBDV administration.	
	Cannabidivarin (only data from purified CBDV is reported here)	1 h pretreatment 50, 100, and 200 mg·kg^{-1} i.p. (rats) 10–200 mg·kg^{-1} i.p. (mice)	PTZ seizures (85 mg·kg^{-1}) or pilocarpine (380 mg·kg^{-1}).	M Wistar Kyoto rats, M MF1 mice, DBA/2 mice 3–4 weeks	*n* = 10 mice *n* = 15 rats	CBDV significantly affected observed seizure severity >50 mg·kg^{-1}. Mortality was reduced by CBDV administration and suppressed seizure activity (100 mg·kg^{-1})	Hill et al. (2013)
	Cannabidivarin (CBDV)	2, 20, and 100 mg·kg^{-1} versus vehicle control, daily intraperitoneally for 14 consecutive days	Rett syndrome	5-month-old MeCP2–308 (B6.129S-MeCP2tm1Heto/J	*n* = 70	20 mg·kg^{-1} CBDV improved motor learning ability. Brain weight was increased with CBDV treatment. CBDV had no effect on GPR55 levels and neurotrophin levels.	Vigli et al. (2018)
	Cannabidivarin (96.4% CBDV, 3.6% CBD; started on postnatal day 28, lasting until day 67)	0.2, 2, 20, and 200 mg·kg^{-1} i per day i.p. initiated postnatal day (PND) 28 until PND 67.	Rett syndrome model; (WT vs. Mecp2 KO)	Mecp2–mouse (WT vs. KO).	*n* ≥ 5 per treatment group total *n* = 112	2–200 mg·kg^{-1} per day CBDV reduced tremors, and 0.2 mg·kg^{-1} per day was ineffective. CBDV reduced hind limb clasping but again not at the lowest dose tested. CBDV improved breathing and gait abnormalities, reduced total symptom score, and improved neurological motor deficits.	Zamberletti et al. (2019)
Cannabichromene (CBC)	CBC	0.01 ml·g^{-1} and 25, 50, and 75 mg·kg^{-1} CBC (mice), 1.0 ml·kg^{-1}, 10–75 mg·kg^{-1} CBC (rats) i.p. for 1 h prior to electroshock	Electroshock seizure test: 50 mA intensity for 0.2 s	M ICR albino mice or male Sprague–Dawley rats	*n* = 90 (mice), 193 mice, 106 rats	CBC/THC had no effect on tonic hindlimb extension. CBC did not alter latency. CBC (lowest dose) shortened the duration of extension. All doses of CBC depressed motor activity (first 10-min interval).	Davis and Hatoum (1983)
Cannabinol (CBN)	CBN	5 mg·kg^{-1}·day^{-1} subcutaneous pouch (25-g mouse). 28 days up to 12 weeks	Amyotrophic lateral sclerosis (ALS) SOD1 model	M Tg (SOD1-G93A) 2Gur (11) mice. Assigned 6 weeks of age	*n* = 18	Motor abnormalities were delayed by CBN versus vehicle.	Weydt et al. (2005)

TABLE 2 (Continued)

Phytocannabinoid	Compound	Dose/route/time	Neuro model	Animals[a]	*n* number	Results	Study
						No significant difference for PaGE test assessment or the age at which animals reached end stage.	
Tetrahydrocannabidivarin (Δ^9-THCV)	Δ^9-THCV	2 mg·kg^{-1} i.p. for 14 days	Parkinson's disease (by 6-hydroxytryptamine-6-HT) or LPS	**M** Sprague–Dawley rats/CB2 knockout mice	*n* = 5–6 rats per group	THCV improved motor activities, reduced neuronal loss and reduced microglial activation. THCV was able to preserve TH positive neurons (LPS model).	García et al. (2011)
	Δ^9-THCV	0.025, 0.25, and 2.5 mg·kg^{-1} i.p. + vehicle prior to initiating seizures	Seizures induced by 80 mg·kg^{-1} PTZ	**M** Wistar rats	64 rats in total; *n* = 16 per group	Median seizure severity, duration, progression, or latency was unaffected by any dose of THCV. 33% of animals exhibited a complete absence of seizures at a dose of 0.25 mg·kg^{-1} THCV.	Hill et al. (2010)
Tetrahydrocanvnabidiolc acid (Δ^9-THCA)	Δ^9-THCA	20 mg·kg^{-1} i.p. 30 min before 3NPA, every 24 h for 4 days	Huntington's disease (3 NPA model)	**M** C57BL/6 mice	*n* = 70; 9 animals per group	THCA improved hindlimb dystonia and locomotor activity. THCA down-regulated all pro-inflammatory mediators.	Nadal et al. (2017)

[a]Animal sex denoted by **F** (female) and **M** (male).

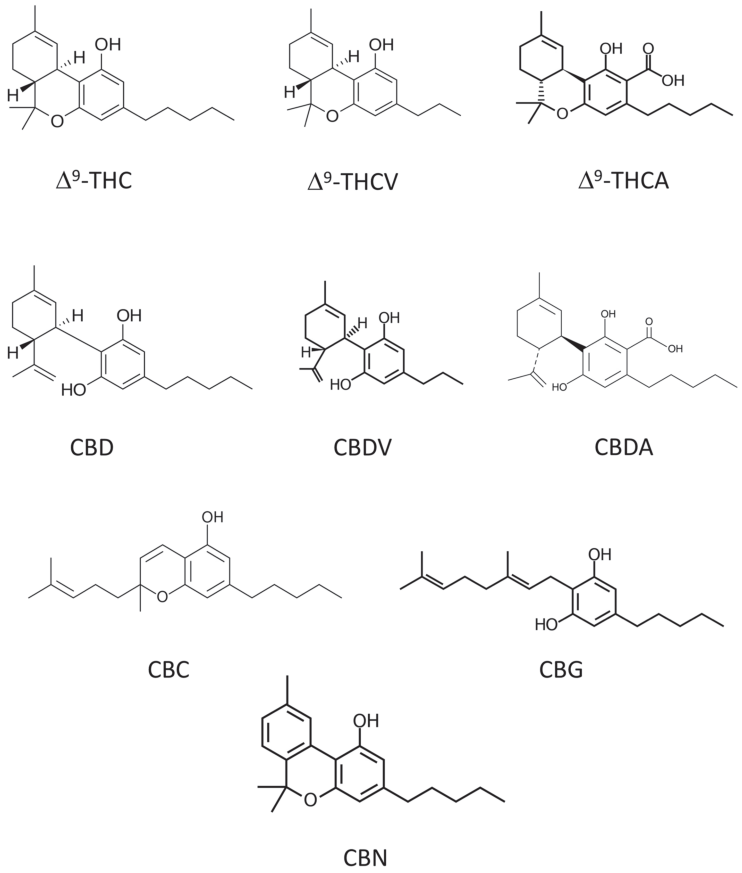

FIGURE 2 Structured and pharmacological profiles of some of the minor phytocannabinoids with cannabidiol (CBD) and tetrahydrocannabidiol (Δ^9-THC) included for reference: Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), Δ^9-tetrahydrocannabinolic (Δ^9-THCV), cannabidivarin (CBDV), cannabidiolic acid (CBDA), cannabichromene (CBC), cannabigerol (CBG), and cannabinol (CBN)

administered prior to the initiation of pentylenetetrazole (PTZ) seizures; however, despite being able to block Nav channel activity, CBG had no effect on seizure severity. No antagonist experiments were conducted in these studies, but Valdeolivas et al. (2015) did show that CBG dose-dependently activated PPARγ in cultured striatal cells (WT and mutant huntingtin; supplementary data). Four studies reported that the CBG derivatives VCE-003 (5 mg·kg^{-1} i.p.) and VCE-003.2 (10 mg·kg^{-1} p.o./i.p.) successfully reduced immune cell activation in macrophages, microglia, and infiltrating neutrophils in models of EAE (to model MS) and Huntington's and LPS-induced Parkinson's disease (PD; Aguareles et al., 2019; Carrillo-Salinas et al., 2014; Díaz-Alonso et al., 2016; García et al., 2018). In the in vivo Parkinson's disease model, García et al. (2018) found that PPARγ antagonist **T0070907** (5 mg·kg^{-1}) blocked VCE-003.2-mediated decreases in TNF-α, IL-1β, and iNOS mRNA levels, but no other antagonists were investigated. In a follow-up study by the same group, 20 mg·kg^{-1} (but not 10 mg·kg^{-1}) oral VCE-003.2 promoted a trend towards recovery in the basal ganglia of LPS-lesioned mice and was associated with decreases in IL-1β gene expression, lysosomal-associated membrane protein-1 (LAMP-1), and glial fibrillary acidic protein (GFAP) immunostaining (Burgaz et al., 2019). Orally dosed VCE-003.2 (10 mg·kg^{-1}) promoted neurogenesis in mice subjected to mutant Huntingtin expression in a Huntington's disease model (Aguareles et al., 2019). In another model of Huntington's disease VCE-003.2 (10 mg·kg^{-1} i.p.) prevented neuronal loss, indicated by increases in Nissl and NeuN staining and at the same dose improved RotaRod performance and reduced astrogliosis in mice, measured by attenuated levels of GFAP and ionized calcium binding adaptor molecule 1 (Iba-1; Díaz-Alonso et al., 2016). Rodríguez-Cueto et al. (2018) found that VCE-003.2 10 mg·kg^{-1} i.p. successfully improved neuropathological deterioration and normalized CB_2 receptor and **IL-1β** levels, in an experimental model of amyotrophic lateral sclerosis, but again no mechanisms of action were probed.

In vitro, Schubert et al. (2019) reported that CBG (100 nM) prevented MC65 neurons from accumulating toxic **amyloid β** (Aβ) protein in an Alzhiemer's disease model. CBG also preserved neuronal trophic factors in primary rat cortical neurons (EC_{50} 1.5 μM) and prevented oxytosis in mouse HT22 hippocampal nerve cells (EC_{50} 1.9 μM). Although no mechanisms were explored in this study, neither MC65 neurons nor HT22 cells express CB_1 or CB_2 receptors, leading the authors to conclude that these effects were mediated independently of these receptors. In N2a cells, VCE-003.2 (10 and 25 μM) prevented excitotoxicity induced by glutamate and in models of LPS induced Parkinson's disease and amyotrophic lateral sclerosis (García et al., 2018; Rodríguez-Cueto et al., 2018). Similarly, VCE-003 (0.1–25 μM) dose dependently protected neuronal cells in a model of MS, while VCE-003.2 (500 nM) promoted neuronal differentiation when dosed for 21 days in an *in vitro* model of Huntington's disease, but no antagonist experiments were conducted to explain these effects (Aguareles et al., 2019; Granja et al., 2012). In a model of neuroinflammation, pretreatment with CBG (7.5 μM) improved viability in cells treated medium from LPS-stimulated macrophages and, while

TABLE 3 Summary of the conditions where emerging cannabinoids have been studied

	Cannabigerol (CBG)/derivatives	Cannabidivarin (CBDV)	Cannabichromene (CBC)	Cannabinol (CBN)	Cannabidiolic acid (CBDA)	Δ^9-THCV	Δ^9-THCA
Huntington's	✓	-	-	✓	X	-	✓ PPARγ[a]
Multiple sclerosis	✓	-	-	-	-	-	-
Autoimmune encephalomyelitis	✓PPARγ/CB_2[a]	-	-	-	-	-	-
Parkinson's	✓PPARγ[a]	-	-	-	-	✓	✓
Neuroinflammation /neuroprotection	✓	✓	✓	✓	-	✓	✓
Epilepsy/seizure	x	✓TRPV1[a]	✓	-	-	✓	-
Amyotrophic lateral sclerosis (ALS)	✓	-	-	✓	-	-	-
Oxidative stress	-	-	-	✓	-	-	-
Rett syndrome	-	✓	-	-	-	-	-
Alzheimer's disease	✓	✓	✓	-	-	-	-

Note. A tick or cross represents whether a cannabinoid showed efficacy in a condition or not. A dash means that a cannabinoid has yet to be studied in a condition.
[a]Some of the compounds neuroprotective effects were mediated by this receptor, but no other receptors were probed.

authors reported that CBG treatment resulted in PPARγ down-regulation, no direct mechanistic probing was conducted (Gugliandolo, Pollastro, Grassi, Bramanti, & Mazzon, 2018). Granja et al. (2012) and Carrillo-Salinas et al. (2014) found that treatment with VCE-003 (1 and 2.5 μM and 1, 10, and 25 μM) blocked the secretion of a number pro-inflammatory mediators including IL-6, TNF-α, IL-1β, and **CCL3** in macrophages and primary microglia. VCE-003.2 also attenuated TNF-α and L-1β secretion but from BV2 mouse microglial cells (5 μM) and astroglial cells (1 and 5 μM; Díaz-Alonso et al., 2016; García et al., 2018; Rodríguez-Cueto et al., 2018). Díaz-Alonso et al. (2016) and García et al. (2018) deduced that VCE-003.2 did not mediate its protective effects via CB_1 or CB_2 receptors due to poor binding affinity (K_i > 40 μM) and both groups found that VCE-003.2 was an agonist at PPARγ (IC_{50} of 1.2 μM).

3.2 | Cannabidivarin (CBDV)

All *in vivo* cannabidivarin (CBDV) studies evaluated the anti-epileptic properties of the compound in models of Rett syndrome and MES seizures (Amada, Yamasaki, Williams, & Whalley, 2013; Hill et al., 2012; Hill et al., 2013; Vigli et al., 2018; Zamberletti et al., 2019). Doses in these studies ranged from 0.2 to 400 $mg{\cdot}kg^{-1}$ per day in rodents with efficacy in reducing tremors was observed between 2 and 200 $mg{\cdot}kg^{-1}$ per day. Two studies reported that 200 $mg{\cdot}kg^{-1}$ i.p. CBDV significantly decreased PTZ seizure severity and mortality in rats (A. J. Hill et al., 2012; Hill et al., 2013). Hill et al. (2012) found that 90% of animals remained seizure free at a dose of 200 $mg{\cdot}kg^{-1}$ CBDV i.p. per day; however, lower concentrations of CBDV were ineffective (0.2 mg·kg), and CBDV had no effect on the severity of pilocarpine convulsions at any tested concentration (50–200 $mg{\cdot}kg^{-1}$ per day). CBDV (400 $mg{\cdot}kg^{-1}$ oral gavage) suppressed PTZ seizures, significantly decreasing seizure severity but had no effect on expression of epilepsy related genes (Amada et al., 2013). Another study reported that 20 $mg{\cdot}kg^{-1}$ i.p. CBDV dosed for 14 days improved brain weight in Rett syndrome (RTT) mice, compared with WT mice, but had no effect on neurotrophin levels (Vigli et al., 2018). None of these *in vivo* studies conducted antagonist experiments to further elucidate the anticonvulsant effects of CBDV.

In HEK293 cells transfected with TRPV1, 2, and 3 channels, CBDV caused a concentration-dependent bidirectional current at TRPV1 channels similar to **capsaicin**, and **capsazepine** (TRPV1 channel antagonist) blocked this effect. Furthermore, **5'-iodoresiniferatoxin (5'-IRTX)**, a selective antagonist of TRPV1 channels counteracted the effect of CBDV in the duration but not amplitude of neuronal burst. These data suggest that CBDV acts as an agonist at these channels, but some of CBDVs effects are mediated independently of this channel. However, no other antagonists were tested to establish which receptors were responsible for the other effects of CBDV (Iannotti et al., 2014). Hill et al. (2012) reported that CBDV (10 and 100 μM) decreased the amplitude and duration of local field potentials in hippocampal brain slices, with an anti-epileptiform effect observed in the

CA1 region (100 μM). CBDV also showed efficacy in an *in vitro* model of Alzheimer's disease, preventing oxytosis and energy loss in HT22 cells (EC_{50} 1.1 μM and 90 nM, respectively), as well as reducing Aβ toxicity (EC_{50} 100 nM) and trophic withdrawal (EC_{50} 350 nM); however, no mechanistic data were reported to determine how these effects were mediated (Schubert et al., 2019).

3.3 | Cannabichromene (CBC)

In a model of electroshock seizure, CBC (10–75 $mg \cdot kg^{-1}$ i.p. per day) significantly depressed motor activity during the first 10-min interval, but subsequently only the highest dose was effective (Davis & Hatoum, 1983). *In vitro*, Shinjyo and Di Marzo (2013) found that 1μM CBC increased viability of adult nestin-positive neuronal stem cells when applied in medium without growth factors (B27 medium), by inducing **ERK** phosphorylation. No antagonist data were presented in these studies.

3.4 | Cannabinol (CBN)

Only one *in vivo* study assessed CBN (5 $mg \cdot kg^{-1}$ per day) in an SOD1 model of amyotrophic lateral sclerosis. CBN delayed motor abnormalities at Day 17 in the chronic treatment regimen, compared with vehicle control, but disease progression was not affected (Weydt et al., 2005). In a model of Huntington's disease, Aiken et al. (2004) found that CBN reduced LDH activity in PC12 cells (20 and 100 μM), but the authors did not investigate the mechanism(s) of this effect. CBN displayed potent antioxidant activity in primary cerebral granule cells under oxidative stress conditions; however, no antagonist data were presented on this cannabinoid (Marsicano et al., 2002).

3.5 | Δ^9-THCV

Male Sprague–Dawley rats and **CB_2** receptor knockout mice were dosed with 2 $mg \cdot kg^{-1}$ per day Δ^9-THCV over a period of 14 days in a model of Parkinson's disease, induced by 6-hydroxydopamine (6-OHDA) or LPS (García et al., 2011). Δ^9-THCV reduced slow motor movements induced by 6-OHDA and enhanced mean velocity of movement with a potency similar to **rimonabant**. Chronic Δ^9-THCV dosing reduced microglial activation and preserved nigrostriatal dopaminergic neurons after 6-OHDA application and in the LPS model of Parkinson's disease, Δ^9-THCV preserved TH positive neurons, mirroring the effects of the **CB_2** receptor agonist **HU-308**. Thus, authors speculated that Δ^9-THCV mediated at least some of its effects in the LPS model via **CB_2** receptors (García et al., 2011). Also, 2 $mg \cdot kg^{-1}$ Δ^9-THCV blocked the effects of the CB_1 receptor agonist, **CP55,940**, suggesting it acts as an antagonist at this receptor. However, no data were presented assessing if such antagonistic properties of Δ^9-THCV at CB_1 receptors mediated its protective effects in the 6-OHDA or LPS models of Parkinson's disease. Hill et al. (2010) studied Δ^9-THCV in a seizure model induced by 80 $mg \cdot kg^{-1}$ PTZ and found that at a dose of 0.25 $mg \cdot kg^{-1}$ i.p. Δ^9-THCV, with 33% of animals having a complete absence of seizures. Although no direct mechanistic probing was investigated, receptor binding assays were performed on rat cortical membranes, and Δ^9-THCV was found to act as a CB_1 receptor ligand (CB_1 $K_i \sim 290$ nM; **[^{3}H]SR141716A** displacement but no agonist stimulation using [^{35}S] GTPγS binding; Hill et al., 2010).

3.6 | Δ^9-THCA

In an acute 3-NP model of Huntington's disease, Nadal et al. (2017) observed a significant improvement in hindlimb dystonia (uncontrollable hindlimb muscle contraction) and locomotor activity in male, C57BL/6 mice treated with Δ^9-THCA (20 $mg \cdot kg^{-1}$ per day i.p.). Δ^9-THCA also prevented astrogliosis and microgliosis and attenuated the up-regulation of pro-inflammatory mediators induced by 3-NP. These effects were blocked when mice were co-administered with the PPARγ antagonist T0070903 (with the exception of IL-6; Nadal et al., 2017). *In vitro*, N2a cells infected with the huntingtin polyQ repeats resulted in toxicity, which was significantly reduced by treatment with Δ^9-THCA, as well as decreased expression of inflammatory mediators: TNF-α, iNOS, IL-6, and COX-2. Δ^9-THCA also improved neuronal viability post-serum deprivation, and this effect was prevented by **GW9662**, a PPARγ antagonist. No other antagonists were used in this study (Nadal et al., 2017).

Δ^9-THCA (0.01–10 μM) displayed no pro-survival effect on dopaminergic neurons but had a significant, positive effect on cell count (123%) when compared to the control, in an *in vitro* model of Parkinson's disease (Moldzio et al., 2012).

4 | DISCUSSION

To our knowledge, this is the first systematic review on the neuroprotective effects of lesser-known, minor phytocannabinoids in various models of neurological disease. Data obtained from our search revealed that CBG, VCE.003, VCE.003.2, and CBDV were the most promising candidates as neuroprotectants, while Δ^9-THCV, Δ^9-THCA, CBC, and CBN have limited but encouraging data as neuroprotectants. CBG, VCE.003, VCE.003.2, and Δ^9-THCA mediated their neuroprotective effects at least in part by the nuclear receptor PPARγ. CBDV was found to mediate some of its antiepileptic effects via TRPV1 channels, and Δ^9-THCV was found to be a CB_1 receptor ligand and a possible CB_2 receptor agonist, but no experiments were conducted to establish whether its neuroprotective action was mediated by CB_1 or CB_2 receptors. No other receptors were investigated, and no studies assessed the neuroprotective potential of CBDA, CBGA, CBGV, CBCV, CBGVA, or CBDVA.

CBG was first isolated in 1964 by the same group that reported the structure of Δ^9-THC (Gaoni & Mechoulam, 1964). It exhibited

antioxidant and anti-inflammatory properties, while displaying no psychotropic effects, as it is a poor CB_1 receptor agonist (Gauson et al., 2007; Navarro et al., 2018; Rosenthaler et al., 2014). CBG is a partial agonist at CB_2 receptors, a potent α_2-adrenoceptor agonist (EC_{50} 0.2 nM) and a moderate 5-HT_{1A} receptor antagonist, as well as interacting with various TRP isoforms including TRPV1 and 2 channels (Cascio, Gauson, Stevenson, Ross, & Pertwee, 2010; De Petrocellis et al., 2012). Studies included here show that these compounds have significant anti-inflammatory effects, including attenuating cytokine release and decreasing the activation of immune cells, an effect observed in both *in vitro* and *in vivo* models.

CBG and its derivatives were particularly effective in models of Huntington's disease, targeting multiple facets of the disease including gene expression, easing motor symptoms, reducing microglial activation, and attenuating the inflammatory response. Huntington's disease pathophysiology, like other neurodegenerative disorders, exhibits uncontrolled microglial activation, which is a key part of the neuroinflammatory response. In early stages of this disease, PET imaging has revealed marked microglial activation, which was correlated with impairments of neuronal activity (Tai et al., 2007). Microglial activation along with increases in pro-inflammatory mediators has also been detected in post-mortem Huntington's disease brains (Palpagama, Waldvogel, Faull, & Kwakowsky, 2019). Interestingly, microglial mediated neuroinflammation was suppressed with the activation of CB_2 receptors (Ehrhart et al., 2005). However, given VCE-003 and VCE.003.2's protective effects were likely to be CB_1 and CB_2 receptor-independent, their effects on microglial activation are likely to be via a different mechanism, possibly through the activation of PPARγ, which has an important role in regulating the inflammatory response, especially in the CNS (see Bright, Kanakasabai, Chearwae, & Chakraborty, 2008; Villapol, 2018). It is also worth noting that microglial activation can be protective, preserving neurons by secreting anti-inflammatory cytokines such as IL-4 and IL-10 as well as various trophic factors (see Le, Wu, & Tang, 2016, and Pöyhönen, Er, Domanskyi, & Airavaara, 2019). In line with these observations, there effectively needs to be a balancing act between enabling some degree of microglial activation to protect neurons, while limiting their overactivation that would ultimately lead to damage. Given that the symptoms of Huntington's disease are currently managed using VMAT inhibitors (such as TBZ) to decrease levels of monoamines, it would be useful to assess whether CBG and its derivates have any efficacy as VMAT inhibitors, or whether their protective effects in models of Huntington's disease are independent of this mechanism. If the latter is the case, future studies should investigate low-dose VMATs (to minimize neuropsychiatric side effects) together with CBG or its derivatives as an adjuvant therapy to assess if there is an additive, or even synergistic, protective effect of these compounds.

Long-term dose tolerability and a lack of accumulation in tissue are both essential features of neuroprotective agents as these drugs are typically taken for life after disease onset. In a study conducted by Deiana et al. (2012), CBG was found to have similar PK profiles in rats and mice but exhibited slower brain penetration in mice. Both animals also had higher concentrations of CBG following i.p. injection compared to oral administration, but interestingly in rats, this did not equate to higher concentrations in brain tissue (Deiana et al., 2012). From the results in our review, treatment with CBG, VCE-003, and VCE.003.2 was well tolerated and ranged from just 3 days to 10 weeks with two studies dosing CBG orally and seven studies dosing intraperitoneally. Deiana et al. (2012) reported that animals tolerated CBG better after i.p. administration, compared with the oral route. In humans, i.p. dosing is not a viable means of regular administration, and all drugs given orally have a larger side effect profile. Moreover, patients receiving certain oral therapies for neurological conditions, such as levodopa for Parkinson's disease, must also take medications to minimize peripheral effects (Fahn, 2008). Therefore, dose formulation and route of administration for these compounds should be carefully assessed, based on thorough ADME profiling and feasibility of long-term dosing.

CBG exhibited positive effects in two Huntington's disease models, despite one study using oral and the other i.p., administration. Of note, CBD has already been trialled in Huntington's disease patients; CBD (10 $mg{\cdot}kg^{-1}$; 700 mg average daily dose) was given for 6 weeks and resulted in a consistent plasma level of 5.9–11 $ng{\cdot}mL^{-1}$. Once treatment had stopped, elimination was between 2 and 5 days, suggesting CBD did not accumulate and remain in plasma longer than 5 days in these Huntington's disease patients (Consroe et al., 1991). Further studies should elucidate whether CBG and its derivatives display efficacy in humans and clarify whether their activation of PPARγ corresponds to their neuroprotective properties and if other receptors are involved. More data are also needed on the PK profiles of CBG and its derivatives in older mice and larger mammals and to establish whether it exhibits a similar elimination to CBD in humans. These factors would aid in the translation of this compound as a treatment for neurodegenerative conditions.

Cannabidivarin (CBDV) is a structural analogue of CBD, with the molecule shortened by two methylene bridges (Morales, Hurst, & Reggio, 2017; Vollner, Bieniek, & Korte, 1969). From our search, *in vivo* studies consistently reported 200 $mg{\cdot}kg^{-1}$ i.p. CBDV having anti-epileptic effects and a 400 $mg{\cdot}kg^{-1}$ oral dose also showing promise. Like CBD, CBDV is a agonist at TRPV1/2 and **TRPA1** channels, and an antagonist at **TRPM8** channels, which may explain similarities in their neuroprotective properties, particularly the action of CBDV as an agonist at TRPV1 channels (De Petrocellis et al., 2011; Iannotti et al., 2014; Scutt & Williamson, 2007). In our review, studies showed that CBDV did not affect neurotrophic levels or epilepsy-related gene expression. Thus, it can be assumed that CBDV mediates its protective effects independent of these pathways (Amada et al., 2013; Vigli et al., 2018). Deiana et al. (2012) reported that CBDV was rapidly absorbed in mice and rats, but there was a higher drug concentration in plasma and brain following oral treatment in rats compared to mice. Furthermore, while i.p. injection resulted in similar PK profiles in the two species, brain concentrations in rats were higher. This brings into question the differences in the amount of CBDV delivered to the brain in the studies conducted in mice compared with rats presented in this review and whether this influenced study outcomes. Only two studies reported chronic CBDV dosing both in models of Rett

syndrome, highlighting the need for future studies to assess the long-term tolerability of CBDV as an anti-epileptic agent and how different species exhibit different bioavailability of this compound, as these will both affect the translatability of CBDV to humans.

Although out of the scope of this review, it is worth noting that CBDV has already been trialled as an anti-convulsant by GW Pharmaceuticals in a phase IIa, placebo-controlled study of 162 adult patients (clinical trial number: NCT02369471/NCT02365610). The drug GWP42006 (which contains CBDV as its main ingredient) was dose titrated (over 2 weeks) up to a 800 mg twice daily dose for a 6-week stable treatment period. However, focal seizures were inadequately controlled with this dose and GWP42006 displayed no difference in efficacy to the placebo control group (Schultz, 2018). While this may cast doubt on the translatability of the evidence presented in this review, it is worth highlighting that the maximum dose in humans from the GW study would be considerably less than if the same dose regimens as the *in vivo* studies were followed for a 60-kg human. Furthermore, Morano et al. (2020) have suggested that the inability of CBDV to control seizures was in part due to an extremely high response from the placebo group and that the use of purified CBDV may have also influenced the study outcome. Therefore, it is important to exercise caution when extrapolating the findings from the *in vitro* and *in vivo* data presented here and what doses may be effective in clinical trials.

Cannabichromene (CBC) was first isolated in 1966 by Gaoni and Mechoulam and is a non-psychotropic cannabinoid that does not interact with CB_1 receptors (Gaoni & Mechoulam, 1966). CBC is an agonist at CB_2 receptors and TRP channels, acting potently at TRPA1 as well as displaying some activity at TRPV3 and TRPV4 channels (Cascio & Pertwee, 2015; De Petrocellis et al., 2008, 2011; de Petrocellis et al., 2012; Udoh, Santiago, Devenish, McGregor, & Connor, 2019). CBC (0.001–1 μM) exhibited promising anti-inflammatory effects in an *in vitro* model of colitis, decreasing LPS increased nitrite levels and attenuating **IFN-γ** and IL-10 secretion in peritoneal macrophages (Romano et al., 2013). More recently CBC acted as a CB_2 receptor agonist in AtT20 cells transfected with these receptors and was confirmed by application of the CB_2 receptor antagonist AM630, which blocked the effects of CBC (Udoh et al., 2019). We found only two papers related to neuroprotective effects of CBC; *in vivo* CBC suppressed motor activity while *in vitro* CBC improved viability of neural stem cells (Davis & Hatoum, 1983; Shinjyo & Di Marzo, 2013). The anti-inflammatory effects of CBC may play a pivotal role in its ability to act as a neuroprotectant, as inflammation and overactivation of the immune response are important features of neurodegenerative conditions.Thus, further research should assess this compound in neuro-inflammatory conditions, where it may have potential.

Cannabinol (CBN) is an oxidation product of Δ^9-THC and was the first cannabinoid to be discovered and isolated (Wood, Spivey, & Easterfield, 1899). Like Δ^9-THC, it has been shown to activate CB_1 receptors (K_i 211.2 nM) but with lower potency, as well as acting as an agonist at TRPV2 channels (Rhee et al., 1997; Russo & Marcu, 2017). CBN (1 $mg{\cdot}mL^{-1}$) was recently shown to reduce mechanical sensitization and sensitivity of afferent muscle fibres in an *in vivo* model of myofascial pain, but no mechanism of action was investigated (Wong & Cairns, 2019). From our search, limited data showed that CBN decreased cell damage and acted as a potent antioxidant in a cell-based Huntington's disease model (Aiken et al., 2004). The antioxidant activity of CBN is a characteristic feature of cannabinoids, which as previously mentioned, is thought to be due to the presence of the phenolic ring and carboxyl moieties, as well as the ability to increase antioxidant defences. CBD has already shown extensive antioxidant properties, including increasing the levels and activity of antioxidants, capturing ROS, and transforming them into less active forms, as well as activating nuclear erythroid 2-related factor (NrF2) that governs the transcription of many antioxidant genes (see Atalay, Jarocka-karpowicz, & Skrzydlewskas, 2020). Oxidative stress is a key feature of neurodegenerative disorders including Parkinson's and Alzheimer's disease. In the latter condition, Aβ deposits contain a significant number of binding sites for biometals (zinc, copper, and iron) that contribute to oxidative stress in patients (Huang, Zhang, & Chen, 2016; Kozlowski et al., 2009). Furthermore, Alzheimer's disease patients have decreased levels of antioxidant enzymes and increased products of oxidative stress, such as peroxidised lipids and oxidized proteins in brain tissue (Kim et al., 2006; Sultana et al., 2011). Also, large amounts of ROS are generated by reactive microglial cells, with studies showing superoxide produced by microglia directly contributing to the death of dopaminergic neurons in Parkinson's disease (Hernandes, Café-Mendes, & Britto, 2013). It is clear that more information is needed on the pharmacology of CBN, especially its antioxidant potential. Moreover, the ability of CBDV, CBG, CBC, and CBN to reduce Aβ deposits *in vitro* is also noteworthy and it is clearly of interest to examine the antioxidant and anti-inflammatory potential of these compounds in Alzheimer's disease models *in vivo* and whether these compounds act through mechanisms, similar to those of CBD.

Δ^9-THCV is a homologue of Δ^9-THC differing by just a propyl side chain, and studies have suggested that Δ^9-THCV acts as a CB_1 receptor agonist, sharing properties with Δ^9-THC, albeit with less potency (Gill, Paton, & Pertwee, 1970; Pertwee, 2008). They exhibit similarities in their *in vivo* effects such as inducing catalepsy in mice and Δ^9-THC-like effects in humans (Gill et al., 1970; Hollister, 1974). We found two studies where Δ^9-THCV showed promise as an anti-epileptic agent and protected neurons in two models of Parkinson's disease, while García et al. (2011) suggested Δ^9-THCV mediated some of its protective effects by acting at CB_1 and CB_2 receptors, the possible mechanisms of action of Δ^9-THCV was largely unexplored (García et al., 2011; Hill et al., 2010). In an earlier study, Δ^9-THCV displaced [^{3}H]CP55940 from specific sites in mouse brain and CHO-hCB_2 cell membranes (K_i values 75.4 nM and 62.8 nM, respectively), and along with data from GTPγS-binding experiments, the authors concluded Δ^9-THCV acted as a CB_1 and CB_2 receptor antagonist (Thomas et al. 2005). Other groups have shown Δ^9-THCV can block CB_1 receptor activity in murine cerebellar slices and, at 5.8 μM, increased GABA release from neurons, sharing the same properties as AM251, a CB_1

receptor antagonist (Ma, Weston, Whalley, & Stephens, 2008; Pertwee, 2008). Thus, while there is evidence to suggest Δ^9-THCV mediates some of its protective effects via CB_1 and CB_2 receptors, the data remain largely unclear, and there is also a lack of investigation into the potential of Δ^9-THCV to act at other known cannabinoid targets.

Microglial activation and the presence of neuroinflammatory factors are well known characteristics of Parkinson's disease and well documented among patients (Mogi et al., 1994; Qian et al., 2011). Moreover, studies have demonstrated that microglial overactivation leads to deleterious effects and the exacerbation of the immune response, especially the release of pro-inflammatory mediators. As observed with the CBG derivative VC-003.2, microglial activation was decreased by Δ^9-THCV, inducing a protective effect by dampening the immune response. Studies have already demonstrated the ability of CBD to modulate the immune response by acting an agonist of PPARγ and altering NF-κB signalling, which is up-regulated in both microglia and astrocytes of Parkinson's disease patients. Furthermore, activation of PPARγ leads to inhibition of NF-κB signalling and decreases mRNA levels of proinflammatory mediators TNF-a, IL-1β, IL-6, and iNOS (Vallée, Lecarpentier, Guillevin, & Vallée, 2017). Therefore, it would be of interest to determine whether Δ^9-THCV is able to reduce microglial activation through the same mechanism as CBD, involving the activation of PPARγ.

Limited pharmacokinetic data on Δ^9-THCV have shown it exhibits rapid absorption in rats and mice when administered either i.p. or orally but is rapidly eliminated when orally administered (<1.5 h) compared to i.p administration where its elimination rate is >5 h (Deiana et al., 2012). Interestingly, Δ^9-THCV exhibited extensive brain penetration (exceeding plasma levels), regardless of the route of administration, meaning it can effectively cross the BBB. At 24 h, Δ^9-THCV was no longer detected, suggesting that it exhibits a lack of accumulation in brain tissue (Deiana et al., 2012). Altogether, these features, along with evidence collected in this study, support Δ^9-THCV as a neuroprotective agent. However, clearly, more data with Δ^9-THCV are needed, especially to assess safety after chronic dosing and whether this compound exhibits tolerance with long-term use.

Δ^9-THCA is the acidic precursor of Δ^9-THC, and competition binding assays revealed that this compound was unable to achieve displacement of [^{3}H]-CP55,940 (CB_1 and CB_2 receptor agonist) up to 10 μM, suggesting Δ^9-THCA exhibits poor affinity for CB_1 or CB_2 receptors (McPartland et al., 2017). Results from this study also showed that Δ^9-THCA has little efficacy at these receptors as it exhibited no inhibition of forskolin-mediated cAMP, compared to Δ^9-THC that acted as an agonist in this assay. Our search revealed that Δ^9-THCA had anti-inflammatory effects that improved neural viability in a model of Huntington's disease, but interestingly, it did not affect the survival of dopaminergic neurons in a model of Parkinson's disease (Moldzio et al., 2012; Nadal et al., 2017). In a recent study, Anderson, Low, Banister, McGregor, and Arnold (2019) reported that Δ^9-THCA had extremely poor brain penetration (an optimistic brain–plasma ratio of 0.15) in both vehicles tested. Furthermore, studies have shown that Δ^9-THCA has poor stability and rapidly decarboxylates to Δ^9-THC, bringing into question whether the ability of Δ^9-THCA to act as a neuroprotectant in the studies presented here is actually due to nearly unavoidable contamination with Δ^9-THC (Anderson et al., 2019; McPartland et al., 2017). Overall, these data warrant further investigation into Δ^9-THCA as a potential neuroprotective and anti-inflammatory agent, but with caution, and such studies should include purity data on Δ^9-THCA to enhance the robustness of the experimental data.

There were no studies identified in this review that looked at the potential neuroprotective effects of other cannabinoid varins or their acidic forms such as CBGV, CBGVA, CBDVA, CBCV, and CBCVA. This may be due to the lack of commercial availability of these compounds due to their low concentrations in the plant, costly synthetic production or that these compounds are not very stable. CBDA was only used in one study on Huntington's disease, where it had no protective effects. This compound, however, has shown promise in other conditions including breast cancer migration, inflammatory pain and nausea (Bolognini et al., 2013; Rock, Limebeer, & Parker, 2018; Takeda et al., 2012), with groups suggesting that CBDA is 1,000 times more potent at the 5-HT_{1A} receptor than CBD (Bolognini et al., 2013). Activation of the 5-HT_{1A} receptor is protective both *in vitro* in Parkinsonian models and *in vivo* in models of hypoxia ischaemia (Miyazaki et al., 2013; Pazos et al., 2013). Although Anderson et al. (2019) concluded that CBDA displayed poor brain penetration in an oil-based formulation, uptake was increased when CBDA was formulated in a Tween-based vehicle. Also, CBDA was anti-convulsant at 10 and 30 $mg{\cdot}kg^{-1}$ displaying greater potency compared to CBD (100 $mg{\cdot}kg^{-1}$). These data support CBDA's efficacy in the brain, as well as highlighting its potential as an anticonvulsant (Anderson et al., 2019). Considering these points, CBDA may be also protective in conditions such as ischaemic stroke and Parkinson's disease and warrants further investigation. Recent studies have also shown that CBDA, CBGV, and CBGA interact with various TRP channel isoforms including TRPV1, TRPV2, TRPA1, and TRPM8 channels. Of note, CBGV and CBGA were also potent desensitizers of TRPV3 and TRPV4 channels, respectively (De Petrocellis et al., 2012). While the extent of the role of TRP channels in neuroprotection has yet to be fully understood, these receptors are involved in a wide range of neurological disorders. For example, TRPA1-deficient mice were more likely to sustain damage post ischaemia and TRPA1 channel activation in Alzheimer's disease may have a crucial role in regulating astrocyte-mediated inflammation (Lee et al., 2016; Pires & Earley, 2018). Conversely, TRPV1 channel activity has been implicated in epilepsy having a role in neuronal excitability and synaptic transmission (Nazıroglu, 2015). Therefore, CBDA, CBGV, and CBGA interactions at TRP channels may be beneficial in conditions that involve these channels in their pathophysiology.

Translatability of these data and the viability of minor phytocannabinoids as neuroprotectants will also rely on understanding and perhaps manipulating their bioavailability and pharmacokinetic properties. In a recent systematic review conducted by our group, Millar, Stone, Yates, and O'Sullivan (2018) highlighted discrepancies regarding CBD bioavailability, C_{max}, T_{max}, and half-life ($t_{1/2}$) in humans depending on the route of administration and formulation and

whether CBD was dosed in a fed or fasted state. That being said, studies conducted in piglets (Garberg et al., 2017) and rodents (Hammell et al., 2016; Long et al., 2012) have shown a dose-dependent relationship between CBD administration and brain and plasma concentrations. Limited data extracted by Millar et al. (2018) showed that administration of CBD in humans also led to dose-dependent increases in plasma concentrations, suggesting the same may apply to brain concentrations in man.

Information on the human metabolites of CBD, Δ^9-THC, and other phytocannabinoids is scarce, with the majority of research focusing on the extensive first pass metabolism of CBD and the identification of its urinary metabolites. Of interest, a patent filed by Mechoulam et al. (2010) described that two major metabolites of CBD, 7-hydroxy (7-OH) CBD and 7-carboxy (7-COOH), are both anti-inflammatory and dose dependently inhibit TNF-a, NO, and ROS. However, these data have yet to be confirmed in academic studies or found to be true of other phytocannabinoids. In addition, the cytochrome P450 (CYP) superfamily is responsible for metabolizing 60%–80% of CNS-acting drugs, 23% by CYP3A4 and 38% CYP2C19, both of which accept CBD as a substrate (Cacabelos, 2010; Iffland & Grotenhermen, 2017). Altogether, these findings highlight that there are major gaps in the ADME of phytocannabinoids, as well as a lack of identification of metabolites and whether they have biological effects. In phase II trials, the minor phytocannabinoids presented in this review will, in all likelihood, be used alongside current therapies to see if they can augment survival of neurons and/or symptom burden, rather than being used as a single agent. In light of the above, it will be essential to consider the interactions that these compounds may have when administered in conjunction with conventional drug therapies (where they exist) and to establish potential synergistic or deleterious effects. Looking forward, initial ADME data will be essential to determine whether these compounds have true clinical potential and for their subsequent formulation and administration.

5 | CONCLUSIONS

This review aimed to collate and summarise all current data on the neuroprotective potential of phytocannabinoids other than Δ^9-THC and CBD. Despite the lack of studies available in this area, we found that all phytocannabinoids tested displayed neuroprotective properties in a range of disorders. CBG and its derivatives displayed significant anti-inflammatory effects and were particularly effective in Huntington's disease models. CBDV, Δ^9-THCV, and CBC were effective as anti-seizure agents, while CBN displayed antioxidant activity and Δ^9-THCA had anti-inflammatory effects. CBG and Δ^9-THCA, like CBD, mediate their anti-inflammatory effects through PPARγ. Many of the studies were screening studies that conducted no mechanistic probing, suggesting that research into these compounds is still in its early stages. Extensive pharmacokinetic and pharmacodynamic data in larger mammals are also necessary on these compounds, given that all *in vivo* studies in this review were conducted in mice and rats. This would provide more evidence for the facilitation of these compounds as therapies in humans. Further studies are required to investigate the full neuroprotective potential of these compounds particularly the mechanisms underlying their protective effects, as well as exploring whether their combinations may enhance their capabilities as neuroprotectants. While we have focused on a select number of minor phytocannabinoids, based predominantly on their shared physical and biological similarities to CBD, there are over 100 phytocannabinoids and terpenes present in the *Cannabis* plant that could potentially display neuroprotective potential.

5.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Cidlowski et al., 2019; Alexander, Fabbro et al., 2019; Alexander, Mathie, et al., 2019).

AUTHOR CONTRIBUTIONS

N.S. and S.O.S. wrote the paper. N.S. and A.M. conducted the searches. All authors contributed to editing the paper and approving the final version.

CONFLICT OF INTEREST

S.O.S. is the Chief owner of CanPharmaConsulting. She is on the advisory board for Artelo Biosciences, as well as acting as the science lead for the Centre for Medicinal Cannabis (CMC). Other authors declare that they have no conflict of interest in relation to this review.

ORCID

Nicole L. Stone https://orcid.org/0000-0002-7572-7429

REFERENCES

Aguareles, J., et al. (2019). Oral administration of the cannabigerol derivative VCE-003.2 promotes subventricular zone neurogenesis and protects against mutant huntingtin-induced neurodegeneration. *Translational Neurodegeneration*, *8*(9). https://doi.org/10.1186/s40035-019-0148-x

Aiken, C. T., Tobin, A. J., Schweitzer, E. S. (2004). A cell-based screen for drugs to treat Huntington's disease. *Neurobiology of Disease*, *16*(3), 546–555. https://doi.org/10.1016/j.nbd.2004.04.001

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peters, J. A., … CGTP Collaborators. (2019). THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: G protein-coupled receptors. *British Journal of Pharmacology*, *176*, S21–S141. https://doi.org/10.1111/bph.14748

Alexander, S. P. H., Cidlowski, J. A., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., … CGTP Collaborators. (2019). THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: Nuclear hormone receptors. *British Journal of Pharmacology*, *176*, S229–S246. https://doi.org/10.1111/bph.14750

Alexander, S. P. H., Fabbro, D., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., … Sharman, J. L. (2019). The concise guide to

pharmacology 2019/20: Enzymes. *British Journal of Pharmacology*, *176*, S297–S396. https://doi.org/10.1111/bph.14752

Alexander, S. P. H., Mathie, A., Peters, J. A., Veale, E. L., Striessnig, J., Kelly, E., … CGTP Collaborators. (2019). THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: Ion channels. *British Journal of Pharmacology*, *176*, S142–S228. https://doi.org/10.1111/bph.14749

Amada, N., Yamasaki, Y., Williams, C. M., & Whalley, B. J. (2013). Cannabidivarin (CBDV) suppresses pentylenetetrazole (PTZ)-induced increases in epilepsy-related gene expression. *PeerJ*, *1*(e214), e214. https://doi.org/10.7717/peerj.214

Anderson, L. L., Low, I. K., Banister, S. D., McGregor, I. S., & Arnold, J. C. (2019). Pharmacokinetics of phytocannabinoid acids and anticonvulsant effect of cannabidiolic acid in a mouse model of Dravet syndrome. *Journal of Natural Products*, *82*(11), 3047–3055. https://doi.org/10.1021/acs.jnatprod.9b00600

Atalay, S., Jarocka-karpowicz, I., & Skrzydlewskas, E. (2020). Antioxidative and anti-inflammatory properties of cannabidiol. *Antioxidants*, *9*(1), 21. https://doi.org/10.3390/antiox9010021

Bolognini, D., Rock, E. M., Cluny, N. L., Cascio, M. G., Limebeer, C. L., Duncan, M., … Pertwee, R. G. (2013). Cannabidiolic acid prevents vomiting in *Suncus murinus* and nausea-induced behaviour in rats by enhancing 5-HT1A receptor activation. *British Journal of Pharmacology*, *168*(6), 1456–1470. https://doi.org/10.1111/bph.12043

Borges, R. S., et al. (2013). Understanding the molecular aspects of tetrahydrocannabinol and cannabidiol as antioxidants. *Molecules*, *18*(10), 12663–12674. https://doi.org/10.3390/molecules181012663

Bright, J. J., Kanakasabai, S., Chearwae, W., & Chakraborty, S. (2008). PPAR Regulation of Inflammatory Signaling in CNS Diseases. *PPAR Research*, *2008*, 658520. https://doi.org/10.1155/2008/658520

Burgaz, S., García, C., Gómez-Cañas, M., Muñoz, E., & Fernández-Ruiz, J. (2019). Development of An Oral Treatment with the PPAR-γ-Acting Cannabinoid VCE-003.2 Against the Inflammation-Driven Neuronal Deterioration in Experimental Parkinson's Disease. *Molecules*, *24*(15), 2702. https://doi.org/10.3390/molecules24152702

Cacabelos, R. (2010). Pharmacogenomic protocols in CNS disorders and dementia. *Neurodegenerative Diseases*, *7*(1–3), 167–169. https://doi.org/10.1159/000289230

Carrillo-Salinas, F. J., Navarrete, C., Mecha, M., Feliú, A., Collado, J. A., Cantarero, I., … Guaza, C. (2014). A cannabigerol derivative suppresses immune responses and protects mice from experimental autoimmune encephalomyelitis. *PLoS ONE*, *9*(4), e94733. https://doi.org/10.1371/journal.pone.0094733

Cascio, M. G., Gauson, L. A., Stevenson, L. A., Ross, R. A., & Pertwee, R. G. (2010). Evidence that the plant cannabinoid cannabigerol is a highly potent α 2-adrenoceptor agonist and moderately potent 5HT 1A receptor antagonist. *British Journal of Pharmacology*, *159*(1), 129–141. https://doi.org/10.1111/j.1476-5381.2009.00515.x

Cascio, M. G., & Pertwee, R. G. (2015). Known pharmacological actions of nine nonpsychotropic phytocannabinoids. In *Handbook of cannabis*. https://doi.org/10.1093/acprof:oso/9780199662685.003.0007

Consroe, P., Laguna, J., Allender, J., Snider, S., Stern, L., Sandyk, R., … Schram, K. (1991). Controlled clinical trial of cannabidiol in Huntington's disease. *Pharmacology, Biochemistry and Behavior*, *40*(3), 701–708. https://doi.org/10.1016/0091-3057(91)90386-G

Davis, W. M., & Hatoum, N. S. (1983). Neurobehavioral actions of cannabichromene and interactions with Δ^9-tetrahydrocannabinol. *General Pharmacology*, *14*(2), 247–252. https://doi.org/10.1016/0306-3623(83)90004-6

De Petrocellis, L., et al. (2008). Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *Journal of Pharmacology and Experimental Therapeutics*, *325*(3), 1007–1015. https://doi.org/10.1124/jpet.107.134809

De Petrocellis, L., et al. (2011). Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *British Journal of Pharmacology*, *163*(7), 1479–1494. https://doi.org/10.1111/j.1476-5381.2010.01166.x

De Petrocellis, L., et al. (2012). Cannabinoid actions at TRPV channels: Effects on TRPV3 and TRPV4 and their potential relevance to gastrointestinal inflammation. *Acta Physiologica, Pp.*, *204*, 255–266. https://doi.org/10.1111/j.1748-1716.2011.02338.x

Deiana, S., Watanabe, A., Yamasaki, Y., Amada, N., Arthur, M., Fleming, S., … Riedel, G. (2012). Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Δ^9-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behaviour. *Psychopharmacology*, *219*(3), 859–873. https://doi.org/10.1007/s00213-011-2415-0

Díaz-Alonso, J., Paraíso-Luna, J., Navarrete, C., Del Río, C., Cantarero, I., Palomares, B., … Muñoz, E. (2016). VCE-003.2, a novel cannabigerol derivative, enhances neuronal progenitor cell survival and alleviates symptomatology in murine models of Huntington's disease. *Scientific Reports*, *19*(6). https://doi.org/10.1038/srep29789

Ehrhart, J., Obregon, D., Mori, T., Hou, H., Sun, N., Bai, Y., … Shytle, R. (2005). Stimulation of cannabinoid receptor 2 (CB2) suppresses microglial activation. *Journal of Neuroinflammation*, *2*(29), 29. https://doi.org/10.1186/1742-2094-2-29

ElSohly, M. A., & Gul, W. (2015). Constituents of *Cannabis sativa*. *Handbook of Cannabis*. https://doi.org/10.1093/acprof:oso/9780199662685.003.0001

Fahn, S. (2008). The history of dopamine and levodopa in the treatment of Parkinson's disease. *Movement Disorders*, *23*(SUPPL. 3), s497–s508. https://doi.org/10.1002/mds.22028

Fernández-Ruiz, J., Sagredo, O., Pazos, M. R., García, C., Pertwee, R., Mechoulam, R., & Martínez-Orgado, J. (2013). Cannabidiol for neurodegenerative disorders: Important new clinical applications for this phytocannabinoid? *British Journal of Clinical Pharmacology*, *75*(2), 323–333. https://doi.org/10.1111/j.1365-2125.2012.04341.x

Gammon, K. (2014). Neurodegenerative disease: Brain windfall. *Nature*, *515*(7526), 299–300. https://doi.org/10.1038/nj7526-299a

Gaoni, Y., & Mechoulam, R. (1964). Isolation, structure, and partial synthesis of an active constituent of hashish. *Journal of the American Chemical Society*, *86*, 1646–1647. https://doi.org/10.1021/ja01062a046

Gaoni, Y., & Mechoulam, R. (1966). Cannabichromene, a new active principle in hashish. *Chemical Communications (London).*, 20. https://doi.org/10.1039/C19660000020

Garberg, H. T., Solberg, R., Barlinn, J., Martinez-Orgado, J., Løberg, E. M., & Saugstad, O. D. (2017). High-dose cannabidiol induced hypotension after global hypoxia-ischemia in piglets. *Neonatology*, *112*(2), 143–149. https://doi.org/10.1159/000471786

García, C., Gómez-Cañas, M., Burgaz, S., Palomares, B., Gómez-Gálvez, Y., Palomo-Garo, C., … Fernández-Ruiz, J. (2018). Benefits of VCE-003.2, a cannabigerol quinone derivative, against inflammation-driven neuronal deterioration in experimental Parkinson's disease: Possible involvement of different binding sites at the PPARγ receptor. *Journal of Neuroinflammation*, *15*(1), 19. https://doi.org/10.1186/s12974-018-1060-5

García, C., Palomo-Garo, C., García-Arencibia, M., Ramos, J. A., Pertwee, R. G., & Fernández-Ruiz, J. (2011). Symptom-relieving and neuroprotective effects of the phytocannabinoid Δ^9-THCV in animal models of Parkinson's disease. *British Journal of Pharmacology*, *163*(7), 1495–1506. https://doi.org/10.1111/j.1476-5381.2011.01278.x

Gauson, L. A., et al. (2007). Cannabigerol behaves as a partial agonist at both CB1 and CB2 receptors. In *Proceedings of the 17th annual symposium on the cannabinoids* (p. 206).

Gill, E. W., Paton, W. D. M., & Pertwee, R. G. (1970). Preliminary experiments on the chemistry and pharmacology of cannabis. *Nature*, *228*(5267), 134–136. https://doi.org/10.1038/228134a0

Granja, A. G., Carrillo-Salinas, F., Pagani, A., Gómez-Cañas, M., Negri, R., Navarrete, C., … Muñoz, E. (2012). A cannabigerol quinone alleviates

neuroinflammation in a chronic model of multiple sclerosis. *Journal of Neuroimmune Pharmacology*, *7*(4), 1002–1016. https://doi.org/10.1007/s11481-012-9399-3

Gugliandolo, A., Pollastro, F., Grassi, G., Bramanti, P., & Mazzon, E. (2018). In vitro model of neuroinflammation: Efficacy of cannabigerol, a non-psychoactive cannabinoid. *International Journal of Molecular Sciences*, *19*(7). https://doi.org/10.3390/ijms19071992

GW Pharmaceuticals. (2019) Pharmaceutical development pipeline. Available at: https://www.gwpharm.co.uk/healthcare-professionals/product-pipeline

Hammell, D. C., Zhang, L. P., Ma, F., Abshire, S. M., McIlwrath, S. L., Stinchcomb, A. L., & Westlund, K. N. (2016). Transdermal cannabidiol reduces inflammation and pain-related behaviours in a rat model of arthritis. *European Journal of Pain (United Kingdom)*, *20*(6), 936–948. https://doi.org/10.1002/ejp.818

Hampson, A. J., Grimaldi, M., Axelrod, J., & Wink, D. (1998). Cannabidiol and (−)Δ^9-tetrahydrocannabinol are neuroprotective antioxidants. In *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 95) (pp. 8268–8273). https://doi.org/10.1073/pnas.95.14.8268

Hayden, M. R., Leavitt, B. R., Yasothan, U., & Kirkpatrick, P. (2009). Tetrabenazine. *Nature Reviews Drug Discovery*, *8*(17–18), 17–18. https://doi.org/10.1038/nrd2784

Hernandes, M. S., Café-Mendes, C. C., & Britto, L. R. G. (2013). NADPH oxidase and the degeneration of dopaminergic neurons in Parkinsonian mice. *Oxidative Medicine and Cellular Longevity*, *2013*(157857), 1–13. https://doi.org/10.1155/2013/157857

Hill, A., et al. (2010). Δ^9-Tetrahydrocannabivarin suppresses in vitro epileptiform and in vivo seizure activity in adult rats. *Epilepsia*, *51*(8), 1522–1532. https://doi.org/10.1111/j.1528-1167.2010.02523.x

Hill, A. J., Jones, N. A., Smith, I., Hill, C. L., Williams, C. M., Stephens, G. J., & Whalley, B. J. (2014). Voltage-gated sodium (NaV) channel blockade by plant cannabinoids does not confer anticonvulsant effects per se. *Neuroscience Letters*, *566*, 269–274. https://doi.org/10.1016/j.neulet.2014.03.013

Hill, A. J., Mercier, M. S., Hill, T. D. M., Glyn, S. E., Jones, N. A., Yamasaki, Y., … Whalley, B. J. (2012). Cannabidivarin is anticonvulsant in mouse and rat. *British Journal of Pharmacology*, *167*(9), 1629–1642. https://doi.org/10.1111/j.1476-5381.2012.02207.x

Hill, T. D. M., Cascio, M. G., Romano, B., Duncan, M., Pertwee, R. G., Williams, C. M., … Hill, A. J. (2013). Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism. *British Journal of Pharmacology*, *170*(3), 679–692. https://doi.org/10.1111/bph.12321

Hollister, L. E. (1974). Structure activity relationships in man of cannabis constituents, and homologs and metabolites of Δ^9-tetrahydrocannabinol. *Pharmacology*, *11*, 3–11. https://doi.org/10.1159/000136462

Huang, W. J., Zhang, X., & Chen, W. W. (2016). Role of oxidative stress in Alzheimer's disease. *Biomedical Reports*, *4*(5), 519–522. https://doi.org/10.3892/br.2016.630

Iannotti, F. A., Hill, C. L., Leo, A., Alhusaini, A., Soubrane, C., Mazzarella, E., … Stephens, G. J. (2014). Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: Potential for the treatment of neuronal hyperexcitability. *ACS Chemical Neuroscience*, *5*(11), 1131–1141. https://doi.org/10.1021/cn5000524

Ibeas Bih, C., Chen, T., Nunn, A. V. W., Bazelot, M., Dallas, M., & Whalley, B. J. (2015). Molecular targets of cannabidiol in neurological disorders. *Neurotherapeutics*, *12*(4), 699–730. https://doi.org/10.1007/s13311-015-0377-3

Iffland, K., & Grotenhermen, F. (2017). An update on safety and side effects of cannabidiol: A review of clinical data and relevant animal studies. *Cannabis and Cannabinoid Research*, *2*(1), 139–154. https://doi.org/10.1089/can.2016.0034

Iuvone, T., Esposito, G., De Filippis, D., Scuderi, C., & Steardo, L. (2009). Cannabidiol: A promising drug for neurodegenerative disorders? *CNS Neuroscience and Therapeutics*, *15*(1), 65–75. https://doi.org/10.1111/j.1755-5949.2008.00065.x

Kaur, N., Kumar, P., Jamwal, S., Deshmukh, R., & Gauttam, V. (2016). Tetrabenazine: Spotlight on drug review. *Annals of Neurosciences*, *23*(3), 176–185. https://doi.org/10.1159/000449184

Kiaei, M. (2013). New hopes and challenges for treatment of neurodegenerative disorders: Great opportunities for young neuroscientists. *Basic and Clinical Neuroscience*, *4*(1), 3–4.

Kim, T. S., Pae, C. U., Yoon, S. J., Jang, W. Y., Lee, N. J., Kim, J. J., … Lee, C. U. (2006). Decreased plasma antioxidants in patients with Alzheimer's disease. *International Journal of Geriatric Psychiatry*, *21*(4), 344–348. https://doi.org/10.1002/gps.1469

Kovac, G. G. (2018). In G. G. Kovac & I. Alafuzoff (Eds.), *Handbook of clinical neurology chapter 21—Concepts and classification of neurodegenerative diseases* (Vol. 145).

Kozlowski, H., Janicka-Klos, A., Brasun, J., Gaggelli, E., Valensin, D., & Valensin, G. (2009). Copper, iron, and zinc ions homeostasis and their role in neurodegenerative disorders (metal uptake, transport, distribution and regulation). *Coordination Chemistry Reviews*, *253*(21–22), 2665–2685. https://doi.org/10.1016/j.ccr.2009.05.011

Le, W., Wu, J., & Tang, Y. (2016). Protective microglia and their regulation in Parkinson's disease. *Frontiers in Molecular Neuroscience*, *9*(89). https://doi.org/10.3389/fnmol.2016.00089

Lee, K. I., et al. (2016). Role of transient receptor potential ankyrin 1 channels in Alzheimer's disease. *Journal of Neuroinflammation*, *13*(1), 1–16. https://doi.org/10.1186/s12974-016-0557-z

Long, L. E., Chesworth, R., Huang, X. F., Wong, A., Spiro, A., McGregor, I. S., … Karl, T. (2012). Distinct neurobehavioural effects of cannabidiol in transmembrane domain neuregulin 1 mutant mice. *PLoS ONE*, *7*(4), e34129. https://doi.org/10.1371/journal.pone.0034129

Ma, Y. L., Weston, S. E., Whalley, B. J., & Stephens, G. J. (2008). The phytocannabinoid Δ^9-tetrahydrocannabivarin modulates inhibitory neurotransmission in the cerebellum. *British Journal of Pharmacology*, *154*(1), 204–215. https://doi.org/10.1038/bjp.2008.57

Maroon, J., & Bost, J. (2018). Review of the neurological benefits of phytocannabinoids. *Surgical Neurology International*, *9*(1), 91. https://doi.org/10.4103/sni.sni_45_18

Marsicano, G., Moosmann, B., Hermann, H., Lutz, B., & Behl, C. (2002). Neuroprotective properties of cannabinoids against oxidative stress: Role of the cannabinoid receptor CB1. *Journal of Neurochemistry*, *80*(3), 448–456. https://doi.org/10.1046/j.0022-3042.2001.00716.x

McPartland, J. M., MacDonald, C., Young, M., Grant, P. S., Furkert, D. P., & Glass, M. (2017). Affinity and efficacy studies of tetrahydrocannabinolic acid A at cannabinoid receptor types one and two. *Cannabis and Cannabinoid Research*, *2*(1), 87–95. https://doi.org/10.1089/can.2016.0032

Mechoulam, P., Tchilibon, S., Fride, E., Hanus, L., Breuer, A., & Galily, R.. US Patent No. 7,759,526. Jerusalem: Yissum Research Development.

Millar, S. A., Stone, N. L., Bellman, Z. D., Yates, A. S., England, T. J., & O'Sullivan, S. E. (2019). A systematic review of cannabidiol dosing in clinical populations. *British Journal of Clinical Pharmacology*, *85*(9), 1888–1900. https://doi.org/10.1111/bcp.14038

Millar, S. A., Stone, N. L., Yates, A. S., & O'Sullivan, S. E. (2018). A systematic review on the pharmacokinetics of cannabidiol in humans. *Frontiers in Pharmacology*, *9*, 1365. https://doi.org/10.3389/fphar.2018.01365

Miyazaki, I., Asanuma, M., Murakami, S., Takeshima, M., Torigoe, N., Kitamura, Y., & Miyoshi, K. (2013). Targeting 5-HT1A receptors in astrocytes to protect dopaminergic neurons in parkinsonian models. *Neurobiology of Disease*, *59*, 244–256. https://doi.org/10.1016/j.nbd.2013.08.003

Mogi, M., Harada, M., Riederer, P., Narabayashi, H., Fujita, K., & Nagatsu, T. (1994). Tumor necrosis factor-α (TNF-α) increases both in

the brain and in the cerebrospinal fluid from parkinsonian patients. *Neuroscience Letters*, *165*(1–2), 208–210. https://doi.org/10.1016/0304-3940(94)90746-3

Moher, D., Liberati, A., & Tetzlaff, J. A. D. (2009). PRISMA 2009 flow diagram. *The PRISMA Statement*.. https://doi.org/10.1371/journal.pmed1000097

Moldzio, R., Pacher, T., Krewenka, C., Kranner, B., Novak, J., Duvigneau, J. C., & Rausch, W. D. (2012). Effects of cannabinoids Δ^9-tetrahydrocannabinol, Δ^9-tetrahydrocannabinolic acid and cannabidiol in MPP+ affected murine mesencephalic cultures. *Phytomedicine*, *19*(8–9), 819–842. https://doi.org/10.1016/j.phymed.2012.04.002

Morales, P., Hurst, D. P., & Reggio, P. H. (2017). Molecular targets of the phytocannabinoids: A complex picture. *Progress in the Chemistry of Organic Natural Products*, *103*, 103–131. https://doi.org/10.1007/978-3-319-45541-9_4

Morano, A., Fanella, M., Albini, M., Cifelli, P., Palma, E., Giallonardo, A. T., & Di Bonaventura, C. (2020). Cannabinoids in the treatment of epilepsy: Current status and future prospects. *Neuropsychiatric Disease and Treatment*, *16*, 381–396. https://doi.org/10.2147/NDT.S203782

Nadal, X., Del Río, C., Casano, S., Palomares, B., Ferreiro-Vera, C., Navarrete, C., … Muñoz, E. (2017). Tetrahydrocannabinolic acid is a potent PPARγ agonist with neuroprotective activity. *British Journal of Pharmacology*, *174*(23), 4263–4276. https://doi.org/10.1111/bph.14019

Navarro, G., Reyes-Resina, I., Rivas-Santisteban, R., Sánchez de Medina, V., Morales, P., Casano, S., … Franco, R. (2018). Cannabidiol skews biased agonism at cannabinoid CB1 and CB2 receptors with smaller effect in CB1-CB2 heteroreceptor complexes. *Biochemical Pharmacology*, *157*, 148–158. https://doi.org/10.1016/j.bcp.2018.08.046

Nazıroglu, M. (2015). TRPV1 channel: A potential drug target for treating epilepsy. *Current Neuropharmacology*, *13*(2), 239–247. https://doi.org/10.2174/1570159x13666150216222543

Palpagama, T. H., Waldvogel, H. J., Faull, R. L. M., & Kwakowsky, A. (2019). The role of microglia and astrocytes in Huntington's disease. *Frontiers in Molecular Neuroscience*, *12*(258), 1–15. https://doi.org/10.3389/fnmol.2019.00258

Pazos, M. R., Mohammed, N., Lafuente, H., Santos, M., Martínez-Pinilla, E., Moreno, E., … Martínez-Orgado, J. (2013). Mechanisms of cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: Role of 5HT1A and CB2 receptors. *Neuropharmacology*, *71*, 282–291. https://doi.org/10.1016/j.neuropharm.2013.03.027

Pertwee, R. (2008). The diverse CB_1 and CB_2 receptor pharmacology of three plant cannabinoids: Δ^9-tetrahydrocannabinol, cannabidiol and Δ^9-tetrahydrocannabivarin. *British Journal of Pharmacology*, *153*(2), 199–215. https://doi.org/10.1038/sj.bjp.0707442

Pertwee, R. G., & Cascio, M. G. (2015). Known pharmacological actions of Δ-9-tetrahydrocannabinol and of four other chemical constituents of cannabis that activate cannabinoid receptors. In *Handbook of cannabis*. https://doi.org/10.1093/acprof:oso/9780199662685.003.0006

Pires, P. W., & Earley, S. (2018). Neuroprotective effects of trpa1 channels in the cerebral endothelium following ischemic stroke. *eLife*, *7*, e35316. https://doi.org/10.7554/eLife.35316

Pöyhönen, S., Er, S., Domanskyi, A., & Airavaara, M. (2019). Effects of neurotrophic factors in glial cells in the central nervous system: Expression and properties in neurodegeneration and injury. *Frontiers in Physiology*, *10*(448). https://doi.org/10.3389/fphys.2019.00486

Qian, L., Wu, H. M., Chen, S. H., Zhang, D., Ali, S. F., Peterson, L., … Flood, P. M. (2011). β2-adrenergic receptor activation prevents rodent dopaminergic neurotoxicity by inhibiting microglia via a novel signaling pathway. *The Journal of Immunology*, *186*(7), 4443–4454. https://doi.org/10.4049/jimmunol.1002449

Rhee, M. H., Vogel, Z., Barg, J., Bayewitch, M., Levy, R., Hanuš, L., … Mechoulam, R. (1997). Cannabinol derivatives: Binding to cannabinoid receptors and inhibition of adenylylcyclase. *Journal of Medicinal Chemistry*, *40*(20), 3228–3233. https://doi.org/10.1021/jm970126f

Rock, E. M., Limebeer, C. L., & Parker, L. A. (2018). Effect of cannabidiolic acid and Δ^9-tetrahydrocannabinol on carrageenan-induced hyperalgesia and edema in a rodent model of inflammatory pain. *Psychopharmacology*, *235*(11), 3259–3271. https://doi.org/10.1007/s00213-018-5034-1

Rodríguez-Cueto, C., Santos-García, I., García-Toscano, L., Espejo-Porras, F., Bellido, M. L., Fernández-Ruiz, J., … De Lago, E. (2018). Neuroprotective effects of the cannabigerol quinone derivative VCE-003.2 in SOD1G93A transgenic mice, an experimental model of amyotrophic lateral sclerosis. *Biochemical Pharmacology*, *157*, 217–226. https://doi.org/10.1016/j.bcp.2018.07.049

Romano, B., Borrelli, F., Fasolino, I., Capasso, R., Piscitelli, F., Cascio, M. G., … Izzo, A. A. (2013). The cannabinoid TRPA1 agonist cannabichromene inhibits nitric oxide production in macrophages and ameliorates murine colitis. *British Journal of Pharmacology*, *169*(1), 213–229. https://doi.org/10.1111/bph.12120

Rosenthaler, S., Pöhn, B., Kolmanz, C., Nguyen Huu, C., Krewenka, C., Huber, A., … Moldzio, R. (2014). Differences in receptor binding affinity of several phytocannabinoids do not explain their effects on neural cell cultures. *Neurotoxicology and Teratology*, *46*, 49–56. https://doi.org/10.1016/j.ntt.2014.09.003

Russo, E. B., & Marcu, J. (2017). Cannabis pharmacology: The usual suspects and a few promising leads. *Advances in Pharmacology*, *80*, 67–134. https://doi.org/10.1016/bs.apha.2017.03.004

Schubert, D., Kepchia, D., Liang, Z., Dargusch, R., Goldberg, J., & Maher, P. (2019). Efficacy of cannabinoids in a pre-clinical drug-screening platform for Alzheimer's disease. *Molecular Neurobiology*, *56*(11), 7719–7730. https://doi.org/10.1007/s12035-019-1637-8

Schultz, S. (2018). GWP42006 trial in focal seizures. In *GW pharmaceuticals*. Available at:. http://ir.gwpharm.com/news-releases/news-release-details/gw-pharmaceuticals-announces-preliminary-results-phase-2a-study

Scutt, A., & Williamson, E. M. (2007). Cannabinoids stimulate fibroblastic colony formation by bone marrow cells indirectly via CB2 receptors. *Calcified Tissue International*, *80*(1), 50–59. https://doi.org/10.1007/s00223-006-0171-7

Shakir, R. (2018). The struggle for stroke reclassification. *Nature Reviews Neurology*, *14*(8), 447–448. https://doi.org/10.1038/s41582-018-0036-5

Shamseer, L., Moher, D., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., … the PRISMA-P Group. (2015). Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: Elaboration and explanation. *BMJ (Online)*, *349*. https://doi.org/10.1136/bmj.g7647

Shinjyo, N., & Di Marzo, V. (2013). The effect of cannabichromene on adult neural stem/progenitor cells. *Neurochemistry International*, *63*(5), 432–437. https://doi.org/10.1016/j.neuint.2013.08.002

Sultana, R., Mecocci, P., Mangialasche, F., Cecchetti, R., Baglioni, M., & Butterfield, D. A. (2011). Increased protein and lipid oxidative damage in mitochondria isolated from lymphocytes from patients with Alzheimer's disease: Insights into the role of oxidative stress in Alzheimer's disease and initial investigations into a potential biomarker for this. *Journal of Alzheimer's Disease*, *24*(1), 77–84. https://doi.org/10.3233/JAD-2011-101425

Tai, Y. F., Pavese, N., Gerhard, A., Tabrizi, S. J., Barker, R. A., Brooks, D. J., & Piccini, P. (2007). Microglial activation in presymptomatic Huntington's disease gene carriers. *Brain*, *130*(Pt 7), 1759–1766. https://doi.org/10.1093/brain/awm044

Takeda, S., Okajima, S., Miyoshi, H., Yoshida, K., Okamoto, Y., Okada, T., … Aramaki, H. (2012). Cannabidiolic acid, a major cannabinoid in fiber-type cannabis, is an inhibitor of MDA-MB-231 breast cancer cell migration. *Toxicology Letters*, *214*(3), 314–319. https://doi.org/10.1016/j.toxlet.2012.08.029

Thomas, A., Stevenson, L. A., Wease, K. N., Price, M. R., Baillie, G., Ross, R. A., & Pertwee, R. G. (2005). Evidence that the plant

cannabinoid Δ^9-tetrahydrocannabivarin is a cannabinoid CB1 and CB2 receptor antagonist. *British Journal of Pharmacology*, *146*(7), 917–926. https://doi.org/10.1038/sj.bjp.0706414

Tóth, F., Schumacher, J., Castro, F., & Perkins, J. (2010). Full-thickness skin grafting to cover equine wounds caused by laceration or tumor resection. *Veterinary Surgery*, no–no. https://doi.org/10.1111/j.1532-950x.2010.00691.x

Turner, S. E., Williams, C. M., Iversen, L., & Whalley, B. J. (2017). Molecular pharmacology of phytocannabinoids. *Progress in the Chemistry of Organic Natural Products*, *103*, 61–101. https://doi.org/10.1007/978-3-319-45541-9_3

Udoh, M., Santiago, M., Devenish, S., McGregor, I. S., & Connor, M. (2019). Cannabichromene is a cannabinoid CB2 receptor agonist. *British Journal of Pharmacology*, *176*(23), 4537–4547. https://doi.org/10.1111/bph.14815

Valdeolivas, S., Navarrete, C., Cantarero, I., Bellido, M. L., Muñoz, E., & Sagredo, O. (2015). Neuroprotective properties of cannabigerol in Huntington's disease: Studies in R6/2 mice and 3-nitropropionate-lesioned mice. *Neurotherapeutics*, *12*(1), 185–199. https://doi.org/10.1007/s13311-014-0304-z

Vallée, A., Lecarpentier, Y., Guillevin, R., & Vallée, J. N. (2017). Effects of cannabidiol interactions with Wnt/β-catenin pathway and PPARγ on oxidative stress and neuroinflammation in Alzheimer's disease. *Acta Biochimica et Biophysica Sinica*, *49*(10), 853–866. https://doi.org/10.1093/abbs/gmx073

Vigli, D., Cosentino, L., Raggi, C., Laviola, G., Woolley-Roberts, M., & De Filippis, B. (2018). Chronic treatment with the phytocannabinoid cannabidivarin (CBDV) rescues behavioural alterations and brain atrophy in a mouse model of Rett syndrome. *Neuropharmacology*, *140*, 121–129. https://doi.org/10.1016/j.neuropharm.2018.07.029

Villapol, S. (2018). Roles of Peroxisome Proliferator-Activated Receptor Gamma on Brain and Peripheral Inflammation. *Cellular and Molecular Neurobiology*, *38*(1), 121–132. https://doi.org/10.1007/s10571-017-0554-5

Vollner, L., Bieniek, D., & Korte, F. (1969). Hashish. XX. Cannabidivarin, a new hashish constituent. *Tetrahedron Letters*, *10*(3), 145–147. https://doi.org/10.1016/S0040-4039(01)87494-3

Weydt, P., Hong, S., Witting, A., Möller, T., Stella, N., & Kliot, M. (2005). Cannabinol delays symptom onset in SOD1 (G93A) transgenic mice without affecting survival. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders*, *6*(3), 182–184. https://doi.org/10.1080/14660820510030149

Wong, H., & Cairns, B. E. (2019). Cannabidiol, cannabinol and their combinations act as peripheral analgesics in a rat model of myofascial pain. *Archives of Oral Biology*, *104*, 33–39. https://doi.org/10.1016/j.archoralbio.2019.05.028

Wood, T. B., Spivey, W. T. N., & Easterfield, T. H. (1899). *Journal of the Chemical Society, Transactions*, *75*, 20.

World Health Organization. (2017) Cannabidiol (CBD) pre-review report, expert committee on drug dependence thirty-ninth meeting, Geneva, 6–10 November 2017, Meeting Pre-Review Report.

Wyant, K. J., Ridder, A. J., & Dayalu, P. (2017). Huntington's disease—Update on treatments. *Current Neurology and Neuroscience Reports*, *17*(4), 33. https://doi.org/10.1007/s11910-017-0739-9

Yamaori, S., Ebisawa, J., Okushima, Y., Yamamoto, I., & Watanabe, K. (2011). Potent inhibition of human cytochrome P450 3A isoforms by cannabidiol: Role of phenolic hydroxyl groups in the resorcinol moiety. *Life Sciences*, *88*, 730–736. https://doi.org/10.1016/j.lfs.2011.02.017

Zamberletti, E., Gabaglio, M., Piscitelli, F., Brodie, J. S., Woolley-Roberts, M., Barbiero, I., … Parolaro, D. (2019). Cannabidivarin completely rescues cognitive deficits and delays neurological and motor defects in male Mecp2 mutant mice. *Journal of Psychopharmacology*, *33*(7), 894–907. https://doi.org/10.1177/0269881119844184

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3. Protective Effects of Cannabidivarin (CBDV) and Cannabigerol (CBG) on Cells of the Blood-Brain Barrier under Ischaemic Conditions

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Protective Effects of Cannabidivarin and Cannabigerol on Cells of the Blood–Brain Barrier Under Ischemic Conditions

Nicole L. Stone,[1,*] Timothy J. England,[1,2] and Saoirse E. O'Sullivan[2,3]

Abstract

Background and Objectives: Preclinical studies have shown cannabidiol is protective in models of ischemic stroke. Based on results from our recent systematic review, we investigated the effects of two promising neuroprotective phytocannabinoids, cannabigerol (CBG) and cannabidivarin (CBDV), on cells of the blood–brain barrier (BBB), namely human brain microvascular endothelial cells (HBMECs), pericytes, and astrocytes.

Experimental Approach: Cultures were subjected to oxygen-glucose deprivation (OGD) protocol to model ischemic stroke and cell culture medium was assessed for cytokines and adhesion molecules post-OGD. Astrocyte cell lysates were also analyzed for DNA damage markers. Antagonist studies were conducted where appropriate to study receptor mechanisms.

Results: In astrocytes CBG and CBDV attenuated levels of interleukin-6 (IL-6) and lactate dehydrogenase (LDH), whereas CBDV (10 nM–10 μM) also decreased vascular endothelial growth factor (VEGF) secretion. CBDV (300 nM–10 μM) attenuated levels of monocyte chemoattractant protein (MCP)-1 in HBMECs. In astrocytes, CBG decreased levels of DNA damage proteins, including p53, whereas CBDV increased levels of DNA damage markers. Antagonists for CB_1, CB_2, PPAR-γ, PPAR-α, 5-$HT1_A$, and TRPV1 had no effect on CBG (3 μM) or CBDV (1 μM)-mediated decreases in LDH in astrocytes. GPR55 and GPR18 were partially implicated in the effects of CBDV, but no molecular target was identified for CBG.

Conclusions: We show that CBG and CBDV were protective against OG mediated injury in three different cells that constitute the BBB, modulating different hallmarks of ischemic stroke pathophysiology. These data enhance our understanding of the protective effects of CBG and CBDV and warrant further investigation into these compounds in ischemic stroke. Future studies should identify other possible neuroprotective effects of CBG and CBDV and their corresponding mechanisms of action.

Keywords: blood–brain barrier; cannabidivarin; cannabigerol; cannabinoids; ischemia; neuroprotection

Introduction

The blood–brain barrier (BBB) is a unique interface that separates the central nervous system (CNS) and the periphery, protecting the brain from damaging components found in general circulation, namely peripheral leukocytes, macromolecules, and xenobiotics.[1,2] The barrier itself is formed by microvascular endothelial cells, which are encompassed by pericytes, and altogether surrounded by astrocyte end feet, which cover 99% of BBB endothelia.[3] Cerebral ischemia–reperfusion (IR) initiates a plethora of inflammatory signaling pathways, cytotoxic glutamate release, and oxidative stress, all of which contribute to increases in BBB permeability.[4] This loss of BBB integrity

[1]Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital, Derby, United Kingdom.
[2]University Hospitals of Derby and Burton NHS Foundation Trust, Royal Derby Hospital, Derby, United Kingdom.
[3]CanPharmaConsulting, Nottingham, United Kingdom.

*Address correspondence to: Nicole L. Stone, PhD, Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital, Derby DE22 3DT, United Kingdom, E-mail: nicolestone@hotmail.co.uk

ultimately causes uncontrolled immune infiltration into the CNS that perpetuates neuronal injury and hinders poststroke recovery. Although administration of tissue plasminogen activator (tPA) and mechanical thrombectomy are effective licensed therapies to dissolve or remove the culpable clot, at present, there are no available approved therapies that mitigate poststroke injury.[5]

Cannabidiol (CBD), one of the chemicals found in *Cannabis sativa*, has displayed a range of neuroprotective qualities, preventing neuronal loss,[6,7] attenuating astrocyte reactivity,[8] and dampening the neuroinflammatory response.[9] Unlike delta^9-tetrahydrocannabinol (Δ^9-THC), CBD does not activate the central cannabinoid receptors, CB_1 or CB_2, but activates a plethora of other targets including PPAR-γ, TRPV1, and 5-HT_{1A} receptors.[10–13] CBD has formulations (alone and with Δ^9-THC) licensed by GW pharmaceuticals to treat rare childhood epilepsies and spasticity associated with multiple sclerosis. The protective effects of CBD in stroke models has been well documented,[14] specifically CBD has been shown to reduce infarct volume,[15,16] reduce glutamate toxicity,[9,17] attenuate mitochondrial dysfunction[18] and glial activation.[6,19] In a co-culture BBB model CBD preserved barrier integrity after oxygen-glucose deprivation (OGD), which was mediated at least in part by PPAR-γ and 5-HT_{1A} receptors.[12]

Cannabigerol (CBG) and cannabidivarin (CBDV) are neutral cannabinoids present in cannabis and studies have found these compounds share similar pharmacological characteristics to CBD. Like CBD, they do not produce feelings of euphoria and display antioxidant and anti-inflammatory properties, as well as interacting with a range of target proteins including TRPV1,[13] PPAR-γ,[20] 5-HT_{1A}, and CB_2.[21] Recently our group conducted a systematic review focusing on the neuroprotective properties of minor phytocannabinoids (other than Δ^9-THC or CBD) and found that CBG and CBDV show efficacy in models of Huntington's disease, Alzheimer's, and epilepsy, with CBG mediating its protective effects through PPAR-γ activation,[22] the same mechanism by which we have shown that CBD protects BBB integrity.[12] However, despite these compounds having neuroprotective effects in other models, no studies have been conducted to test whether CBG or CBDV are protective in IR injury.

In light of the above, we hypothesized these compounds may exhibit protective properties at the BBB in a stroke model. To test this, we treated cells of the BBB with CBG or CBDV *in vitro* before an OGD protocol and measured various proinflammatory cytokines, adhesion molecules, and cell damage markers.

Materials and Methods

Materials

CBG and CBDV were kindly gifted by STI pharmaceuticals. Both compounds were dissolved in 100% ethanol to 10 mM and were stored at −20°C. AM251, AM630, GW6471, GW9962, O1918, CID16020046, SB366791 (Tocris, United Kingdom) were dissolved in dimethyl sulfoxide as stock solutions of 10 mM. (S)-WAY100135 was dissolved in deionized water. Antagonists were stored at −20°C and dilutions were made fresh as required.

General cell culture

Human brain microvascular endothelial cells (HBMECs), astrocytes, and pericytes (passages 3–6) were grown in their respective medium and maintained at 37°C in a humidified incubator supplemented with 5% CO_2. HBMECs were cultured on fibronectin-coated plasticware (2 μg/cm^2), as per supplier recommendations. Primary cells and medium were purchased from ScienCell, United Kingdom.

OGD protocol

To simulate ischemic conditions, normal medium was replaced with glucose free RPMI medium (Gibco, United Kingdom) containing either CBG or CBDV (10 nM to 10 μM), alongside a vehicle control (0.01% ethanol). Cell culture plates were then placed in an anoxic bag (BD GasPak™, anaerobe) for 4 h (8 h for astrocyte experiments) plus an additional 20 min to ensure anaerobic conditions. For vehicle normoxia, ethanol (0.01%) was added to the respective medium of each cell type (ScienCell) and maintained in normal oxygenated conditions. After OGD, medium was aspirated and replaced with each cell types respective medium (ScienCell) containing the relevant concentrations of CBG or CBDV for a 20-h/16-h reperfusion period. At 24 h, the medium was sampled, and cells were lysed with RIPA buffer containing protease and phosphatase inhibitors (Sigma, United Kingdom; ThermoFisher, United Kingdom). Medium and lysates were stored at −80°C for future analysis.

Total protein

To quantify total protein, a bicinchoninic acid (BCA) protein assay was performed on cell lysates. A working reagent of copper II sulfate and BCA (Sigma-Aldrich) was prepared in a 1:50 ratio and added to wells. After a 30-min incubation at 37°C, plates were read at

562 nm. Unknowns were extrapolated from a standard curve of known concentrations of bovine serum album. Unless otherwise stated, all secreted and intracellular proteins were normalized to total protein.

Enzyme-linked immunosorbent assay

Medium samples were analyzed for various proinflammatory cytokines including interleukin (IL)-6, IL-8, and adhesion molecules including intracellular adhesion molecule (ICAM)-1, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein (MCP)-1 using duoset enzyme-linked immunosorbent assay (ELISA) by R&D systems, United Kingdom (DY206, DY208, DY720, DY293B, and DY279). Raw values at 570 nm were subtracted from values obtained at 450 nm, sample concentrations were determined by extrapolating unknowns from the 8-point standard curve (known concentrations).

Lactate dehydrogenase assay

A lactate dehydrogenase (LDH) assay was performed to determine nonspecific damage induced by the OGD protocol. A standard curve of known concentration of nicotinamide adenine dinucleotide was constructed as per manufacturer's instructions. Fifty microliters of standard or sample was aliquoted into a 96-well plate and 50 μL of assay mix was added. Plate absorbance was read at 450 nm and unknown values were obtained from a standard curve.

DNA damage/genotoxicity assay

Astrocyte lysates post-OGD were analyzed using the Milliplex DNA damage/Genotoxicity multiplex assay kit (Millipore, 48–621MAG) to detect changes in DNA damage markers ataxia-telangiectasia mutated (ATR-Total), checkpoint kinases 1, 2 (Chk1, Ser345 and Chk2, and Thr68), histone family member X (H2A.X, Ser139), mouse double minute 2 homolog (MDM2, total), cyclin-dependent kinase inhibitor 1 (p21, total), tumour protein (p53, Ser15). Kits were performed according to manufacturer's instructions.

Statistical analysis

All data are represented as the mean ± standard error of the mean, data were assessed for normality using the D'Agostino–Pearson normality test and subsequently analyzed using one-way analysis of variance with Dunnett's *post hoc* analysis. All statistical analyses were conducted using GraphPad prism (7/8) (Version 7.01; GraphPad Software, Inc.), comparing either vehicle normoxia or vehicle OGD with all other treatments. A value of $p < 0.05$ was considered significant.

Results

HBMEC monocultures

Protein levels from HBMEC lysates were significantly lower post-OGD compared with vehicle normoxia wells ($p < 0.001$). This was not affected by pretreatment with CBDV or CBG (Supplementary Fig. S1C, F).

IL-6, ICAM-1, and MCP-1 were significantly increased in cell culture medium 24 h after 4-h OGD compared with normoxia vehicle ($p < 0.05$; Fig. 1A–F). Pretreatment with CBG (10 nM–10 μM) displayed an overall trend to decrease IL-6 and 100 nM, 300 nM, and 10 μM CBG-treated wells were not statistically significant to vehicle normoxia (Fig. 1A). CBG pretreatment did not alter ICAM-1 and MCP-1 secretion in response to OGD (Fig. 1B, C).

Pretreatment with CBDV (10 nM–1 μM and 10 μM) did not attenuate IL-6 levels 24-h post-OGD. However, 3 μM CBDV was not significantly different from vehicle normoxia (Figure 1D). Pretreatment with 3 and 10 μM CBDV significantly increased levels of ICAM-1 24-h post-OGD ($p < 0.05$, Fig. 1E). CBDV (100 nM–10 μM) concentration-dependently reduced levels of MCP-1, an effect that was significantly different to vehicle OGD at 3 and 10 μM ($p < 0.05$; Fig. 1F).

Pericyte monocultures

Protein levels from pericyte monocultures were not significantly altered by the OGD protocol or drug treatment (Supplementary Fig. S1A, D). A 4-h OGD increased levels of IL-6, VEGF, and IL-8 measured in cell culture medium 24-h post-OGD (Fig. 2A–F).

In pericyte monocultures, neither CBG nor CBDV (10 nM–3 μM) altered IL-6 levels post-OGD; however, both compounds increased IL-6 levels at 10 μM ($p < 0.0001$; Fig. 2A, D). Pretreatment with CBG and CBDV (10 nM–10 μM) did not alter levels of VEGF (Fig. 2B, E).

At the lowest and highest concentrations tested, CBG pretreatment increased IL-8 levels compared with vehicle normoxia and vehicle OGD ($p < 0.05$; Fig. 2C). At 100 and 300 nM, CBG did not alter increased levels of IL-8 produced by OGD (Fig. 2C). CBDV did not affect IL-8 levels post-OGD, although there was a trend to produce an increase in IL-8 at 10 μM (Fig. 2F).

Astrocyte monocultures

IL-6 levels were not statistically different to vehicle normoxia 24 h after 4-h OGD (70.03 pg·mL normoxia vs. 65.29 pg·mL OGD, data not shown), but levels of IL-6

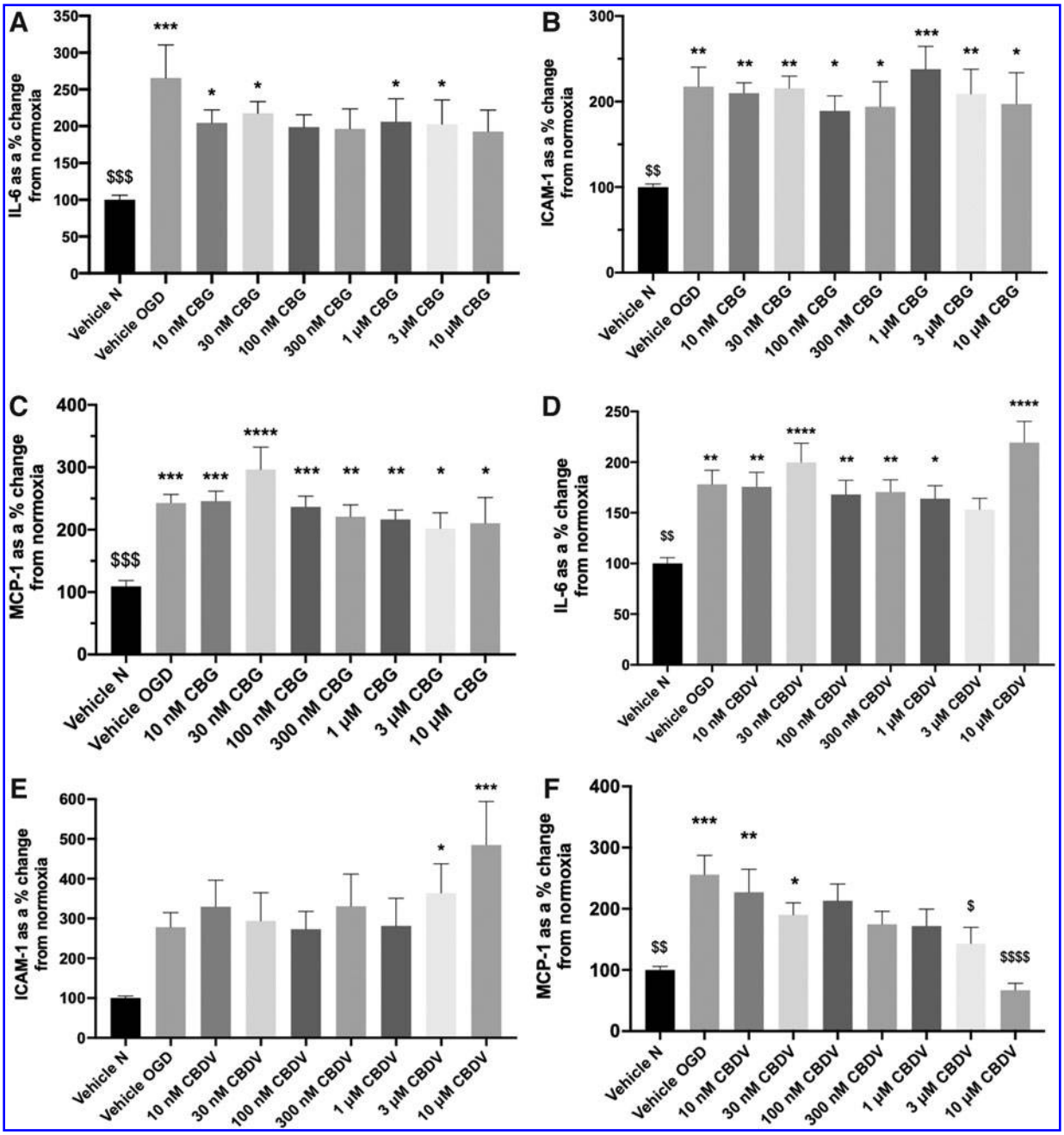

FIG. 1. The effects of CBG and CBDV on HBMEC monocultures. Medium was analyzed for IL-6 **(A, D)**, ICAM-1 **(B, E),** and MCP-1 **(C, F)** 24 h after 4-h OGD. Data were normalized to total protein (calculated using a BCA assay) and are given as a % change from the normoxia vehicle presented as means with error bars representing SEM. $n = 6–9$ from three experimental repeats. *, Significant difference compared with vehicle normoxia (vehicle N) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, and $^{\$\$\$\$}p < 0.0001$) significant difference to vehicle OGD, one-way ANOVA with Dunnett's *post hoc* analysis. ANOVA, analysis of variance; BCA, bicinchoninic acid; CBDV, cannabidivarin; CBG, cannabigerol; HBMEC, human brain microvascular endothelial cell; ICAM-1, intracellular adhesion molecule-1; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; OGD, oxygen-glucose deprivation; SEM, standard error of the mean.

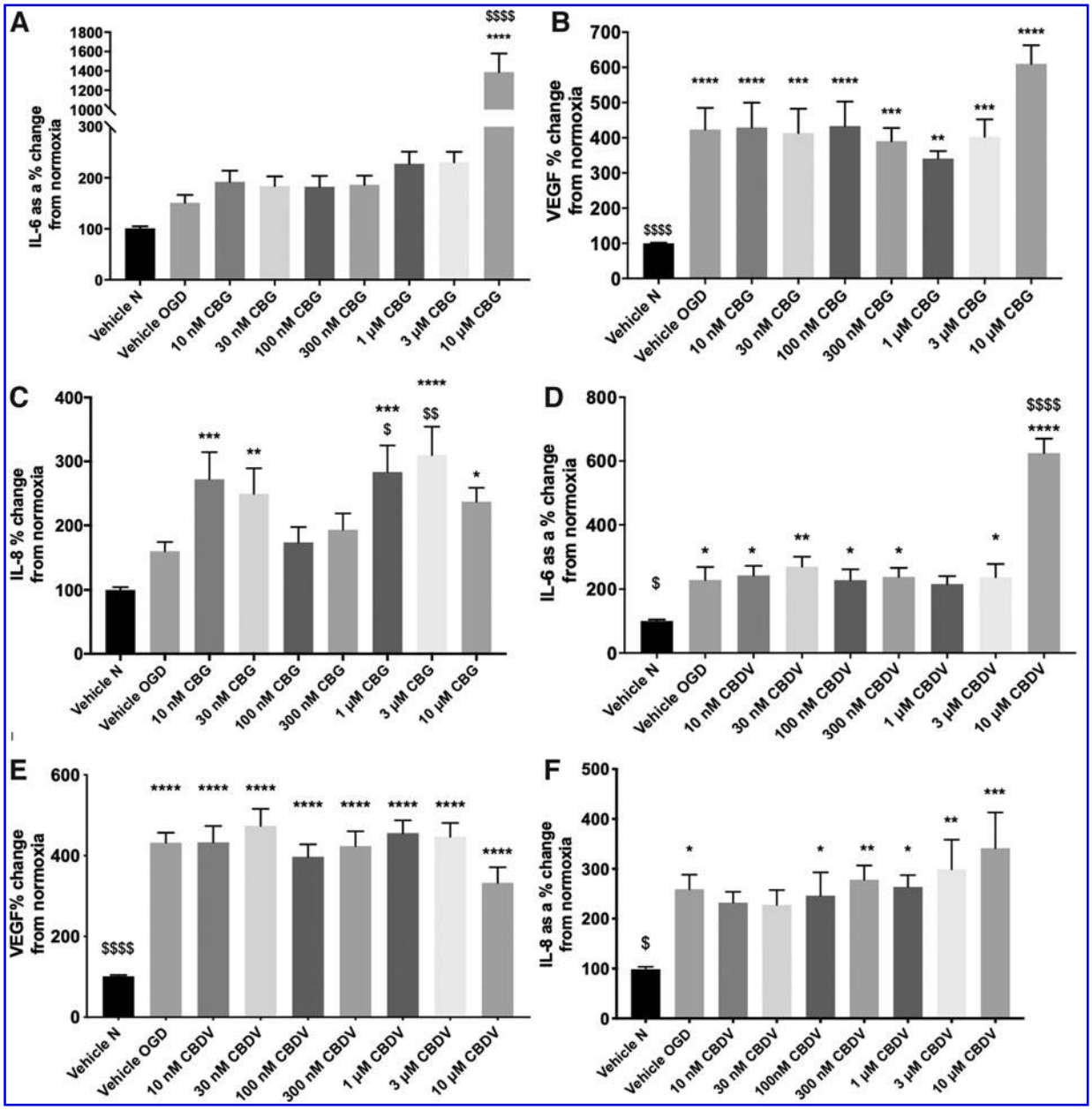

FIG. 2. The effects of CBDV and CBG on pericyte monocultures. Medium 24 h after 4-h OGD was analyzed for IL-6, VEGF, and IL-8 **(A–F)**. Data were normalized to total protein and are given as a % change from the normoxia vehicle, presented as means with error bars representing SEM. $n = 6–9$ from 3 experimental repeats. *, Significant difference compared with vehicle normoxia (vehicle N) ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$). $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, and $^{\$\$\$\$}p < 0.0001$) significant difference to vehicle OGD, one-way ANOVA with Dunnett's *post hoc* analysis. VEGF, vascular endothelial growth factor.

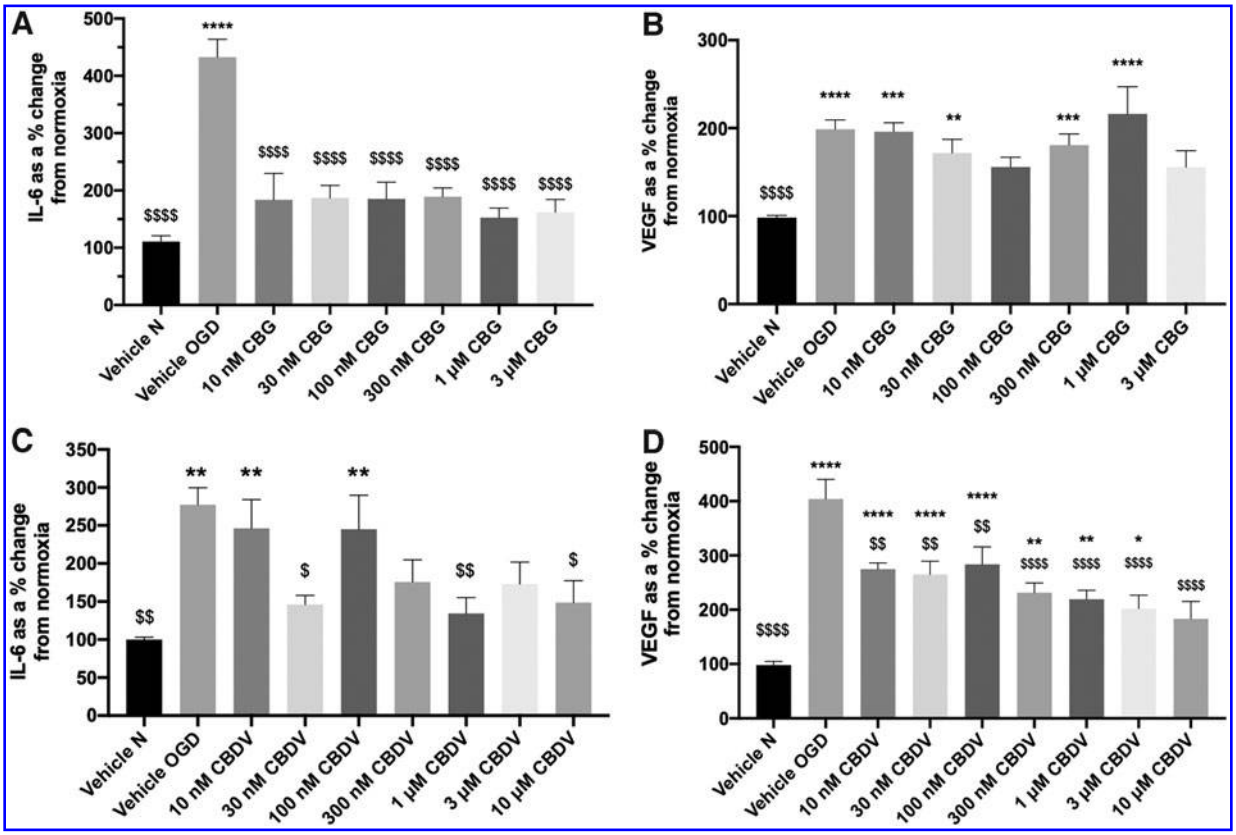

FIG. 3. The effects of CBG and CBDV on astrocyte monocultures. **(A-D)** Medium 24 h after 8-h OGD were analyzed for IL-6 and VEGF. Data were normalized to total protein and are given as a % change from the normoxia vehicle, presented as means with error bars representing SEM. $n = 5$–9 from 3 experimental repeats. *, Significant difference compared with vehicle normoxia (vehicle N) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, and $^{\$\$\$\$}p < 0.0001$) significant difference to vehicle OGD, one-way ANOVA with Dunnett's *post hoc* analysis.

were significantly increased 24 h after 8-h OGD ($p < 0.01$; Figure 3A and C). Therefore, subsequent experiments in astrocytes were conducted using an 8-h OGD protocol.

An 8-h protocol significantly decreased protein levels in astrocyte cell lysates ($p < 0.01$ versus vehicle normoxia; Supplementary Fig. S1B, E). Treatment with 10 μM CBG decreased protein content compared with both vehicle OGD and vehicle normoxia ($p < 0.0001$; Supplementary Fig. S1B). Pretreatment with CBDV did not prevent the decrease in protein content caused by the 8-h OGD protocol ($p < 0.05$ vs. vehicle normoxia); however, 30 nM, 1 and 10 μM CBDV did not exhibit a significant difference compared with vehicle normoxia (Supplementary Figure S1E).

Pretreatment with CBG 10 nM–3 μM attenuated astrocytic IL-6 levels ($p > 0.0001$ vs. vehicle OGD; Fig. 3A); however, at 10 μM CBG significantly increased IL-6 (Supplementary Fig. S2A). CBDV reduced levels of IL-6 compared with vehicle OGD at 30 nM ($p < 0.05$), 1 μM ($p < 0.01$), and 10 μM ($p < 0.05$; Fig. 3C). CBDV at 300 nM and 3 μM also appeared to decrease IL-6 levels, exhibiting no statistical difference to vehicle normoxia.

Astrocytic VEGF levels were significantly increased post-OGD ($p < 0.0001$; Fig. 3B, D). CBG pretreatment appeared to attenuate VEGF levels at 100 nM and 3 μM, but this did not reach significance to vehicle OGD (Fig. 3B). Conversely, 10 μM CBG significantly increased VEGF compared with both vehicle normoxia and vehicle OGD ($p < 0.001$; Supplementary Fig. S2B). Pretreatment with CBDV (10 nM–10 μM) decreased

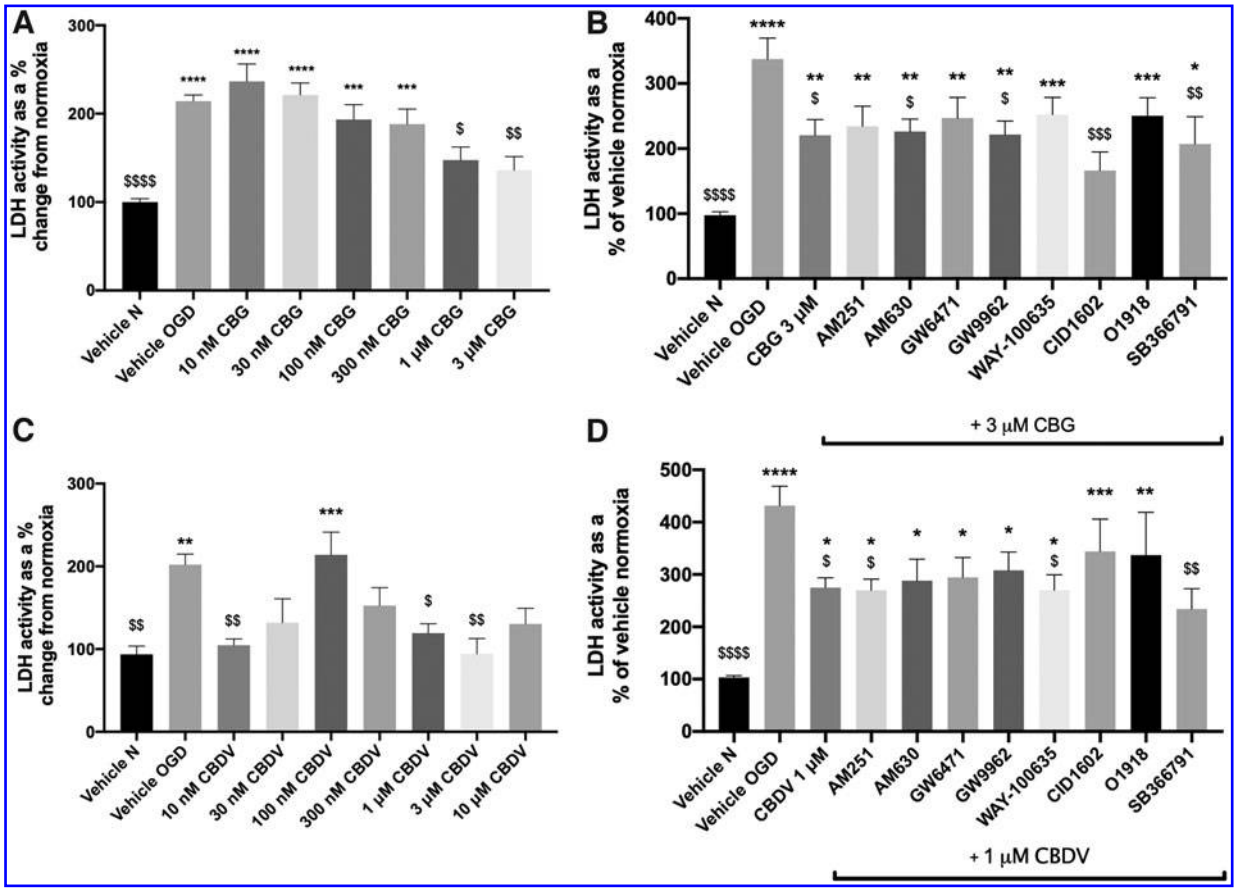

FIG. 4. The effects of CBG and CBDV treatment alone **(A, B)** and with antagonists **(C, D)** on LDH release from astrocyte monocultures. Medium 24 h after 8-h OGD were analyzed LDH. Data were normalized to total protein and are given as a % change from the normoxia vehicle, presented as means with error bars representing SEM. $n = 5–6$ from 3 experimental repeats. *, Significant difference compared with vehicle normoxia (vehicle N) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, and $^{\$\$\$\$}p < 0.0001$) significant difference to vehicle OGD, one-way ANOVA with Dunnett's *post hoc* analysis. LDH, lactate dehydrogenase.

VEGF levels in a concentration-dependent manner. At 10 μM this was not significantly different to vehicle normoxia and significantly different to vehicle OGD ($p < 0.0001$; Fig. 3D).

LDH was significantly elevated in astrocyte medium post-OGD ($p < 0.01$; Fig. 4A, C). Pretreatment with 1 and 3 μM CBG significantly attenuated LDH activity ($p < 0.05$; Fig. 4A); however, at 10 μM CBG significantly increased LDH activity (Supplementary Fig. S2C). CBDV exhibited a biphasic concentration response, decreasing LDH activity at lower (10 nM; $p < 0.01$) and higher concentrations ($p < 0.05$; 1 and 3 μM), but increasing levels at 100 nM ($p < 0.001$; Fig. 4C).

None of the antagonists tested blocked CBG (3 μM)-mediated decreases in LDH; however, application of CID1602 (antagonist for GPR55) appeared to potentiate the effects of CBG ($p < 0.001$; 3 μM CBG + CID1602 vs. vehicle OGD; Fig. 4B). In the presence of antagonists for GPR55, CID1602 and O1918, CBDV (1 μM)-mediated decreases in LDH were no longer significantly different to vehicle OGD (Fig. 4D). In addition, SB366791 appeared to potentiate the LDH-reducing effects of CBDV ($p < 0.01$; 1 μM CBDV SB366791 vs. vehicle OGD, Fig. 4D).

As CBDV and CBG reduced cell damage in astrocytes, we next investigated whether these compounds

(at the most efficacious lower and higher concentrations tested) influenced levels of DNA damage proteins. Levels of DNA damage proteins, ATR, Chk1, Chk2, H2A.X, and p53 were increased in astrocyte cell lysates 24 h after 8-h OGD. MDM2 showed a trend for increasing post-OGD, but levels of p21 were not affected (Fig. 5A–N). Application of CBG (1 μM) before OGD significantly reduced levels of Chk1 and Chk2 compared with OGD vehicle ($p > 0.01$, $p < 0.05$; Fig. 5B, C). In addition, CBG pretreatment at 10 nM, 1 μM, and 3 μM decreased H2A.X levels that displayed trend for increasing post-OGD ($p > 0.05$; Fig. 5D). Levels of p53 were also increased post-OGD ($p > 0.05$) and attenuated by CBG in a concentration-dependent manner that was significant at 1 μM ($p > 0.05$; Fig. 5K). By contrast, CBDV (10 nM, 100 nM) increased levels of ATR ($p > 0.0001$, $p < 0.01$; Fig. 5E) as well as increasing levels of Chk1 at 100 nM ($p < 0.05$) and Chk2 at 10 nM ($p < 0.01$; Fig.5F). CBDV (100 nM and 1 μM) also increased levels of H2A.X, p53 and MDM2 ($p < 0.05$; Fig. 5H, L, N).

Discussion

In this study we assessed whether non-euphoric phytocannabinoids CBG and CBDV protected cells of the BBB in a cellular model of ischemic stroke. Despite promising preclinical data, drugs developed for one or more of the hallmarks of stroke have failed once they have reached clinical trials.[23,24] Poor translational efficacy is likely to stem from the multifactorial pathophysiology of ischemic stroke and complicating factors among elderly patients, which are often overlooked in ischemic stroke modeling.[25] These points emphasize the need to generate new, effective therapies for patients, which target multiple aspects of stroke pathogenesis.[26]

CBD has been widely studied as a neuroprotectant, partly because of its promiscuous pharmacology, tolerable safety profile in humans and absence of euphoric effects.[10,27,28] However, other phytocannabinoids are beginning to gain significant interest as therapeutic agents. CBG has displayed prominent anti-inflammatory and antioxidant capabilities[20,29,30] and the antiepileptic properties of CBDV have been well documented.[31–33] Recently, CBDV has been shown to reduce inflammatory cytokine release in a model of intestinal inflammation.[34] Our results demonstrate that CBDV and CBG exhibit protective properties against OGD-induced damage in astrocytes and HBMECs, modulating a range of biochemical parameters measured post-OGD. For CBDV, its cytoprotective effects appeared to partially involve GPR55, but a target for CBG was not identified. These data warrant their further investigation into these compounds as neuroprotectants and to assess their clinical applicability, specifically, their efficacy in *in vivo* models of ischemic stroke and whether they are protective when applied post-OGD.

Post-cerebral ischemia and elevated levels of proinflammatory cytokine IL-6 are associated with increased neuronal cell necrosis and are correlated with stroke severity, increases in mortality rate, poor performance, and functional disability.[35–38] In this study, CBG and CBDV significantly decreased levels of IL-6 in astrocytes, suggesting that like CBD, CBDV and CBG may offer protection against inflammation caused by ischemic stroke.[12] Increases in IL-6 post-ischemia have also been implicated in BBB breakdown and tight junction remodeling, including reduced expression of VE-cadherin, occludin, and claudin-5.[39] Although there was a trend for CBDV and CBG to attenuate IL-6 levels in HBMECs, more pronounced reductions in IL-6 were observed in astrocytes. Astrocytes provide biochemical and mechanical support that help to maintain the BBB, as well as providing neurovascular crosstalk between neurons and cerebral blood vessels.[40] Unlike in monoculture, *in vivo,* astrocyte endfeet are in direct contact with endothelial cells; thus, modulating the astrocyte inflammatory response *in situ* may act to preserve BBB integrity indirectly by soluble factors secreted by astrocytes or by preserving normal astrocyte function.

Mice lacking the receptor for adhesion molecule, MCP-1 (CCR2), have significantly reduced infarct sizes together with reduced BBB permeability and similarly, MCP-1 knockout mice have a reduced influx of hematogenic cells from systemic circulation and improved neurological outcome.[41,42] Bonifačić et al. found a relationship between patients with poor outcomes 90 days after stroke and elevated levels of MCP-1 and a recent meta-analysis revealed that higher baseline circulating levels of MCP-1 correlated with a higher risk of ischemic stroke.[43,44] Our data show that CBDV concentration dependently decreased levels of MCP-1 secreted by HBMECs when applied at the same time as initiating OGD, suggesting that CBDV might offer protection against MCP-1-related damage post-stroke and/or offer protection in individuals at a higher risk of ischemic stroke. These data are also consistent with that of a recent study showing that CBDV treatment attenuated MCP-1 mRNA levels in colonic tissue post-colitis.[34] Of interest, this study also showed that CBDV was able to

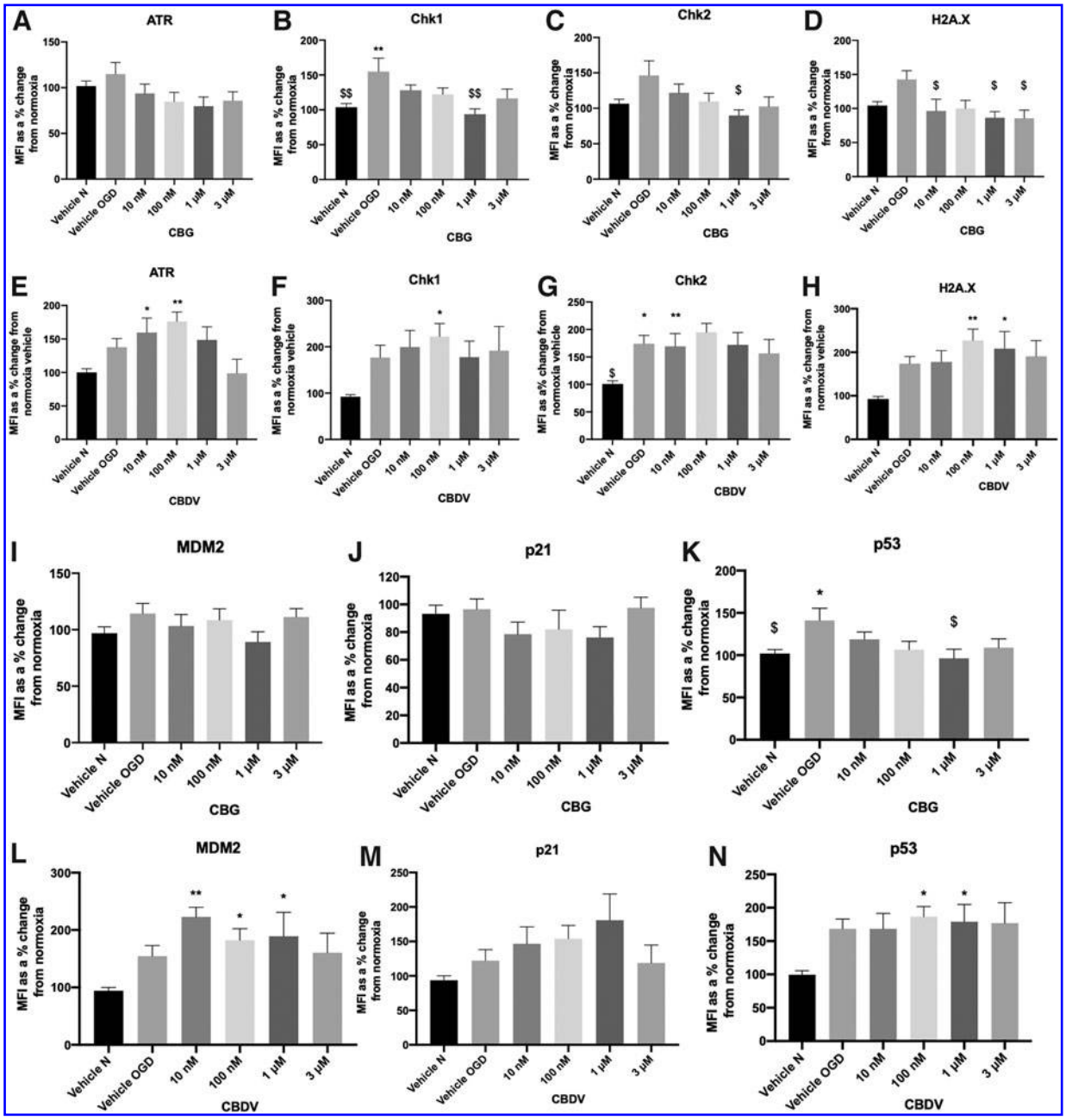

FIG. 5. The effects of CBG and CBDV on DNA damage markers (ATR **[A,E]**, Chk1 **[B,F]**, Chk2 **[C,G]**, H2A.X **[D,H]**, MDM2 **[I,L]**, p21 **[J,M]**, and p53 **[K,N]** from astrocyte cell lysates, 24 h after 8-h OGD. Data were normalized to total protein and are given as MFI as a % change from the normoxia vehicle (vehicle N); means with error bars represent SEM. $n = 6$–8 from 3 experimental repeats. *, Significant difference compared with vehicle normoxia (vehicle N) (*$p < 0.05$, **$p < 0.01$). $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ significant difference to vehicle OGD, one-way ANOVA with Dunnett's *post hoc* analysis. MFI, mean fluorescent intensity.

reduce intestinal permeability, an effect that may be replicated at the BBB, but this has yet to be investigated.

We also measured VEGF secreted by pericytes and astrocytes post-OGD reperfusion as elevations in VEGF are correlated with increased endothelial barrier permeability post-ischemia.[45–47] Li and co-authors found that astrocyte-derived VEGF mediated endothelial barrier disruption, which was associated with decreases in occludin and claudin-5.[47] Interestingly, whilst CBG and CBDV did not affect pericyte-derived VEGF, CBDV decreased VEGF secretion in astrocytes in a concentration-dependent manner and CBG exhibited a trend for decreasing VEGF at 100 nM and 3 μM. As VEGF is known to facilitate BBB opening these compounds may offer protection against BBB breakdown post-ischemia; however, the mechanisms in which these compounds decrease VEGF remains to be elucidated.

During IR injury cells undergo a combination of apoptosis and necrosis, causing various cellular components to be released into the extracellular space. One of these components, LDH, is often used as a marker of cell damage. Previous studies have shown that IR models cause LDH leakage into cell culture medium[48,49] and clinically, LDH has been trialled as a marker of ischemic severity.[50,51] Pretreatment with CBDV and CBG offset increases in LDH, suggesting both compounds mitigate cellular damage produced by OGD reperfusion. Application of receptor antagonists revealed that CBDV appeared to mediate its effects on LDH levels by GPR55; however, none of the antagonists tested blocked the effect of CBG. This could be explained by the nonspecific antioxidant properties of cannabinoids, namely owing to their phenolic rings and hydroxyl moieties.[17,52] Indeed, previous studies have shown that CBD increases antioxidant enzymes in BV2 microglial cells,[53] as well as attenuating oxidative stress and increasing mitochondrial bioenergetics in OGD reperfusion-damaged neurons.[18] Similarly, CBD, CBDV, and CBG were able to prevent oxytosis in a preclinical drug screen for Alzheimer's disease and CBG exhibited antioxidant capacity in neuroblastoma cells.[54,55] More data are clearly needed on the specific and nonspecific mechanisms in which these compounds mediate their protective effects, particularly whether their antioxidant status is responsible for reducing cell damage in the context of ischemia.

Ischemia is a pathophysiological stressor and as a consequence, nonspecific single- and double-strand DNA breaks (ssDNA/dsDNA breaks) and replication-associated DNA damage responses (DDRs) occur. DNA damage can activate the DDR pathway and DDR response proteins ATR, Chk1, Chk2, H2A.X, MDM2, p21 and p53 that govern elements of DNA repair, cell cycle arrest, apoptotic and necrotic cell death.[56–59] These processes are central in IR injury and early studies found that neurons are the first to exhibit signs of DNA damage (0.5–8 h reperfusion) followed by astrocytes (24 h reperfusion).[60] Thus, we next investigated the effect of CBDV and CBG on DDR proteins post-OGD in astrocytes.

In support of previous studies, our OGD protocol (and subsequent reperfusion period) increased levels of almost all measured DDR proteins in astrocyte monoculture lysates.[61] In stroke patients, Huttner and colleagues found evidence of ATM/ATR activity in the penumbra of cortical neurons 7–10 days post-ischemia.[62] Studies have also shown p53 activation is implicated in ischemia-induced neuronal cell death, with elevated levels of p53 also present in reactive astrocytes and microglia.[63,64] Ahn and colleagues found that inhibition of p53 by pifithrin-α reduced OGD-induced cell death in cultured astrocytes, and as a secondary effect reduced elevated levels of glutamate and glial fibrillary acidic protein (GFAP), which were also increased post-OGD.[65] To our knowledge, this is the first study to show that CBG pretreatment reduced levels of Chk1, Chk2, H2A.X, and p53 in astrocytes post-OGD. It is likely that these decreases in DNA damage proteins were caused indirectly, possibly because of the overall reductions in cellular damage and inflammation, as well as the known antioxidant properties of CBG that have both been demonstrated in other studies.[20,66] Nevertheless, direct modulation of these proteins should not be ruled out particularly as PPAR-γ, a known target for phytocannabinoids, has been implicated in ATM signaling and the DDR.[67]

Pretreatment with CBDV significantly increased expression of the majority of DNA damage proteins in astrocytes and exhibited a trend for increasing p21. CBD was recently found to increase protein expression of ATM and p21, but not p53 in an *in vitro* model of gastric cancer, suggesting CBD promotes cell cycle arrest at the G_0–G_1 phase.[68] Our data suggest that CBDV acts in a similar manner; however, it is important to emphasize that p21 has roles in both enhancing and inhibiting apoptosis depending on the type of stressor; thus, generating this response in a cancer cell model will be different to responses of astrocytes subjected to OGD. Low dose *N*-methyl-D-aspartate (NMDA) to

simulate ischemic preconditioning was shown to increase MDM2 protein expression, preventing p53 stabilization in mouse cortical neurons and ischemia-induced apoptotic cell death.[69] CBDV significantly increased levels of MDM2, which is a key protein involved in p53 degradation and thus promotion of cell survival. Future studies should clarify the implications of CBDVs ability to increase levels of DNA damage proteins in ischemia and establish whether modulating DNA damage and repair in astrocytes can influence post-stroke injury and recovery.

Conclusions

This study provides novel data on the neuroprotective and anti-inflammatory properties of CBG and CBDV in an *in vitro* model of IR. These data, together with evidence from other studies, corroborate the protective properties of these compounds and further studies are needed to elucidate the mechanism of action of CBG and CBDV and whether they can modulate BBB permeability in more clinically relevant *in vivo* models of ischemic stroke. There is lack of effective treatments for ischemic stroke, a condition that will increase in prevalence in coming years, to which cannabinoids may offer a unique therapeutic strategy.

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Authors' Contributions

S.O.S. and N.S. designed the experiments that were carried out by N.S. N.S. wrote the article with input from S.O.S. and T.J.E. T.J.E. and S.O.S. supervised the project.

Author Disclosure Statement

S.O.S. is director of CanPharmaConsulting, scientific advisor to pharmaceutical companies developing cannabinoid-based medicines. None of these companies were involved in this research.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

References

1. Abbott NJ, Patabendige AAK, Dolman DEM, et al. Structure and function of the blood-brain barrier. Neurobiol Dis. 2010;37:13–25.
2. McConnell HL, Kersch CN, Woltjer RL, et al. The translational significance of the neurovascular unit. J Biol Chem. 2017;292:762–770.
3. Sosunov AA, Wu X, Tsankova NM, et al. Phenotypic heterogeneity and plasticity of isocortical and hippocampal astrocytes in the human brain. J Neurosci. 2014;34:2285–2298.
4. Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. J Neuroimmunol. 2007;184:53–68.
5. Douglas DR, Luoma V, Reddy U. Acute management of ischaemic stroke. Anaesth Intensive Care Med. 2020;21:1–7.
6. Ceprián M, Jiménez-Sánchez L, Vargas C, et al. Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. Neuropharmacology. 2017;116:151–159.
7. Rodríguez-Muñoz M, Onetti Y, Cortés-Montero E, et al. Cannabidiol enhances morphine antinociception, diminishes NMDA-mediated seizures and reduces stroke damage via the sigma 1 receptor. Mol Brain. 2018; 11:51.
8. Lafuente H, Alvarez FJ, Pazos MR, et al. Cannabidiol reduces brain damage and improves functional recovery after acute hypoxia-ischemia in newborn pigs. Pediatr Res. 2011;70:272–277.
9. Castillo A, Tolón MR, Fernández-Ruiz J, et al. The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB2 and adenosine receptors. Neurobiol Dis. 2010;37:434–440.
10. Russo EB, Marcu J. Cannabis pharmacology: the usual suspects and a few promising leads. Adv Pharmacol. 2017;80:67–134.
11. Russo EB, Burnett A, Hall B, et al. Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochem Res. 2005;30:1037–1043.
12. Hind WH, England TJ, O'Sullivan SE. Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARγ and 5-HT1A receptors. Br J Pharmacol. 2016;173:815–825.
13. Iannotti FA, Hill CL, Leo A, et al. Nonpsychotropic plant cannabinoids, cannabidivarin (CBDV) and cannabidiol (CBD), activate and desensitize transient receptor potential vanilloid 1 (TRPV1) channels in vitro: potential for the treatment of neuronal hyperexcitability. ACS Chem Neurosci. 2014;5:1131–1141.
14. England TJ, Hind WH, Rasid NA, et al. Cannabinoids in experimental stroke: a systematic review and meta-analysis. J Cereb Blood Flow Metab. 2015;35:348–358.
15. Khaksar S, Bigdeli MR. Correlation between cannabidiol-induced reduction of infarct volume and inflammatory factors expression in ischemic stroke model. Basic Clin Neurosci. 2017;8:139–146.
16. Pazos MR, Cinquina V, Gómez A, et al. Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. Neuropharmacology. 2012;63:776–783.
17. Hampson AJ, Grimaldi M, Axelrod J, et al. Cannabidiol and (-)Δ9-tetrahydrocannabinol are neuroprotective antioxidants. Proc Natl Acad Sci U S A. 1998;95:8268–8273.
18. Sun S, Hu F, Wu J, et al. Cannabidiol attenuates OGD/R-induced damage by enhancing mitochondrial bioenergetics and modulating glucose metabolism via pentose-phosphate pathway in hippocampal neurons. Redox Biol. 2017;11:577–585.
19. Mori MA, Meyer E, Soares LM, et al. Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. Prog Neuropsychopharmacol Biol Psychiatry. 2017;75:94–105.
20. Gugliandolo A, Pollastro F, Grassi G, et al. In vitro model of neuroinflammation: efficacy of cannabigerol, a non-psychoactive cannabinoid. Int J Mol Sci. 2018;19:1992.
21. Giacoppo S, Gugliandolo A, Trubiani O, et al. Cannabinoid CB2 receptors are involved in the protection of RAW264.7 macrophages against the oxidative stress: an in vitro study. Eur J Histochem. 2017;61:1–13.
22. Stone NL, Murphy AJ, England TJ, et al. A systematic review of minor phytocannabinoids with promising neuroprotective potential. Br J Pharmacol. 2020;177:4330–4352.
23. O'Collins VE, Macleod MR, Donnan GA, et al. 1,026 Experimental treatments in acute stroke. Ann Neurol. 2006;59:467–477.
24. Schmidt-Pogoda A, Bonberg N, Koecke MHM, et al. Why most acute stroke studies are positive in animals but not in patients: a systematic

comparison of preclinical, early phase, and phase 3 clinical trials of neuroprotective agents. Ann Neurol. 2020;87:40–51.
25. Cai W, Zhang K, Li P, et al. Dysfunction of the neurovascular unit in ischemic stroke and neurodegenerative diseases: an aging effect. Ageing Res Rev. 2017;34:77–87.
26. Suzman R, Beard JR, Boerma T, Chatterji S. Health in an ageing world—what do we know? Lancet. 2015;385:484–486.
27. Ibeas Bih C, Chen T, Nunn AVW, et al. Molecular targets of cannabidiol in neurological disorders. Neurotherapeutics. 2015;12:699–730.
28. Millar SA, Stone NL, Bellman ZD, et al. A systematic review of cannabidiol dosing in clinical populations. Br J Clin Pharmacol. 2019;85:1888–1900.
29. Borrelli F, Fasolino I, Romano B, et al. Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease. Biochem Pharmacol. 2013;85:1306–1316.
30. Echeverry C, Prunell G, Narbondo C, et al. A comparative in vitro study of the neuroprotective effect induced by cannabidiol, cannabigerol, and their respective acid forms: relevance of the 5-HT1A receptors. Neurotox Res. 2021;39:335–348.
31. Hill TDM, Cascio MG, Romano B, et al. Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism. Br J Pharmacol. 2013;170:679–692.
32. Hill AJ, Mercier MS, Hill TDM, et al. Cannabidivarin is anticonvulsant in mouse and rat. Br J Pharmacol. 2012;167:1629–1642.
33. Amada N, Yamasaki Y, Williams CM, et al. Cannabidivarin (CBDV) suppresses pentylenetetrazole (PTZ)-induced increases in epilepsy-related gene expression. PeerJ. 2013;1:e214.
34. Pagano E, Romano B, Iannotti FA, et al. The non-euphoric phytocannabinoid cannabidivarin counteracts intestinal inflammation in mice and cytokine expression in biopsies from UC pediatric patients. Pharmacol Res. 2019;149:104464.
35. Shaafi S, Sharifipour E, Rahmanifar R, et al. Interleukin-6, a reliable prognostic factor for ischemic stroke. Iran J Neurol. 2014;13:70–76.
36. Miao Y, Liao JK. Potential serum biomarkers in the pathophysiological processes of stroke. Expert Review of Neurother. 2014;14:173–185.
37. Armstead WM, Hekierski H, Pastor P, et al. Release of IL-6 after stroke contributes to impaired cerebral autoregulation and hippocampal neuronal necrosis through NMDA receptor activation and upregulation of ET-1 and JNK. Transl Stroke Res. 2019;10:104–111.
38. Beridze M, Sanikidze T, Shakarishvili R, et al. Selected acute phase CSF factors in ischemic stroke: findings and prognostic value. BMC Neurol. 2011;11:41.
39. Rochfort KD, Collins LE, Murphy RP, et al. Downregulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions. PLoS One. 2014;9:e101815.
40. Liu Z, Chopp M. Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke. Prog Neurobiol. 2016;144:103–120.
41. Strecker JK, Minnerup J, Gess B, et al. Monocyte chemoattractant protein-1-deficiency impairs the expression of IL-6, IL-1β and G-CSF after transient focal ischemia in mice. PLoS One. 2011;6:e25863.
42. Stamatovic SM, Shakui P, Keep RF, et al. Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. J Cereb Blood Flow Metab. 2005;25:593–606.
43. Georgakis MK, Malik R, Björkbacka H, et al. Circulating monocyte chemoattractant protein-1 and risk of stroke meta-analysis of population-based studies involving 17180 individuals. Circ Res. 2019;125:773–782.
44. Bonifačić D, Toplak A, Benjak I, et al. Monocytes and monocyte chemoattractant protein 1 (MCP-1) as early predictors of disease outcome in patients with cerebral ischemic stroke. Wien Klin Wochenschr. 2016;128:20–27.
45. Bai Y, Zhu X, Chao J, et al. Pericytes contribute to the disruption of the cerebral endothelial barrier via increasing VEGF expression: implications for stroke. PLoS One. 2015;10:1–20.
46. Argaw AT, Asp L, Zhang J, et al. Astrocyte-derived VEGF-A drives blood-brain barrier disruption in CNS inflammatory disease. J Clin Invest. 2012;122:2454–2468.
47. Li YN, Pan R, Qin XJ, et al. Ischemic neurons activate astrocytes to disrupt endothelial barrier via increasing VEGF expression. J Neurochem. 2014;129:120–129.
48. Wang L, Zhang Y, Asakawa T, et al. Neuroprotective effect of neuroserpin in oxygen-glucose deprivation- and reoxygenation-treated rat astrocytes in vitro. PLoS One. 2015;10:e0123932.
49. Zhang QY, Wang ZJ, Sun DM, et al. Novel therapeutic effects of leonurine on ischemic stroke: new mechanisms of BBB integrity. Oxid Med Cell Longev. 2017;2017:7150376.
50. Lampl Y, Paniri Y, Eshel Y, et al. Cerebrospinal fluid lactate dehydrogenase levels in early stroke and transient ischemic attacks. Stroke. 1990;21:854–857.
51. Karlsson M, Wiberg-Itzel E, Chakkarapani E, et al. Lactate dehydrogenase predicts hypoxic ischaemic encephalopathy in newborn infants: a preliminary study. Acta Paediatr Int J Paediatr. 2010;99:1139–1144.
52. Borges RS, Batista J, Viana RB, et al. Understanding the molecular aspects of tetrahydrocannabinol and cannabidiol as antioxidants. Molecules. 2013;18:12663–12674.
53. Juknat A, Pietr M, Kozela E, et al. Microarray and pathway analysis reveal distinct mechanisms underlying cannabinoid-mediated modulation of LPS-induced activation of BV-2 microglial cells. PLoS One. 2013;8:e61462.
54. Schubert D, Kepchia D, Liang Z, et al. Efficacy of cannabinoids in a pre-clinical drug-screening platform for Alzheimer's disease. Mol Neurobiol. 2019;56:7719–7730.
55. Granja AG, Carrillo-Salinas F, Pagani A, et al. A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis. J Neuroimmune Pharmacol. 2012;7:1002–1016.
56. Liu Q, Guntuku S, Cui XS, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint. Genes Dev. 2000;14:1448–1459.
57. Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science (80-). 1998;282:1893–1897.
58. Savic V, Yin B, Maas NL, et al. Formation of dynamic γ-H2AX domains along broken DNA strands is distinctly regulated by ATM and MDC1 and dependent upon H2AX densities in chromatin. Mol Cell. 2009;34:298–310.
59. Maréchal A, Zou L. DNA damage sensing by the ATM and ATR kinases. Cold Spring Harb Perspect Biol. 2013;5:a012716.
60. Chen J, Jin K, Chen M, et al. Early detection of DNA strand breaks in the brain after transient focal ischemia: implications for the role of DNA damage in apoptosis and neuronal cell death. J Neurochem. 1997;69:232–245.
61. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. J Biol Chem. 2003;278:12207–12213.
62. Huttner HB, Bergmann O, Salehpour M, et al. The age and genomic integrity of neurons after cortical stroke in humans. Nat Neurosci. 2014;17:801–803.
63. Chung YH, Shin CM, Kim MJ, et al. Enhanced expression of p53 in reactive astrocytes following transient focal ischemia. Neurol Res. 2002;24:324–328.
64. Leker RR, Aharonowiz M, Greig NH, et al. The role of p53-induced apoptosis in cerebral ischemia: effects of the p53 inhibitor pifithrin α. Exp Neurol. 2004;187:478–486.
65. Ahn KC, MacKenzie EM, Learman CR, et al. Inhibition of p53 attenuates ischemic stress-induced activation of astrocytes. Neuroreport. 2015;26:862–869.
66. Di Giacomo V, Chiavaroli A, Recinella L, et al. Antioxidant and neuroprotective effects induced by cannabidiol and cannabigerol in rat CTX-TNA2 astrocytes and isolated cortexes. Int J Mol Sci. 2020;21:3575.
67. Li CG, Mahon C, Sweeney NM, et al. PPARγ interaction with UBR5/ATMIN promotes DNA repair to maintain endothelial homeostasis. Cell Rep. 2019;26:1333–1343.e7.
68. Zhang X, Qin Y, Pan Z, et al. Cannabidiol induces cell cycle arrest and cell apoptosis in human gastric cancer SGC-7901 cells. Biomolecules. 2019;9:302.
69. Vecino R, Burguete MC, Jover-Mengual T, et al. The MDM2–p53 pathway is involved in preconditioning-induced neuronal tolerance to ischemia. Sci Rep. 2018;8:1610.

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Abbreviations Used

Δ^9-THC = delta^9-tetrahydrocannabinol
ANOVA = analysis of variance
BBB = blood–brain barrier
BCA = bicinchoninic acid
CBDV = cannabidivarin
CBG = cannabigerol
CNS = central nervous system
DDR = DNA damage response
ELISA = enzyme-linked immunosorbent assay
GFAP = glial fibrillary acidic protein
HA = human astrocytes
HBMECs = human brain microvascular endothelial cells
ICAM-1 = intracellular adhesion molecule-1
IR = ischaemia–reperfusion
LDH = lactate dehydrogenase
MCP-1 = monocyte chemoattractant protein-1
MFI = mean fluorescent intensity
NMDA = N-methyl-D-aspartate
OGD = oxygen-glucose deprivation
TEER = trans epithelial resistance
VEGF = vascular endothelial growth factor
VCAM-1 = vascular adhesion molecule

3.1 Supplemental information

IL-6 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	66.09	191.10	226.59	1037.36	285.33	509.77
SD	11.46	80.32	71.53	359.27	55.13	168.85
SEM	3.82	23.19	20.65	103.71	18.38	56.28

VEGF (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	121.53	225.47	249.61	201.46	208.37	188.32
SD	43.75	57.84	131.13	101.97	105.88	57.92
SEM	14.58	19.28	43.71	33.99	35.29	19.30

IL-8 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	1792.51	2565.71	2688.87	2673.21	2716.32	5258.47
SD	759.68	485.39	875.53	865.50	170.40	876.31
SEM	287.13	183.46	291.84	288.00	98.38	505.94

Table II: Raw data from ELISA of pericyte cell culture medium. Note that these values are taken as an average from experimental repeats with cells at different passages and have not been normalised to total protein.

IL-6 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	66.09	191.10	226.59	1037.36	121.25	167.12
SD	11.46	80.32	71.53	359.27	21.78	43.50
SEM	3.82	23.19	20.65	103.71	8.89	12.76

VEGF (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	98.5	253.6	249.69	232.61	273.47	190.55
SD	60.4	84.82	59.80	170.75	66.78	51.12
SEM	16.75	28.27	19.93	56.92	27.26	29.87

Table III: Raw data from ELISA of astrocyte cell culture medium. Note that these values are taken as an average from experimental repeats with cells at different passages and have not been normalised to total protein.

IL-6 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	248.15	457.63	453.34	189.71	228.72	397.39
SD	55.16	243.32	263.86	119.89	73.79	248.87
SEM	24.67	86.03	87.95	48.95	24.60	82.96

MCP-1 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	2304.50	3573.39	4756.84	3088.78	6861.55	3433.11
SD	1050.72	1150.08	1878.69	1663.37	4206.34	1614.60
SEM	350.24	383.36	626.23	743.88	1717.23	659.16

ICAM-1 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBG 10 nM	CBG 10 µM	CBDV 10 nM	CBDV 10 µM
Mean	520.69	882.82	799.72	645.74	362.35	333.93
SD	342.50	448.88	422.61	295.39	72.88	85.87
SEM	139.82	183.25	172.53	120.59	29.75	35.06

Table IV: Raw data from ELISA of human brain microvascular endothelial cell (HBMEC) cell culture medium. Note that these values are taken as an average from experimental repeats with cells at different passages and have not been normalised to total protein.

4. CBDA modulates blood brain barrier (BBB) *in vitro* post oxygen-glucose deprivation

Abstract

Background and purpose

Cannabidiol (CBD) has demonstrated a plethora neuroprotective effects and previously our group showed CBD reduced blood-brain barrier (BBB) permeability in an *in vitro* model of ischaemic stroke. We aimed to investigate whether CBD's acidic precursor, cannabidiolic acid (CBDA), also reduced BBB permeability and protected cells against oxygen-glucose deprivation (OGD) induced damage.

Experimental Approach

Human brain microvascular endothelial cells (HBMECs), pericytes, astrocytes and neurons were grown in a four-cell transwell style BBB model. Pericytes and neurons were also grown in monoculture. Oxygen-glucose deprivation (OGD) was used to model ischaemia. Medium was analysed for proinflammatory mediators and heat shock proteins post OGD. Propidium iodide staining was also conducted in neurons to evaluate neuronal cell death. Mechanisms of action were probed in pericytes using appropriate receptor antagonists.

Results

CBDA 3 μM prevented an increase in permeability post OGD between 4 h (post OGD) and 24 h (p<0.05). CBDA (1 μM) also protected neurons from OGD induced damage as shown by propidium iodide staining and by visual assessment using a light microscope (72 h post OGD).

In pericyte monocultures, CBDA (10 nM- 1 μM) attenuated increases in interleukin 6 (IL-6) 24 h post 4 h OGD (p<0.01). CBDA did not modulate levels of VEGF, ICAM-1 but increased IL-8 at 10 μM (p<0.001). The 5-HT_{1A} antagonist, WAY-100635, inhibited

CBDAs ability to reduce IL-6 secretion. CBDA (100 nM) also reduced levels of HSP27(ps78) and HSP90 in neuronal monocultures.

Conclusions and implications

These data suggest that CBDA, like CBD, displays anti-inflammatory and neuroprotective properties, as well as modulating BBB permeability in a four-cell model. CBDA appears to mediate some of its anti-inflammatory effects in pericytes in a similar mechanism to CBD; via the 5-HT_{1A} receptor. Overall, further research is warranted into CBDAs neuroprotective effects in *in vivo* models of ischaemic stroke and to elucidate potential interactions at other receptors.

6.1 Introduction

The development and maintenance of the BBB is governed by specialised cell types namely human brain microvascular cells (HBMECs), astrocytes and pericytes. Collectively they regulate the formation of tight junctions, barrier polarization, paracellular permeability and expression of transporter proteins, which are characteristic features of the BBB (Abbott, 2002; Abbott *et al.*, 2010). The BBB also has significant interaction with surrounding cells, namely neurons and microglia, and altogether form the neurovascular unit (NVU) (Reviewed in Iadecola, 2017; McConnell *et al.*, 2017). Crosstalk between neurons and the cells of BBB is essential for functional hyperaemia, BBB maintenance, immune responses and metabolic waste clearance (Nippert, Biesecker and Newman, 2018). In addition, pericytes are key players in regulating cerebral blood flow as well as tight junction formation and maintenance in HBMECS (Bell *et al.*, 2010; Daneman *et al.*, 2010).

Ischaemia-reperfusion (IR) ensues significant damage upon the BBB, specifically oxidative stress, glutamate release and immune cell activation, which stimulate the release of pro-inflammatory cytokines, disruption of endothelial cell tight-junctional complexes, as well as increases in the expression of adhesion molecules. These pathological mechanisms increase BBB permeability and facilitate leukocyte infiltration into the CNS, perpetuating neuronal damage in the potentially salvageable penumbral region. Only two treatments, tissue plasminogen activator (tPA) and surgical clot removal, are licenced to treat ischaemic stroke (Minnerup *et al.*, 2012). Recently it was reported in a study where patients were receiving thrombolysis treatment, good functional outcome decreased by 75% for every 1% increase in BBB permeability measured 5 days post stroke (Nadareishvili *et al.*, 2019). Currently, there are no successful treatments to mitigate BBB permeability which can dramatically affect neurological outcome and functional recovery post stroke.

Cannabidiol (CBD) does not activate the cannabinoid receptors CB_1 and CB_2 but can activate a plethora of other pharmacological targets (Russo and Marcu, 2017). CBD

possesses significant anti-inflammatory, antioxidant and neuroprotective properties (Esposito *et al.*, 2007; Ruiz-Valdepeñas *et al.*, 2011) and studies have shown CBD protects against hypoxia induced neuronal cell damage (Alvarez *et al.*, 2008; Castillo *et al.*, 2010; Lafuente *et al.*, 2011). At the BBB, CBD mediates its protective effects at least in part through the activation of TRPV2, A2A, PPARy and 5-HT_{1A} (Mecha *et al.*, 2013; Hind, England and O'Sullivan, 2016; Luo *et al.*, 2019). Despite extensive interest in CBD, data on CBDs acidic precursor cannabidiolic acid (CBDA, particularly its neuroprotective effects, is extremely limited (Formato *et al.*, 2020; Stone *et al.*, 2020). CBDA has displayed efficacy as an anti-hyperalgesic, antiemetic, an antiepileptic, an anti-cancer agent and as an inhibitor and down-regulator of the cyclooxygenase enzyme, COX-2 (Takeda *et al.*, 2008; E M Rock and Parker, 2017; Rock, Limebeer and Parker, 2018; Anderson *et al.*, 2019) Bolognini *et al.*, (2013) showed that CBDA is 1000 times more potent than CBD as an antiemetic, via activation of 5-HT_{1A}. Additionally, CBDA has been shown to activate various transient receptor ion channels, albeit with a lower potency than CBD (De Petrocellis *et al.*, 2008). Given these data it is possible that CBDA acts similarly to CBD in other conditions, such as ischaemic stroke. Recently Anderson *et al.*, (2019) characterised the pharmacokinetics and CNS penetration of acidic phytocannabinoids and the poor brain: plasma ratio of CBDA was positively improved by administration with a Tween 80 vehicle, highlighting that CBDA can penetrate the brain when administered in the right formulation.

In light of the above, together with previous data generated by our group, we hypothesised that CBDA may also be protective at the BBB through a similar mechanism to CBD. The aim of this study was to assess the effects of pre-treatment with CBDA in pericyte and neuronal cell monocultures and an *in vitro* BBB model (Stone et al., 2018). BBB permeability was assessed using transepithelial resistance (TEER) and levels various cytokines and chemokines were assessed in cell culture medium. Possible mechanisms were probed using appropriate receptor antagonists.

6.2 Materials and Methods

Permeability experiments

HBMECs, astrocytes, pericytes and neurons were grown in a contact four-cell model in their respective media (ScienCell, UK). HBMECs were seeded in the apical side of collagen coated 3.0 µm pore polytetrafluoroethylene membrane Transwell inserts (12 well type; Corning Costar, USA). Astrocytes and pericytes were seeded as a mixed culture on the underside of the insert. Neurons were cultured on poly-L-lysine coated coverslips which were placed on the cell culture well bottom. For full methodology see Stone et al., (2018). Cells were grown to confluency and the resistance across the membrane was measured using STX-3 chopstick electrodes linked to an epithelial voltohmeter (World Precision Instruments, UK). These measurements were used to evaluate barrier integrity. For monoculture experiments pericytes and neurons (P3-P5 and P1 respectively, ScienCell, UK) were cultured until confluency in 24 well cell culture plates or 48 well plates for neurons.

OGD protocol

To simulate ischaemic conditions, medium was replaced with glucose free RPMI medium (Gibco, UK) containing increasing concentrations of cannabidiolic acid (CBDA) or cannabidiol (CBD), alongside a vehicle control (0.01% acetonitrile or ethanol) and then placed in an anoxic bag (BD, anaerobe) for 4 h plus an additional 20 minutes to ensure anaerobic conditions (Hind *et al.*, 2015;2016). For normoxic conditions, the same concentrations were applied but these were prepared in normal pericyte or neuronal medium (Sciencell, UK) and maintained in normal oxygenated conditions. After 4 h OGD, medium was aspirated and replaced with normal pericyte or neuronal medium containing the relevant concentrations of CBDA or CBD for a 20 h reperfusion period. At 24 h, medium from both conditions (OGD and normoxia) was sampled and cells were lysed with RIPA buffer containing protease and phosphatase inhibitors. Medium and lysates were stored at -80°C for future analysis.

Antagonist experiments

Pericytes (P3-P5) were cultured until confluency in 24 well cell culture plates. For OGD conditions the medium was replaced with glucose free RPMI medium (Gibco, UK) containing relevant antagonists, AM251 (100 nM), AM630 (100 nM), GW6471 (100 nM), GW9962 (100 nM), (S)-WAY 100135 (300 nM), O1918 (1 μM), capsazepine (1 μM), CID16020046 (1 μM), SB366791 (1 μM). CBDA 1 μM alone and a vehicle control (0.01% acetonitrile). After 15 minutes, CBDA 1 μM was applied to the antagonist treated wells and then placed in an anoxic bag (0% O_2) for 4 h (BD, anaerobe). After 4 h, medium was replaced with normal pericyte medium containing the relevant antagonists and 15 minutes later CBDA 1 μM was applied to the antagonist treated wells.

At 24 h, medium from both conditions (OGD and normoxia) was sampled and cells were lysed with RIPA buffer containing protease and phosphatase inhibitors. Medium and lysates were stored at -80°C for future analysis.

Biochemical analysis

Medium samples were analysed for Interleukin-6 (IL-6) a pro-inflammatory mediator, intracellular adhesion molecule-1 (ICAM-1), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF) using duo-set ELISA by affymetrix and R&D systems, UK. Data obtained from monolayer experiments were normalised to total protein using a bicinchoninic acid (BCA) assay (Sigma-Aldrich, UK).

Propidium Iodide (PI) Staining

At 72 h post OGD, neurons were washed with 1X PBS and incubated with a 100 μL/mL solution of PI prepared in PBS for 1-2 minutes at room temperature in the absence of light. PI is excluded from live cells but binds to double stranded DNA of dead or dying cells, thus the red staining is proportional to the number of dying cells. Images were obtained immediately using a Nikon DS-Fi1 digital camera linked to an upright

fluorescence microscope (Nikon Eclipse 50i) with a 20x objective. Images were visually assessed to determine proportion of dead cells.

Materials

CBDA was obtained from Sigma, UK and CBD from Tocris, UK. CBDA was dissolved in 100% acetonitrile and CBD in 100% ethanol to a stock concentration of 10 mM and were stored at -20°C. AM251, AM630, GW6471, capsazapine, GW9962, O1918, CID16020046, SB366791 (Tocris, UK) were dissolved in dimethyl sulfoxide as stock solutions of 10 mM. (S)-WAY100635 was dissolved in deionised water. Antagonists were stored at -20°C and dilutions were made fresh as required.

Statistical Analysis

Data are represented as the mean ± S.E.M, data were assessed for normality using the D'Agostino-Pearson normality test and subsequently analysed using one-way ANOVA with *Dunnett's* post hoc analysis. All statistical analyses were conducted using GraphPad prism (7/8) (Version 7.01; GraphPad Software Inc.), comparing either vehicle normoxia or vehicle OGD with all other treatments. TEER and IL-6 data from BBB model were analysed using two-way ANOVA with Turky's multiple comparisons test. A p value of <0.05 was considered significant.

6.3 Results

6.3.1 The effects of CBDA and CBD in a 4-cell BBB model

Subjecting co-cultures to a 4 h OGD increased permeability, as shown by decrease in TEER (0 h-4 h), measured immediately post OGD (Figure 6.1 A, B). Pre-treatment with CBD displayed a trend to prevent permeability increases between 4 h and 24 h post OGD, but this did not achieve statistical significance (Figure 6.1A). There was a significant difference between treatment with 300 nM CBD and 1 μM CBD at 48 h and 72 h. CBDA (300 nM-3 μM) exhibited a trend to decrease permeability between 4 h and 24 h post OGD and was significant at 3 μM ($p<0.05$; Figure 6.1B). There was a trend for CBD (3 μM) and CBDA (1 μM) to decrease levels of IL-6 at 24 h and 48 h but this did not reach significance (Figure 6.2A,B).

CBDA prevented cell death in neurons present in the 4-cell model which had been subjected to 4 h OGD; as shown by fewer PI-stained neuronal nuclei vs vehicle control wells, Figure 6.3.

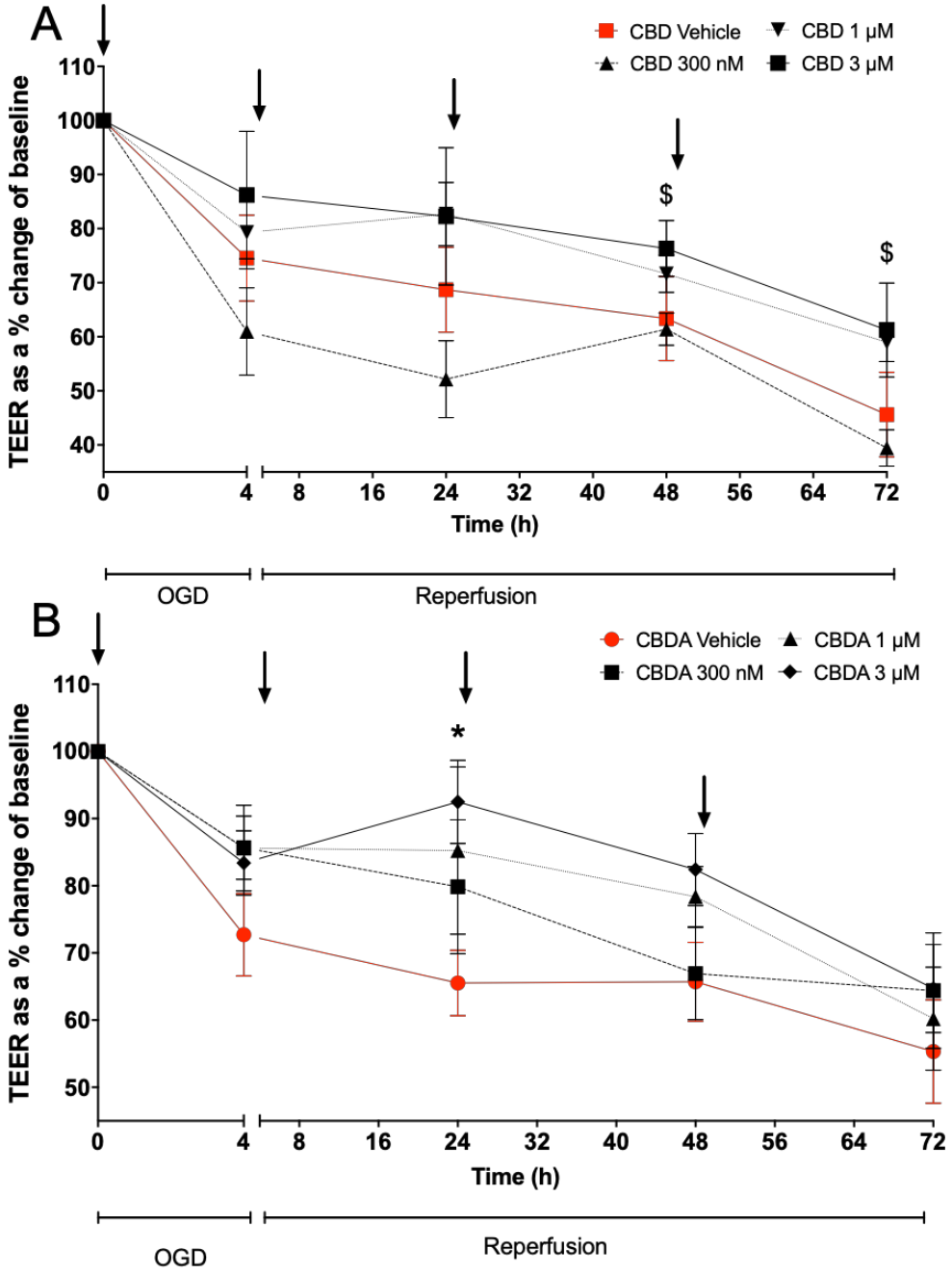

Figure 6.1. *The effects of CBD (A) and CBDA (B) on permeability (transepithelial resistance, TEER in vitro model of the BBB post 4 h oxygen-glucose deprivation (OGD) and a subsequent 20 h reperfusion period.* TEER was measured at the same intervals prior to medium change/ sampling. Compounds were applied prior to OGD, post OGD and at 24 and 48 h post OGD after TEER measurement and medium sampling (depicted by black arrows). Data are given as means with error bars representing S.E.M. n= 6 inserts based on 3 experimental repeats. Statistical analysis was conducted using a two-way ANOVA and multiple comparisons were adjusted for by Turkey's statistical hypothesis test. * denotes a significant difference compared to control (p<0.05), $ denotes a significant difference between 300 nM CBD and 1 µM CBD (p<0.05).

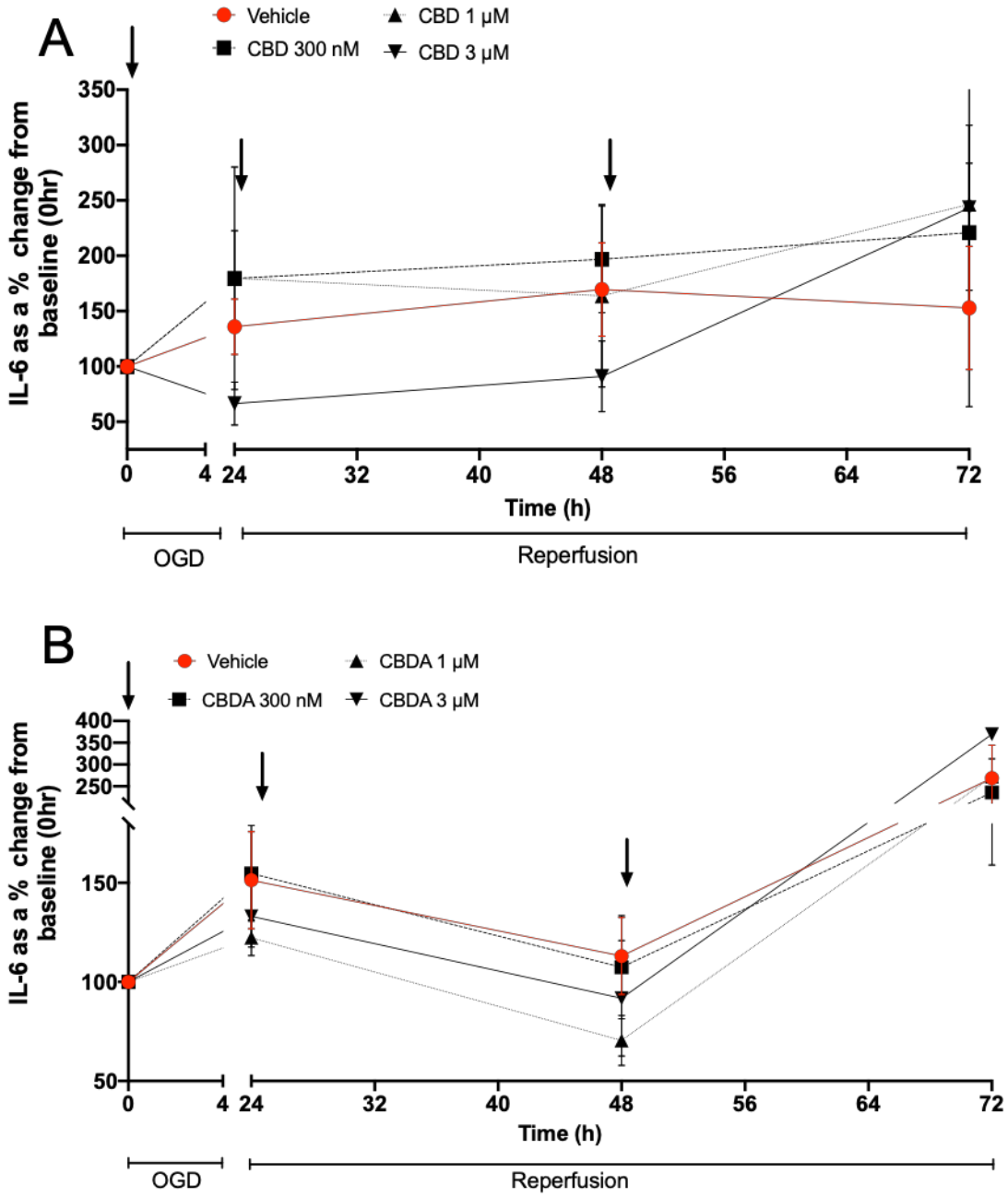

Figure 6.2. *The effects of CBD (A) and CBDA (B) on IL-6 secretion in an in vitro model of the BBB post 4 h oxygen-glucose deprivation (OGD) and a subsequent 20 h reperfusion period.* Medium was sampled before OGD, post OGD and at 24 h intervals before drug reapplication. Compounds were applied prior to OGD, post OGD and at 24 and 48 h post OGD after TEER measurement and medium sampling (depicted by black arrows). Data are given as means with error bars representing S.E.M. n= 6 inserts based on 3 experimental repeats. Statistical analysis was conducted using a two-way ANOVA and multiple comparisons were adjusted for by Turkey's statistical hypothesis test. * denotes a significant difference compared to control (p<0.05), $ denotes a significant difference between 300 nM CBD and 1 µM CBD (p<0.05).

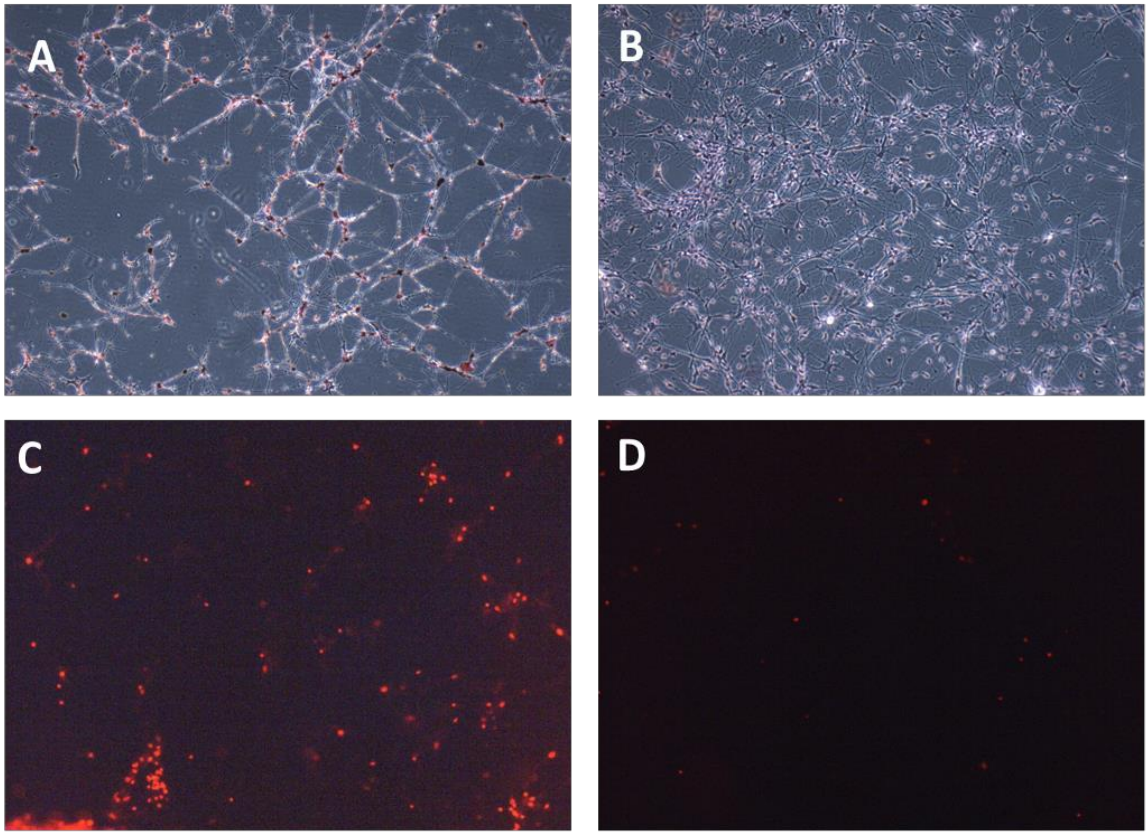

Figure 6.3. Fluorescence and light microscope images of neurons from the 4-cell BBB model stained with propidium iodide at 72 h post 4 h OGD. PI s excluded from live cells but can bind to double stranded DNA of dead or dying cells, thus red staining is proportional to the number of dying cells. 1 µM CBDA (B, D) protected neurons against OGD induced cell death as shown in vehicle control wells (A, C). Images were obtained at 20× objective.

6.3.2 Pericyte monocultures

In pericytes, IL-6, VEGF and ICAM-1 levels, but not IL-8, rose significantly 24 h post 4 h OGD compared to the normoxia vehicle ($p \leq 0.01$; Figure 6.4A-D). Pre-treatment with CBDA (10 nM, 100 nM and 1 µM) significantly decreased IL-6 secretion in pericytes 24 h post OGD compared to OGD vehicle ($p < 0.01$; Figure 6.4A). 10 µM CBDA exhibited a trend for decreasing IL-6 but this did not reach significance. CBDA (10 nM- 1 µM) did not modulate levels of VEGF but exhibited a slight trend to further increase VEGF at 10 µM (Figure 6.4B). CBDA (10 nM- 10 µM) had no effect on levels of elevated levels of ICAM-1 (Figure 6.4C), but significantly increased IL-8 at 10 µM ($p < 0.01$; Figure 6.4D).

BCA results showed that there was no significant difference in protein levels in OGD conditions 24 h post OGD between vehicle and CBDA treated cells (6.6).

Application of 5-HT_{1A} antagonist, WAY-100,635, under OGD conditions inhibited CBDA mediated reduction in IL-6 secretion in 24 h post OGD samples ($p < 0.01$ normoxia vehicle vs WAY+CBDA 1 µM; Figure 6.5A). All other antagonists tested did not block CBDAs ability to reduce IL-6 secretion (Figure 6.5A,B), however PPARα anatagonist, GW6471, appeared to potentiate CBDAs effects ($p < 0.0001$ vs vehicle OGD and $p < 0.05$ vs vehicle normoxia; Figure 6.5A).

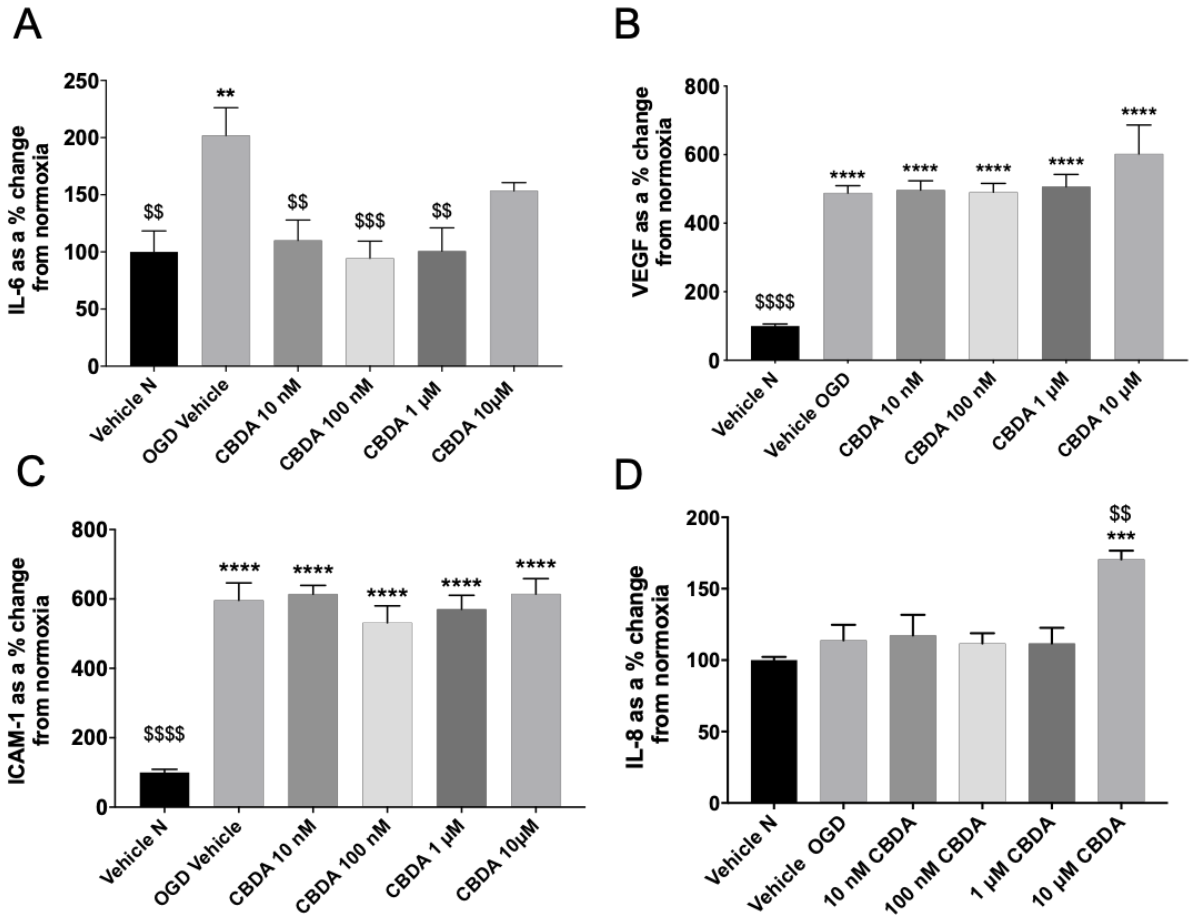

Figure 6.4. *The effects of CBDA on pericyte cytokine signalling.* CBDA attenuated IL-6 secretion (A) 10 µM CBDA increased VEGF secretion (B) but had no effect on ICAM-1 (C). 10 nM-1 µM (C) and increased secretion of IL-8 (D), n=6-10 from 3 experimental repeats. Data are given as means with error bars representing S.E.M. normalised to total protein and expressed as a % change from the normoxia vehicle (vehicle N). Data was analysed using a one-way ANOVA and multiple comparisons were adjusted for by Dunnett's statistical hypothesis test. * denotes a significant difference compared to vehicle normoxia (p<0.05), $ denotes a significant difference to vehicle OGD.

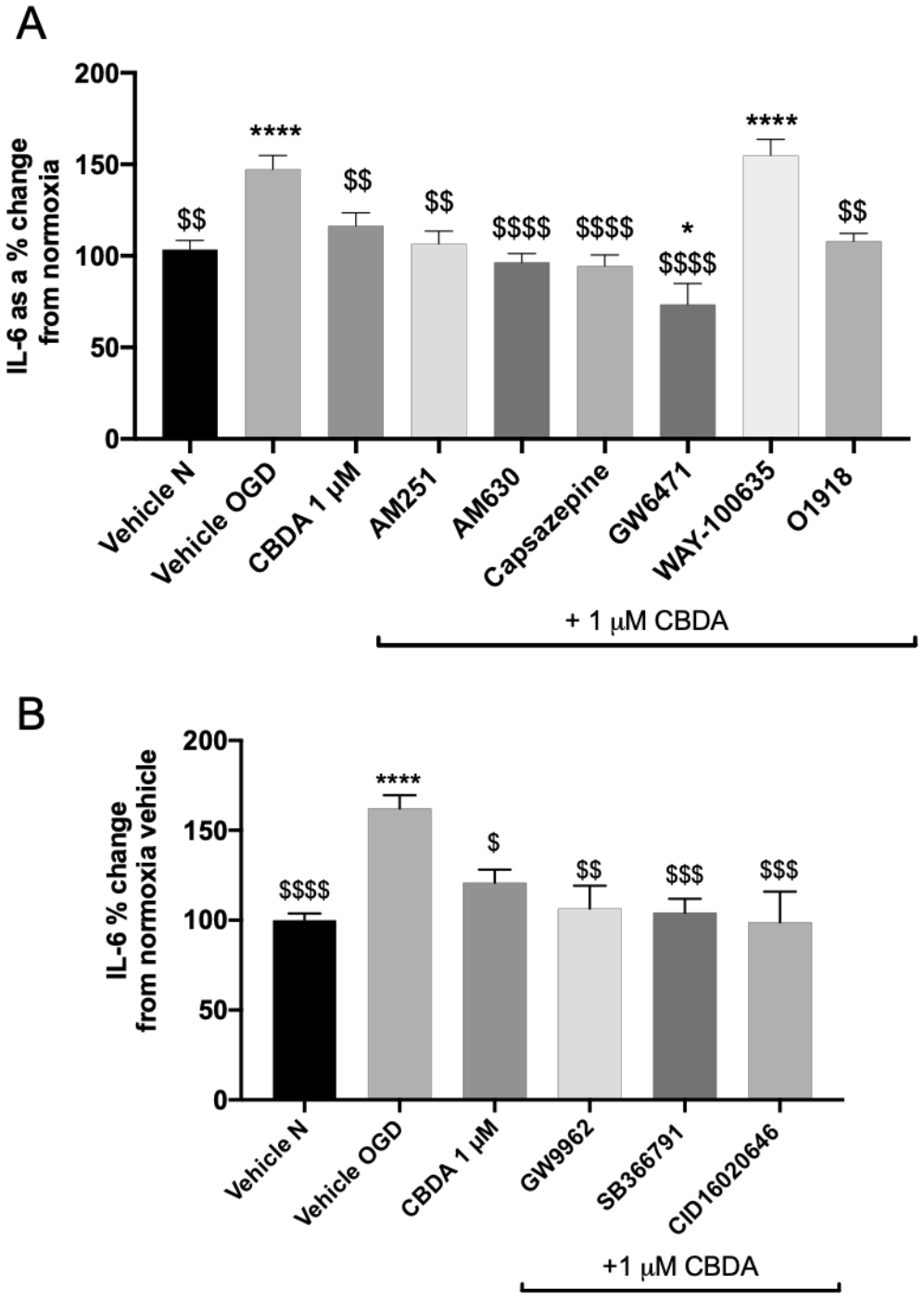

Figure 6.5. *Effect of 1 µM CBDA on IL-6 secretion alongside a range of antagonists*; AM251 (A; 100 nM), AM630 (A; 100 nM), capsazepine (A; 1 µM), GW6471 (A; 100 nM), WAY-100635 (A; 300 nM), O1918 (A; 1 µM), GW9962 (B;100 nM), SB366791 (B; 1 µM) and CID16020646 (B; 1 µM). 5-HT_{1A} antagonist WAY-100,635 blocked the effects of CBDA on IL-6 secretion (A,B) n=6-10 from 3 experimental repeats. Data are given as means with error bars representing S.E.M. normalised to total protein and expressed as a % change from the normoxia vehicle (vehicle N). Data was analysed using a one-way ANOVA and multiple comparisons were adjusted for by Dunnett's statistical hypothesis test. * denotes a significant difference compared to vehicle normoxia (p<0.05), $ denotes a significant difference to vehicle OGD.

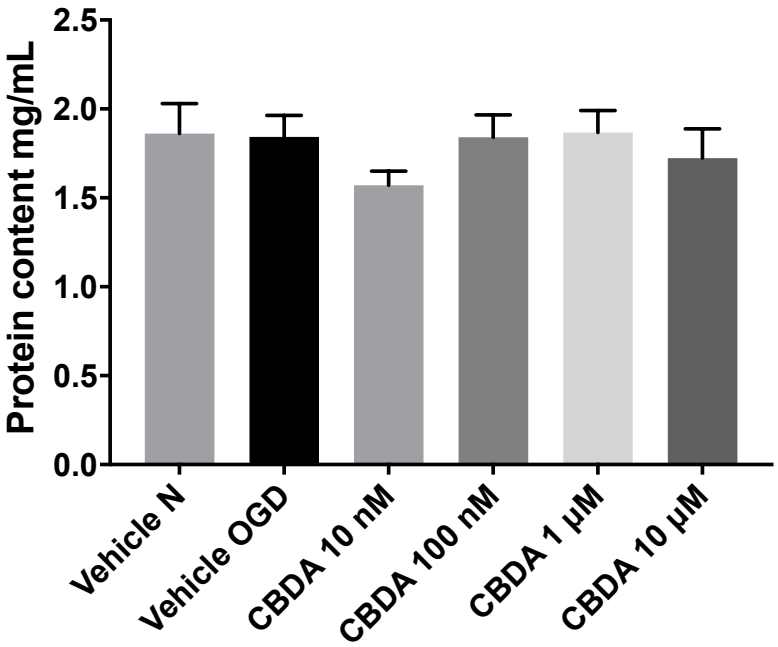

Figure 6.6. *The effects of CBDA on pericyte monoculture protein levels 24 h post 4 h OGD, determined using a BCA.* Data are given as means with error bars representing S.E.M. Data was analysed using a one-way ANOVA and multiple comparisons were adjusted for by Dunnett's statistical hypothesis test, comparing to vehicle normoxia (vehicle N) or vehicle OGD.

6.3.3. Neuronal monocultures

In neuronal monocultures, levels of heat shock proteins (HSP), HSP 27 (ps78) and HSP 90α increased in OGD samples vs normoxia control (p<0.05; Figure 6.7D,H). Levels of HSP27 (total) and HSP60 exhibited a trend for being increased post OGD but this did not reach significance (Figure 6.7A,B,E,F). CBDA (100 nM) significantly decreased HSP27 (ps78) and HSP90 (p>0.05) and exhibited a trend for decreasing HSP27 (total) (Figure 6.7B,D,H).CBDA also displayed a trend to decrease HSP60 at all concentrations (Figure 6.7F). CBD (100 nM, 1 μM) displayed a trend to decreases HSP27 (total) (Figure 6.7A) but not HSP27(ps78). CBD also exhibited a trend to decrease HSP60 at 1 μM (Figure 6.7E) and HSP90α at 10 nM and 1 μM, but this did not reach significance (Figure 6.7G).

Levels of DNA damage markers were not significantly altered 24 h post OGD and were not affected by either CBDA or CBD treatment (Figure 6.8).

Exposing neuronal monocultures to a 4 h OGD protocol significantly increased MCP-1 in 24 h post OGD medium samples and exhibited a trend to increase NADH (Figure 6.9). Neither CBDA nor CBD significantly decreased levels of NADH post OGD (Figure 6.9A,B). CBD (100 nM, 1 μM) and CBDA (1 μM) significantly reduced levels of MCP-1 in 24 h medium samples (p>0.05; Figure 6.9C,D).

Whilst there was a trend for neuronal protein levels to decrease post OGD, this did not reach significance nor were protein levels significantly affected by CBD or CBDA (Figure 6.10).

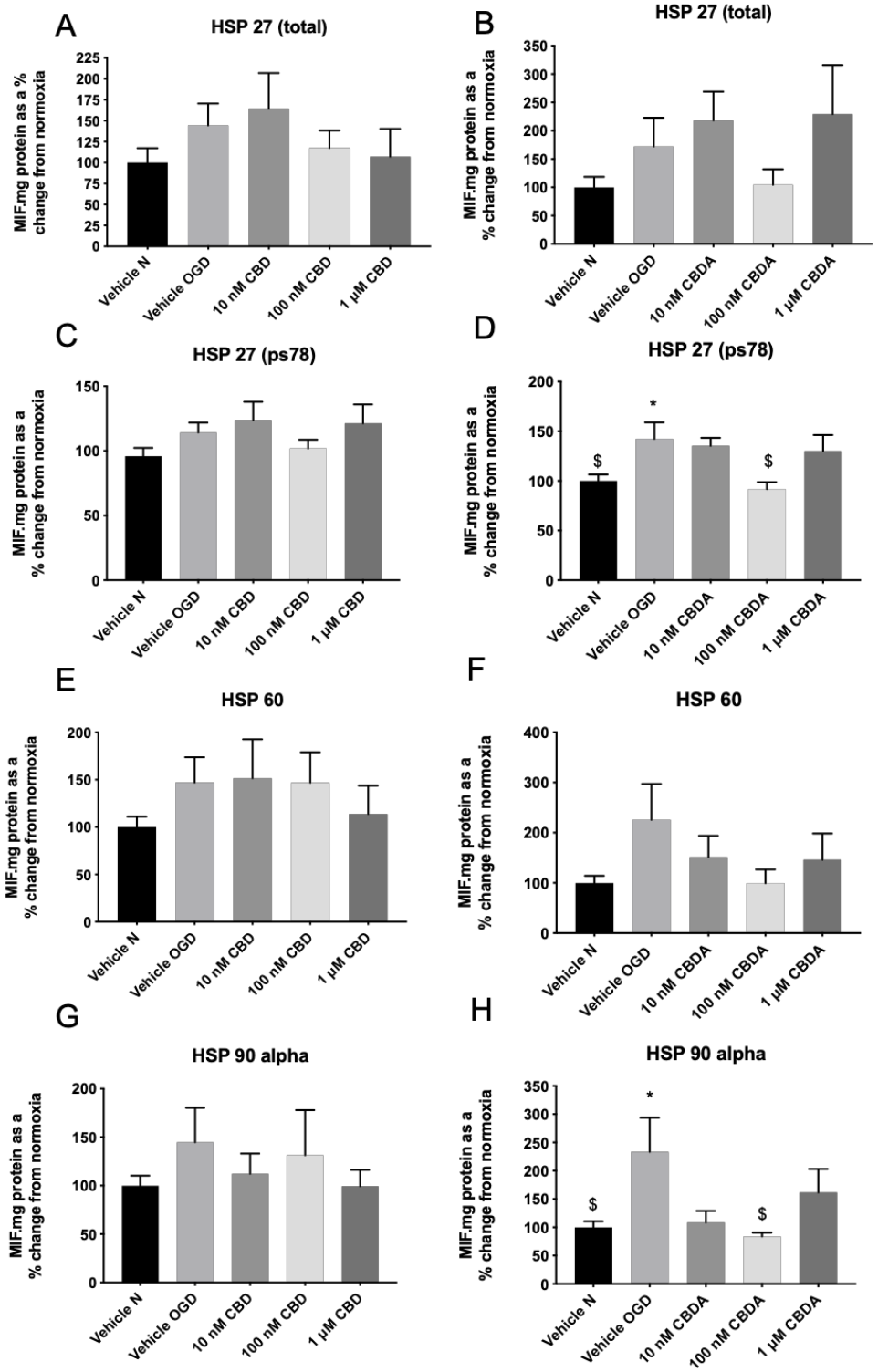

Figure 6.7. *The effects of CBDA and CBD on levels of heat shock proteins in neuronal monocultures post OGD.* A-J A multiplex HSP protein assay (Milliplex™, 48-615MAG, EMD Millipore) was used to detect changes in HSP levels (HSP-27 total, HSP 27, HSP 60, HSP 70 and HSP 90 respectively) in 24 h samples following treatment with CBDA and CBD (10 nM-1 µM) following a 4 h OGD protocol (n=5-6). Data are given as means with error bars representing S.E.M. normalised to total protein and expressed as a % change from the normoxia vehicle (vehicle N). Data was analysed using a one-way ANOVA and multiple comparisons were adjusted for by Dunnett's statistical hypothesis test. * denotes a significant difference compared to vehicle normoxia ($p<0.05$), $ denotes a significant difference to vehicle OGD.

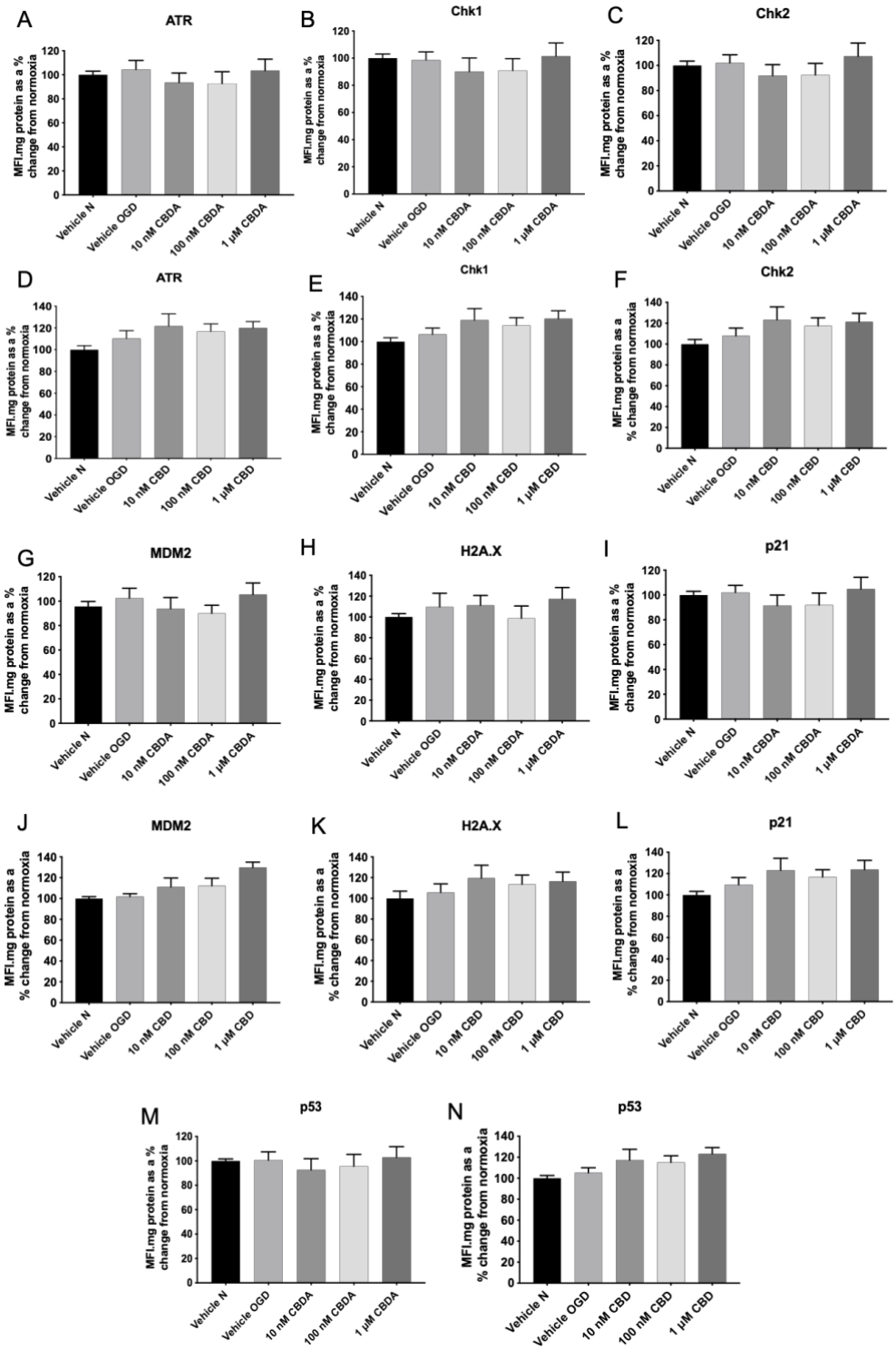

Figure 6.8. *The effects of CBDA and CBD (10 nM-1 µM) on DNA damage proteins in neuronal monocultures post OGD*. A multiplex DNA damage protein assay (Milliplex™, 48-615MAG, EMD Millipore) was used to detect changes in levels of ATR (Total), Chk1 (Ser345), Chk2 (Thr68), H2A.X (Ser139), MDM2 (total), p21 (Total), p53 (Ser15) respectively in 24 h medium samples following treatment with CBDA and CBD and an 4 h OGD protocol (A-N). Data are given as means with error bars representing S.E.M. (n=6 from 2 experimental repeats). Data was analysed by One-way ANOVA. Multiple comparisons were adjusted for by Dunnett's statistical hypothesis test.

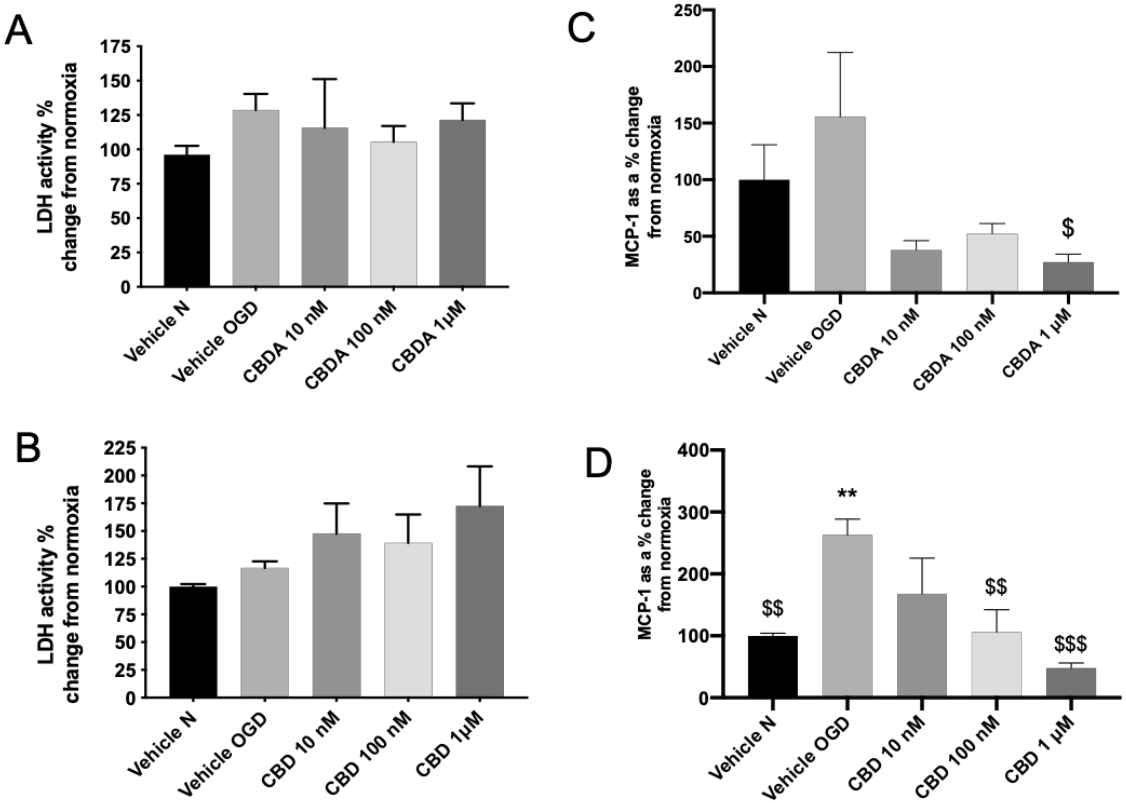

Figure 6.9. *The effects of CBDA and CBD (10 nM-1 µM) on neuronal monocultures subjected to a 4 h OGD protocol.* Following treatment with CBDA and CBD an LDH assay was used to detect changes in NADH (A,B) and a multiplex cytokine panel was used to detect changes in MCP-1 (C,D) in 24 h medium samples following treatment with CBDA and CBD. Data are given as means with error bars representing S.E.M. (n=4 from 1 experimental repeat). Data were analysed by one-way ANOVA. Multiple comparisons were adjusted for by Dunnett's statistical hypothesis test. * denotes a significant difference compared to vehicle normoxia (Vehicle N) (p<0.05), $ denotes a significant difference to vehicle OGD.

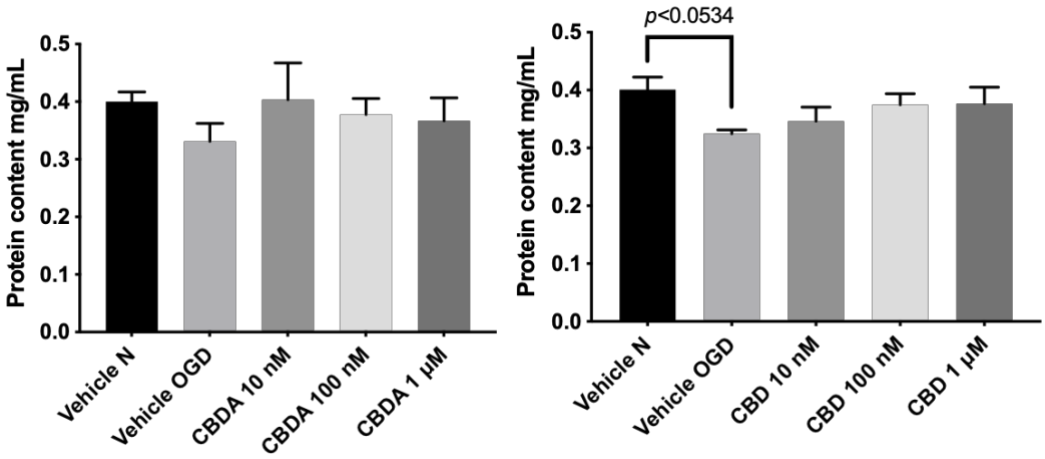

Figure 6.10. *The effects of CBDA on neuronal monoculture protein levels 24 h post 4 h OGD, determined using a BCA.* Data are given as means with error bars representing S.E.M. Data was analysed using a one-way ANOVA and multiple comparisons were adjusted for by Dunnett's statistical hypothesis test, comparing to vehicle normoxia (vehicle N) or vehicle OGD.

6.4 Supplemental information

	Baseline (0h)		post OGD (4h)		24 h post OGD		48 h post OGD		72 h post OGD	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Vehicle CBDA	39.67	3.46	28.56	4.56	29.68	9.48	25.85	4.99	22.03	8.31
CBDA 300 nM	41.72	3.45	32.01	8.20	32.76	8.22	27.53	5.04	26.32	6.93
CBDA 1 µM	44.88	6.46	35.04	7.95	35.60	13.77	35.52	6.04	28.00	8.76
CBDA 3 µM	34.25	7.90	35.00	5.45	34.72	11.72	33.23	5.78	25.11	5.57
Vehicle CBD	38.19	8.22	26.66	7.19	23.97	6.40	26.88	3.31	18.82	5.29
300 nM CBD	44.15	4.89	26.69	9.06	22.68	9.76	53.76	2.40	34.72	3.98
CBD 1 µM	36.49	5.43	28.56	5.59	30.15	6.34	56.93	4.43	43.49	5.38
CBD 3 µM	33.32	3.51	28.65	11.70	27.16	12.10	53.95	5.21	39.57	7.22

Table V: Raw average transepithelial resistance (TEER) values measured in ohms per cm^2 obtained from experiments depicted in Figure 6.1.

VEGF (pg.mL)	Vehicle normoxia	Vehicle OGD	CBDA 10 nM	CBDA 100 nM	CBDA 1 uM	CBDA 10 uM
Mean	118.87	553.46	483.29	471.65	414.39	443.12
SD	40.14	150.30	97.01	130.54	104.27	73.32

Table VI: Raw data from VEGF ELISA of pericyte cell culture medium. Note that these values are taken as an average from experimental repeats with cells at different passages and have not been normalised to total protein.

IL-6 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBDA 10 nM	CBDA 100 nM	CBDA 1 uM	CBDA 10 uM
Mean	121.53	225.47	249.61	201.46	208.37	188.32
SD	43.75	57.84	131.13	101.97	105.88	57.92

Table VII: Raw data from IL-6 ELISA of pericyte cell culture medium. Note that these values are taken as an average from experimental repeats with cells at different passages and have not been normalised to total protein.

ICAM-1 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBDA 10 nM	CBDA 100 nM	CBDA 1 uM	CBDA 10 uM
Mean	75.94	452.93	466.26	403.67	433.33	466.47
SD	15.44	92.86	47.14	90.00	73.94	82.16

Table VIII: Raw data from ICAM-1 ELISA of pericyte cell culture medium. Note that these values are taken as an average from experimental repeats with cells at different passages and have not been normalised to total protein.

IL-8 (pg.mL)	Vehicle normoxia	Vehicle OGD	CBDA 10 nM	CBDA 100 nM	CBDA 1 uM	CBDA 10 uM
Mean	1705.32	2039.62	1877.85	1925.97	1909.49	1822.18
SD	158.84	448.01	291.84	452.47	547.47	239.34

Table IX: Raw data from IL-8 ELISA of pericyte cell culture medium. Note that these values are taken as an average from experimental repeats with cells at different passages and have not been normalised to total protein.

6.5 Discussion

BBB breakdown is one of the major consequences of ischaemic stroke and is accompanied with unwanted infiltration of noxious substances and peripheral immune cell into the brain, which perpetuates neuronal injury (Reviewd in W. Zhang *et al.*, 2020). Pathological processes that mediate BBB disruption post ischaemia have been found to be positively modulated by cannabidiol, including oxidative stress, mitochondrial dysfunction, inflammation, DNA and cellular damage (Iuvone *et al.*, 2004; Ryan *et al.*, 2009; Pazos *et al.*, 2013; Ceprián *et al.*, 2017; Sun *et al.*, 2017). In this study we demonstrate that like CBD, CBDA decreases BBB permeability when given prior to OGD. We also found CBDA displays anti-inflammatory effects in pericytes and exhibits neuroprotective properties in our BBB model and in neuronal monocultures. Our data add to the limited knowledge on the biological properties of CBDA and further investigation is needed into the neuroprotective properties of this compound, particularly in ischaemic stroke.

Pericytes are pivotal in the regulation, maintenance and integrity of the BBB and pericyte loss has drastic consequences to the BBB (Nakagawa *et al.*, 2007; Daneman *et al.*, 2010). During ischaemia-reperfusion (IR), pericytes secrete a range of pro-inflammatory mediators including IL-6, IL-8, ICAM-1 and VCAM-1 (Balabanov, Beaumont and Dore-Duffy, 1999; Pieper, Pieloch and Galla, 2013; Rustenhoven *et al.*, 2016). Specifically, elevations in ICAM-1, VCAM-1 and IL-8 post ischaemia are known to aid the adhesion and diapedesis of leukocytes across the endothelial barrier (Stanimirovic *et al.*, 1997). VEGF also has a significant role in post stroke injury and recovery, with mixed conclusions on its role at the BBB. Interestingly, Zechariah and colleagues (2013) found that VEGF treatment prior to MCA occlusion reduced infarct volume and when pre-treated for 21 days VEGF promoted pericyte coverage of endothelial cells. To our knowledge this is the first study to demonstrate pre-treatment with CBDA attenuated levels of IL-6 in pericytes post OGD, which was blocked by 5-HT_{1A} receptor antagonist WAY-100635. This supports data from other studies showing

CBDA acts as a 5-HT_{1A} receptor agonist (Bolognini *et al.*, 2013; E. M. Rock and Parker, 2017), but contrast to findings from a recent study which showed CBDA increased IL-6 and PPARγ protein levels in mouse prefrontal cortex (Alegre-Zurano, Martín-Sánchez and Valverde, 2020). CBD has been shown to attenuate VEGF secretion in experimental diabetes (El-Remessy *et al.*, 2006) and in a BBB model subjected to OGD (Hind, England and O'Sullivan, 2016). However, in HBMEC monocultures under normal conditions, CBD was found to increase VEGF which was inhibited by PPARγ antagonist GW9662 (Hind, England and O'Sullivan, 2016). Interestingly, CBDA (10 μM) appeared to increase VEGF levels post OGD in pericytes, but this was not tested in our BBB model nor did we determine CBDAs mechanism of action. Rajesh *et al.*, (2007) found that CBD decreased levels of ICAM-1 and VCAM-1 in endothelial cells under high glucose conditions and Hind and colleagues (2016) found CBD decreased levels of VCAM-1 in normoxic conditions, an effect also blocked by PPARγ antagonist GW9662. Unlike CBD, in our model CBDA had little effect on ICAM-1 levels and did not decrease VEGF at any concentration tested. These data suggest that whilst CBDA and CBD share some pharmacological targets, CBDA does not modulate levels of the same proteins in pericytes under OGD conditions in the same manner as CBD in endothelial cells. Thus, it will be important for future studies to assess differences between CBD and CBDA on inflammatory cytokine secretion in multiple cell types and to fully elucidate CBDAs mechanisms of action.

Preserving BBB integrity is vital to protect salvageable neuronal tissue and enhance the probability of positive neurological outcomes post ischaemia. In the present study CBDA (3 μM) reduced BBB permeability and prevented neuronal cell death in our BBB model post OGD. These findings build upon previous data from our group which showed that 10 μM CBD was effective at decreasing BBB permeability in a co-culture BBB model, mediated through 5-HT_{1A} and PPARγ (Hind et al., 2016). Recently Mori *et al.*, (2017) found that CBD improved cognitive function and preserved the number of nissel stained neurons in mice subjected to bilateral common carotid artery occlusion (BCCAO). In addition, CBD reduced glial activation in the hippocampus, decreasing the

number of Iba-1 and GFAP positive cells. Future studies should assess whether CBDA can attenuate glial activation and other inflammatory markers, as well as investigate whether CBDA can modulate BBB permeability *in vivo*.

HSPs are typically referred to as chaperone proteins, helping to stabilise existing proteins and prevent their degradation. Some HSPs are expressed constitutively, but many are induced as a result of cellular stress, for example during hypoxia or neurological disease (Latchman, 2005; Luo *et al.*, 2010). Our data support that from an early study by Wagstaff and colleagues (1996) which found elevations in HSP70, HSP27 and HSP60 mRNA 8 h and 24 h post middle cerebral artery occlusion (MCAO) in rats. Similarly, HSP70 and HSP90 were overexpressed in myocardial ischaemia-reperfusion injury (Nishizawa *et al.*, 1996), which was later found to be triggered by elevations in reactive oxygen species (Nishizawa *et al.*, 1999). Recently, Zhang *et al.*, (2020) found that decreases in HSP90 expression reduced infarct volume, attenuated neuronal apoptosis, and reduced levels of pro-inflammatory cytokines IL-6, TNF-α and IL-1β in a murine model of cerebral ischaemia reperfusion injury. On the other hand, HSP27 is considered to be protective against cellular stresses, namely thermal and ischaemic stress (Latchman, 2005; Stetler *et al.*, 2008). Endothelial specific HSP27 overexpression was also able to attenuate I/R induced BBB disruption, but not the severity of the initial insult (Shi *et al.*, 2017). We found that both CBD and CBDA modulated levels of several HSPs in neuronal lysates 24 h post OGD, with 100 nM CBDA significantly decreasing levels of HSP27 (total) and HSP90α. However, at the lowest and highest concentrations tested, CBDA appeared to increase HSP27, while still decreasing HSP90. CBD also showed a trend for decreasing HSP60 and HSP90 at the highest concentration tested, which is contrast with a study conducted by Scott and colleagues (2015) showing CBD 10 µM enhanced the expression of HSP40,60, 70 and 90 in glioma cell lines, suggesting that CBD appears to exhibit different effects depending on the pathology being investigated. Thus, it will be important for future studies to establish whether CBD or CBDA attenuate HSP levels in *in vivo* models of ischaemia and whether this is protective. The antioxidant properties of phytocannabinoids have been well

documented (Hampson *et al.*, 1998; Borges *et al.*, 2013; Schubert *et al.*, 2019) and may be partly responsible for meditating the effects of CBD and CBDA on levels of heat shock proteins, as oxidative stress is a known regulator of HSP expression. Moreover, as modulation in HSP expression has been linked to alterations in pro-inflammatory mediators, this could be another route in which phytocannabinoids mediate their protective effects and warrants further investigation.

The role of DNA damage and BBB disruption post ischaemia is not fully understood, however emerging evidence has shown that vascular endothelial cells and astrocytes are targets for oxidative DNA damage. Interestingly we did not see any significant changes in DNA damage proteins in neuronal monoculture lysates 24 h post OGD and neither CBD nor CBDA significantly affected the levels of these proteins. This may be due to the DNA damage response being activated earlier as irreversible damage has been detected within 20 minutes of ischaemia (Ordy *et al.*, 1993).

Overall conclusions

In this study we have shown that CBDA is able to decrease BBB permeability when given before an OGD, in a four-cell contact model consisting of HBMECs, astrocytes, pericytes and neurons. This was achieved at a lower concentration (3 µM) compared to CBD (10 µM) in a previous study (Hind, England and O'Sullivan, 2016). CBDA also decreased the secretion of IL-6 after a 4 h OGD protocol in pericyte monocultures and attenuated heat shock proteins in neuronal monocultures. To our knowledge, no study has assessed the effects of CBDA on BBB permeability and its ability to act as a neuroprotectant in a stroke model. We acknowledge that we have only looked at a select number of biological markers associated with IR injury and future studies should assess whether CBDA can modulate other aspects of post stroke injury, particularly in glial cells, as well as fully establish CBDAs mechanism of action. Overall, these pilot data suggest that like CBD, CBDA is protective in an *in vitro* model of ischaemic stroke.

Author Contributions

S.O.S and N.S designed the experiments which were carried out by N.S. N.S wrote the manuscript with input from S.O.S and T.J.E. T.J.E and S.O.S supervised the project.

References

ABBOTT NJ (2002) Astrocyte-endothelial interactions and blood-brain barrier permeability. *Journal of Anatomy* 200(6): 629–638. DOI: 10.1046/j.1469-7580.2002.00064.x.

ABBOTT NJ, PATABENDIGE AAK, DOLMAN DEM, et al. (2010) Structure and function of the blood-brain barrier. *Neurobiology of Disease* 37(1). Elsevier Inc.: 13–25. DOI: 10.1016/j.nbd.2009.07.030.

ALEGRE-ZURANO L, MARTÍN-SÁNCHEZ A AND VALVERDE O (2020) Behavioural and molecular effects of cannabidiolic acid in mice. *Life Sciences* 259. DOI: 10.1016/j.lfs.2020.118271.

ALVAREZ FJ, LAFUENTE H, REY-SANTANO MC, et al. (2008) Neuroprotective Effects of the Nonpsychoactive Cannabinoid Cannabidiol in Hypoxic-Ischemic Newborn Piglets. 64(6): 653–658.

ANDERSON LL, LOW IK, BANISTER SD, et al. (2019) Pharmacokinetics of Phytocannabinoid Acids and Anticonvulsant Effect of Cannabidiolic Acid in a Mouse Model of Dravet Syndrome. *Journal of Natural Products* 82(11): 3047–3055. DOI: 10.1021/acs.jnatprod.9b00600.

BALABANOV R, BEAUMONT T AND DORE-DUFFY P (1999) Role of central nervous system microvascular pericytes in activation of antigen-primed splenic T-lymphocytes. *Journal of Neuroscience Research* 55(5): 578–587. DOI: 10.1002/(SICI)1097-4547(19990301)55:5<578::AID-JNR5>3.0.CO;2-E.

BELL RD, WINKLER EA, SAGARE AP, et al. (2010) Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. *Neuron*. DOI: 10.1016/j.neuron.2010.09.043.

BOLOGNINI D, ROCK EM, CLUNY NL, et al. (2013) Cannabidiolic acid prevents vomiting in Suncus murinus and nausea-induced behaviour in rats by enhancing 5-HT1A receptor activation. *British Journal of Pharmacology* 168(6): 1456–1470. DOI: 10.1111/bph.12043.

BORGES RS, BATISTA J, VIANA RB, et al. (2013) Understanding the molecular aspects of tetrahydrocannabinol and cannabidiol as antioxidants. *Molecules* 18(10): 12663–12674. DOI: 10.3390/molecules181012663.

CASTILLO A, TOLÓN MR, FERNÁNDEZ-RUIZ J, et al. (2010) The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB2 and adenosine receptors. *Neurobiology of Disease* 37(2): 434–440. DOI: 10.1016/j.nbd.2009.10.023.

CEPRIÁN M, JIMÉNEZ-SÁNCHEZ L, VARGAS C, et al. (2017) Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. *Neuropharmacology* 116: 151–159. DOI:

10.1016/j.neuropharm.2016.12.017.
DANEMAN R, ZHOU L, KEBEDE AA, et al. (2010) Pericytes are required for bloodĝ€"brain barrier integrity during embryogenesis. *Nature* 468(7323): 562–566. DOI: 10.1038/nature09513.
DE PETROCELLIS L, VELLANI V, SCHIANO-MORIELLO A, et al. (2008) Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *Journal of Pharmacology and Experimental Therapeutics* 325(3): 1007–15. DOI: 10.1124/jpet.107.134809.
EL-REMESSY AB, AL-SHABRAWEY M, KHALIFA Y, et al. (2006) Neuroprotective and blood-retinal barrier-preserving effects of cannabidiol in experimental diabetes. *The American journal of pathology* 168(1): 235–44. DOI: 10.2353/ajpath.2006.050500.
ESPOSITO G, SCUDERI C, SAVANI C, et al. (2007) Cannabidiol in vivo blunts β-amyloid induced neuroinflammation by suppressing IL-1β and iNOS expression. *British Journal of Pharmacology* 151(8): 1272–1279. DOI: 10.1038/sj.bjp.0707337.
FORMATO M, CRESCENTE G, SCOGNAMIGLIO M, et al. (2020) (–)-Cannabidiolic Acid, a Still Overlooked Bioactive Compound: An Introductory Review and Preliminary Research. *Molecules (Basel, Switzerland)*. DOI: 10.3390/molecules25112638.
HAMPSON AJ, GRIMALDI M, AXELROD J, et al. (1998) Cannabidiol and (-)Δ9-tetrahydrocannabinol are neuroprotective antioxidants. *Proceedings of the National Academy of Sciences of the United States of America* 95: 8268–73. DOI: 10.1073/pnas.95.14.8268.
HIND WH, TUFARELLI C, NEOPHYTOU M, et al. (2015) Endocannabinoids modulate human blood-brain barrier permeability in vitro. *British Journal of Pharmacology* 172(12): 3015–3027. DOI: 10.1111/bph.13106.
HIND WH, ENGLAND TJ AND O'SULLIVAN SE (2016) Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARγ and 5-HT1A receptors. *British Journal of Pharmacology* 173(5): 815–825. DOI: 10.1111/bph.13368.
IADECOLA C (2017) The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron*. DOI: 10.1016/j.neuron.2017.07.030.
IUVONE T, ESPOSITO G, ESPOSITO R, et al. (2004) Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on β-amyloid-induced toxicity in PC12 cells. *Journal of Neurochemistry* 89(1): 134–141. DOI: 10.1111/j.1471-4159.2003.02327.x.
LAFUENTE H, ALVAREZ FJ, PAZOS MR, et al. (2011) Cannabidiol Reduces Brain Damage and Improves Functional Recovery After Acute Hypoxia-Ischemia in Newborn Pigs. 70(3): 272–277.
LATCHMAN DS (2005) Hsp27 and cell survival in neurones. *International Journal of Hyperthermia* 21(5): 393–402. DOI: 10.1080/02656730400023664.
LUO H, ROSSI E, SAUBAMEA B, et al. (2019) Cannabidiol Increases Proliferation, Migration, Tubulogenesis, and Integrity of Human Brain Endothelial Cells through TRPV2 Activation. *Molecular Pharmaceutics* 16(3): 1312–1326. DOI: 10.1021/acs.molpharmaceut.8b01252.

LUO W, SUN W, TALDONE T, et al. (2010) Heat shock protein 90 in neurodegenerative diseases. *Molecular Neurodegeneration*. DOI: 10.1186/1750-1326-5-24.
MCCONNELL HL, KERSCH CN, WOLTJER RL, et al. (2017) The translational significance of the neurovascular unit. *Journal of Biological Chemistry*. DOI: 10.1074/jbc.R116.760215.
MECHA M, FELIÚ A, IÑIGO PM, et al. (2013) Cannabidiol provides long-lasting protection against the deleterious effects of inflammation in a viral model of multiple sclerosis: A role for A2A receptors. *Neurobiology of Disease* 59: 141–150. DOI: 10.1016/j.nbd.2013.06.016.
MINNERUP J, SUTHERLAND BA, BUCHAN AM, et al. (2012) Neuroprotection for stroke: Current status and future perspectives. *International Journal of Molecular Sciences* 13(9): 11753–11772. DOI: 10.3390/ijms130911753.
MORI MA, MEYER E, SOARES LM, et al. (2017) Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 75: 94–105. DOI: 10.1016/j.pnpbp.2016.11.005.
NADAREISHVILI Z, SIMPKINS AN, HITOMI E, et al. (2019) Post-stroke blood-brain barrier disruption and poor functional outcome in patients receiving thrombolytic therapy. *Cerebrovascular Diseases* 47(3–4): 135–142. DOI: 10.1159/000499666.
NAKAGAWA S, DELI MA, NAKAO S, et al. (2007) Pericytes from brain microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. *Cellular and Molecular Neurobiology* 27(6): 687–694. DOI: 10.1007/s10571-007-9195-4.
NIPPERT AR, BIESECKER KR AND NEWMAN EA (2018) Mechanisms Mediating Functional Hyperemia in the Brain. *Neuroscientist* 24(1): 73–83. DOI: 10.1177/1073858417703033.
NISHIZAWA J, NAKAI A, HIGASHI T, et al. (1996) Reperfusion causes significant activation of heat shock transcription factor 1 in ischemic rat heart. *Circulation* 94(9): 2185–2192. DOI: 10.1161/01.CIR.94.9.2185.
NISHIZAWA J, NAKAI A, MATSUDA K, et al. (1999) Reactive oxygen species play an important role in the activation of heat shock factor 1 in ischemic-reperfused heart. *Circulation* 99(7): 934–941. DOI: 10.1161/01.CIR.99.7.934.
ORDY JM, WENGENACK TM, BIALOBOK P, et al. (1993) Selective vulnerability and early progression of hippocampal CA1 pyramidal cell degeneration and GFAP-positive astrocyte reactivity in the rat four-vessel occlusion model of transient global ischemia. *Experimental Neurology* 119(1): 128–139. DOI: 10.1006/exnr.1993.1014.
PAZOS MR, MOHAMMED N, LAFUENTE H, et al. (2013) Mechanisms of cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: Role of 5HT1A and CB2 receptors. *Neuropharmacology* 71: 282–91. DOI: 10.1016/j.neuropharm.2013.03.027.
PIEPER C, PIELOCH P AND GALLA HJ (2013) Pericytes support neutrophil transmigration via interleukin-8 across a porcine co-culture model of the blood-brain barrier. *Brain Research* 1524. Elsevier: 1–11. DOI: 10.1016/j.brainres.2013.05.047.
RAJESH M, MUKHOPADHYAY P, BÁTKAI S, et al. (2007) Cannabidiol attenuates high

glucose-induced endothelial cell inflammatory response and barrier disruption. *American journal of physiology. Heart and circulatory physiology* 293(1): H610-9. DOI: 10.1152/ajpheart.00236.2007.

ROCK E. M. AND PARKER LA (2017) The Role of 5-HT1A Receptor, and Nausea and Vomiting Relief by Cannabidiol (CBD), Cannabidiolic Acid (CBDA), and Cannabigerol (CBG). In: *Handbook of Cannabis and Related Pathologies: Biology, Pharmacology, Diagnosis, and Treatment*, pp. 703–712. DOI: 10.1016/B978-0-12-800756-3.00083-1.

ROCK EM, LIMEBEER CL AND PARKER LA (2018) Effect of cannabidiolic acid and Δ9-tetrahydrocannabinol on carrageenan-induced hyperalgesia and edema in a rodent model of inflammatory pain. *Psychopharmacology* 235(11): 3259–3271. DOI: 10.1007/s00213-018-5034-1.

RUIZ-VALDEPEÑAS L, MARTÍNEZ-ORGADO JA, BENITO C, et al. (2011) Cannabidiol reduces lipopolysaccharide-induced vascular changes and inflammation in the mouse brain: An intravital microscopy study. *Journal of Neuroinflammation* 8. DOI: 10.1186/1742-2094-8-5.

RUSSO EB AND MARCU J (2017) Cannabis Pharmacology: The Usual Suspects and a Few Promising Leads. *Advances in Pharmacology* 80: 67–134. DOI: 10.1016/bs.apha.2017.03.004.

RUSTENHOVEN J, AALDERINK M, SCOTTER EL, ET AL. (2016) TGF-beta1 regulates human brain pericyte inflammatory processes involved in neurovasculature function. *Journal of Neuroinflammation* 13(1). DOI: 10.1186/s12974-016-0503-0.

RYAN D, DRYSDALE AJ, LAFOURCADE C, et al. (2009) Cannabidiol targets mitochondria to regulate intracellular ca2+ levels. *Journal of Neuroscience* 29(7): 2053–2063. DOI: 10.1523/JNEUROSCI.4212-08.2009.

SCHUBERT D, KEPCHIA D, LIANG Z, et al. (2019) Efficacy of Cannabinoids in a Pre-Clinical Drug-Screening Platform for Alzheimer's Disease. *Molecular Neurobiology* 56(11): 7719–7730. DOI: 10.1007/s12035-019-1637-8.

SCOTT KA, DENNIS JL, DALGLEISH AG, et al. (2015) Inhibiting heat shock proteins can potentiate the cytotoxic effect of cannabidiol in human glioma cells. *Anticancer Research*.

SHI Y, JIANG X, ZHANG L, et al. (2017) Endothelium-targeted overexpression of heat shock protein 27 ameliorates blood-brain barrier disruption after ischemic brain injury. *Proceedings of the National Academy of Sciences of the United States of America* 114(7): E1243–E1252. DOI: 10.1073/pnas.1621174114.

STANIMIROVIC DB, WONG J, SHAPIRO A, et al. (1997) Increase in Surface Expression of ICAM-1, VCAM-1 and E-Selectin in Human Cerebromicrovascular Endothelial Cells Subjected to Ischemia-Like Insults. *Acta Neurochirurgica, Supplement* 1997(70): 12–16. DOI: 10.1007/978-3-7091-6837-0_4.

STETLER RA, CAO G, GAO Y, et al. (2008) Hsp27 protects against ischemic brain injury via attenuation of a novel stress-response cascade upstream of mitochondrial cell death signaling. *Journal of Neuroscience* 28(49): 13038–13055. DOI: 10.1523/JNEUROSCI.4407-08.2008.

STONE NL, MURPHY AJ, ENGLAND TJ, et al. (2020) A systematic review of minor phytocannabinoids with promising neuroprotective potential. *British Journal of*

Pharmacology 177(19): 4330–4352. DOI: 10.1111/bph.15185.
SUN S, HU F, WU J, et al. (2017) Cannabidiol attenuates OGD/R-induced damage by enhancing mitochondrial bioenergetics and modulating glucose metabolism via pentose-phosphate pathway in hippocampal neurons. *Redox Biology* 11: 577–585. DOI: 10.1016/j.redox.2016.12.029.
TAKEDA S, MISAWA K, YAMAMOTO I, et al. (2008) Cannabidiolic acid as a selective cyclooxygenase-2 inhibitory component in cannabis. *Drug Metabolism and Disposition* 36(9): 1917–1921. DOI: 10.1124/dmd.108.020909.
WAGSTAFF MJD, COLLAÇO-MORAES Y, ASPEY BS, et al. (1996) Focal cerebral ischaemia increases the levels of several classes of heat shock proteins and their corresponding mRNAs. *Molecular Brain Research* 42(2): 236–244. DOI: 10.1016/S0169-328X(96)00127-1.
ZECHARIAH A, ELALI A, DOEPPNER TR, et al. (2013) Vascular endothelial growth factor promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra. *Stroke* 44(6): 1690–1697. DOI: 10.1161/STROKEAHA.111.000240.
ZHANG E, CHEN Q, WANG J, et al. (2020) Protective role of microRNA-27a upregulation and HSP90 silencing against cerebral ischemia-reperfusion injury in rats by activating PI3K/AKT/mTOR signaling pathway. *International Immunopharmacology* 86. DOI: 10.1016/j.intimp.2020.106635.
ZHANG W, ZHU L, AN C, et al. (2020) The blood brain barrier in cerebral ischemic injury – Disruption and repair. *Brain Hemorrhages* 1(1). Elsevier B.V.: 34–53. DOI: 10.1016/j.hest.2019.12.004.

5. An Analysis of Endocannabinoid Concentrations and Mood Following Singing and Exercise in Healthy Volunteers.

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Endocannabinoids are implicated in a range of physiological and psychological processes including mood, cognition, appetite, and energy consumption. In addition, they help to regulate neuronal plasticity and synaptic function, and have been found to offer protection in cerebral ischaemia. This study enhanced my knowledge of the role of endocannabinoids in health and disease, as well as having relevance to my PhD project.

Statement of author contributions: NS and SM carried out cardiovascular measurements, surveys and blood processing. SOS processed the data and NS drafted the manuscript with input from all authors.

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An Analysis of Endocannabinoid Concentrations and Mood Following Singing and Exercise in Healthy Volunteers

*Nicole L. Stone[1], Sophie A. Millar[1], Philip J. J. Herrod[1], David A. Barrett[2], Catharine A. Ortori[2], Valerie A. Mellon[3] and Saoirse E. O'Sullivan[1]**

[1]Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Nottingham, United Kingdom, [2]Centre for Analytical Bioscience, Advanced Materials and Healthcare Division, School of Pharmacy, University of Nottingham, Nottingham, United Kingdom, [3]BBC Studios "Trust Me I'm a Doctor", BBC Scotland, Glasgow, United Kingdom

The euphoric feeling described after running is, at least in part, due to increased circulating endocannabinoids (eCBs). eCBs are lipid signaling molecules involved in reward, appetite, mood, memory and neuroprotection. The aim of this study was to investigate whether activities other than running can increase circulating eCBs. Nine healthy female volunteers (mean 61 years) were recruited from a local choir. Circulating eCBs, haemodynamics, mood and hunger ratings were measured before and immediately after 30 min of dance, reading, singing or cycling in a fasted state. Singing increased plasma levels of anandamide (AEA) by 42% ($P < 0.05$), palmitoylethanolamine (PEA) by 53% ($P < 0.01$) and oleoylethanolamine (OEA) by 34% ($P < 0.05$) and improved positive mood and emotions ($P < 0.01$), without affecting hunger scores. Dancing did not affect eCB levels or hunger ratings, but decreased negative mood and emotions ($P < 0.01$). Cycling increased OEA levels by 26% ($P < 0.05$) and tended to decrease how hungry volunteers felt, without affecting mood. Reading increased OEA levels by 28% ($P < 0.01$) and increased the desire to eat. Plasma AEA levels were positively correlated with how full participants felt ($P < 0.05$). Plasma OEA levels were positively correlated with positive mood and emotions ($P < 0.01$). All three ethanolamines were positively correlated with heart rate (HR; $P < 0.0001$). These data suggest that activities other than running can increase plasma eCBs associated with changes in mood or appetite. Increases in eCBs may underlie the rewarding and pleasurable effects of singing and exercise and ultimately some of the long-term beneficial effects on mental health, cognition and memory.

Keywords: endocannabinoids, anandamide, human, clinical, high, mood, singing and dancing

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Edited by:
Walter Adriani,
Istituto Superiore di Sanità (ISS), Italy

Reviewed by:
Maria Morena,
University of Calgary, Canada
Antonia Manduca,
Aix-Marseille Université, France

***Correspondence:**
Saoirse E. O'Sullivan
mbzso@nottingham.ac.uk

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; BBB, blood brain barrier; BDNF, brain derived neurotrophic factor; eCBs, endocannabinoids; LC-ESI-MS-MS, electrospray ionization liquid chromatography/mass spectrometry; OEA, oleoylethanolamine; PEA, palmitoylethanolamine.

INTRODUCTION

The classic "runners high" is described as the sense of well-being and mood elevation associated with moderate distance running. Other typical indicators include a decrease in anxious thinking (anxiolytic), positive emotions/mood (euphoria), reduced pain perception (analgesia) and a feeling of increased endurance (Sparling et al., 2003; Dietrich and McDaniel, 2004; Tsatsoulis and Fountoulakis, 2006; Raichlen et al., 2012). To explain these positive effects post-exercise, attention was directed to the endocannabinoid (eCB) system, and a number of groups have found significant correlations between physical activity, mood and elevated eCB levels. Interestingly, the majority of studies have only observed significant rises in the first identified eCB, anandamide (AEA; Sparling et al., 2003; Heyman et al., 2012; Raichlen et al., 2013), whilst the reports analyzing 2-arachidonylglycerol (2-AG) levels post-exercise have been less clear. Heyman et al. (2012) reported no change in circulating 2-AG levels after cycling. However, Brellenthin et al. (2017) showed that 2-AG and AEA were significantly increased in a study analyzing the effects of preferred (self-selected) and prescribed (70%–75% of max) exercise on eCB levels and mood.

The eCB system consists of the cannabinoid receptors 1 and 2 (CB_1 and CB_2), eCBs, and the enzymes that are responsible for their synthesis and breakdown (Devane et al., 1992; Mechoulam et al., 1995; De Petrocellis and Di Marzo, 2009). AEA and 2-AG are partial agonists of CB_1 and CB_2, whilst palmitoylethanolamine (PEA) and oleoylethanolamine (OEA) share similar synthesis and degradation mechanisms, without directly interacting with these receptors themselves (Hansen et al., 2000; Okamoto et al., 2004). Instead, these molecules interact with other receptors, primarily peroxisome proliferator-activated receptor alpha (PPAR-α) and transient receptor potential cation channel subfamily V member 1 (TRPV1; Ahern, 2003; Fu et al., 2003; Lo Verme et al., 2005a,b; Karwad et al., 2017). eCB signaling mediates a number of physiological and psychological processes including emotional responses, cognition, memory, motor behavior, feeding and energy consumption (Berger and Motl, 2000; Cota et al., 2003; Cota, 2007; Brellenthin et al., 2017). Studies have also established prominent roles of eCB signaling in the positive reinforcement in reward driven activities such as masturbation, arousal, binge-eating and social interactions in humans (Klein et al., 2012; Monteleone et al., 2015, 2017; Fuss et al., 2017).

Singing and dancing, especially as a group activity, are associated with positive mood in humans (Zajenkowski et al., 2015; Pearce et al., 2016; Tarr et al., 2016; Schladt et al., 2017). However, little has been studied to elucidate how these positive emotions are mediated. Recently, Hahn et al. (2017) studied the relationship between song practice and the eCB system in European starlings. They found a significant positive correlation between conditioned place preference (a measure of reward and song production), the number of songs a bird produced and the expression of CB_1 in areas of the brain associated with reward, primarily the ventral tegmental area. Therefore suggesting a role for eCB signaling in singing and reward (Hahn et al., 2017; Riters et al., 2017). In humans, singing has been studied as a therapy for long-term disorders such as Alzheimer's (to improve cognition, memory and long-term pain), chronic obstructive pulmonary disease, as well as to improve mood in conditions such as anxiety and depression (Reagon et al., 2016; Kang et al., 2017). Similarly, dancing has been explored as a potential therapy for cognitive and emotional dysfunction in conditions such as depression, dementia and Parkinson's. In a systematic review of 11 studies, Kiepe et al. (2012) found that depression and psychological distress were reduced by dance therapy in patients suffering from Parkinson's, diabetes, breast cancer or heart failure. Dance therapy in a group of 60 students also significantly reduced depression over a period of 12 weeks (Akandere and Demir, 2011). To date, no study has assessed singing or dancing and whether they modulate eCB levels in humans and whether that correlates to an improved mood. Given that mood is central in the measure of overall psychological well-being, low intensity activities that can positively modulate mood could be useful therapeutic tools in numerous conditions such as depression, anxiety and stress, especially if a patient cannot undertake moderate/higher intensity exercise.

The purpose of this study was to investigate whether activities other than running can give you a measurable "high" through changes in circulating eCBs levels. We examined activities that are associated with euphoria (singing and dancing) as well as an exercise regime other than running (cycling), with the hypothesis that these activities would increase plasma eCB levels. Quiet reading was used as a control condition. A secondary objective of this study was to establish whether there was a link between cycling, dancing, singing and reading with regards to mood and hunger ratings.

MATERIALS AND METHODS

Participants

All procedures were approved by the University of Nottingham Faculty of Health Sciences ethics committee, and were carried out according to the declaration of Helsinki. Nine healthy post-menopausal female volunteers (age range 55–67, mean 61 years) were recruited from a local choir as people who enjoyed singing and exercise. The inclusion criteria were that volunteers be non-smokers, in good physical health, accustomed to singing in a group, and also enjoy exercise. Volunteers gave written informed consent prior to participation. The medications taken included antihypertensives (n = 2), antacids (n = 2), antidepressants/anti-anxiety medication (n = 2), HRT (n = 1), and an inhaler for asthma (n = 1).

Subjects arrived fasted (feeding affects plasma eCB levels; Monteleone et al., 2012) with no consumption of caffeine and this was verbally confirmed on arrival at the study facility. Participants were also asked to refrain from any exercise prior to attending the laboratory. Volunteers were unaware of the activity they were to perform on a given day until all baseline measurements were made to avoid any anticipatory effects.

Study Days

Subjects came to the test site on four occasions between 8 am and 10 am in loose fitting sportswear. Each day,

individuals were asked to complete two questionnaires before and after completing the activity. A visual analog scale (VAS) questionnaire was used to assess how hungry subjects were feeling on a scale of 1–10, using the questions "how hungry do you feel?", "how full are you?", "how much food could you eat?" and "how strong is your desire to eat?". A positive and negative affect schedule (PANAS) questionnaire was used to assess subject's mood before and after each activity using the following scoring system: 1 = "very slightly or not at all," 2 = "a little," 2 = "moderately," 4 = "quite a bit" and 5 = "extremely;" Watson et al., 1988; Crawford and Henry, 2004). Positive affect score was calculated by adding the positive emotional responses and the negative affect score was calculated based on the addition of the negative affect scores.

Blood pressure was measured by oscillometry with the participant seated according to the British Hypertension Society guidelines, and heart rate (HR) was taken prior to commencing the activity and immediately after finishing the activity. Blood pressure and HR measurements were taken as the average over three (pre-activity) or 2 (post-activity) measurements. Blood draws (approximately 5 mL) were taken before commencing the activity and immediately after finishing the activity into pre-chilled K2-EDTA (Ethylenediaminetetraacetic acid) tubes and immediately placed on ice. After collection, blood was centrifuged at 2,000 *g* for 15 min at 4°C, plasma was removed and aliquoted, and immediately snap frozen in liquid nitrogen. Samples were stored at −80°C until subsequent analysis.

After the baseline measurements were made, volunteers were informed of the activity they were to perform. On day 1, volunteers did a supervised 30 min dance exercise class preceded by a 5 min warm up, to upbeat music. On day 2, volunteers did 30 min of supervised quiet reading (of boiler and dishwasher catalogs) to classical music. On day 3, volunteers for 30 min choir practice led by their choral director. On day 4, volunteers did a 30 min spin class (cycling) with a qualified instructor from the University of Nottingham Sports facility, with a 5 min warm up to upbeat music. All activities were performed as a group.

eCB Quantification

eCB analysis was based on the method as described by Richardson et al. (2007). Samples were thawed and 100 μL of internal standard of 2-AG-d8 (10 μM) and 15 μL of AEA-d8 (28 μM) were added to a 0.4 mL aliquot of each plasma sample or blank sample (0.4 mL water) vortexed briefly. Ethyl acetate:hexane (9:1 v/v) was added to each sample and subjected for a slow vortex (10 min) and centrifuged for 13,000 rpm, 10 min, 4°C. The supernatants were transferred and the procedure was repeated. Supernatants were then pooled and evaporated using a centrifugal evaporator. Prior to analysis, each sample extract was reconstituted in 100 μL of acetonitrile (ACN). Standards for AEA, 2-AG, PEA, OEA, *N*-(2-hydroxyethyl)-9Z-octadecenamide), arachidonyl ethanolamide-d8 (*N*-(2-Hydroxyethyl)-5Z, 8Z, 11Z, 14Z-eicosatetraenamide-d8, AEA-d8) and 2-arachidonyl glycerol-d8 (2-AG-d8, (5Z, 8Z, 11Z, 14Z)-5, 8, 11, 14-Eicosatetraenoic acid-d8, 2-hydroxy-1-(hydroxymethyl)ethyl ester-d8) were purchased from Cambridge BioSciences, UK.

Following sample preparation, 10 μL of final sample extract was analyzed using liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS/MS). The HPLC system used was a modular Shimadzu Vp series LC (Shimadzu, Milton Keynes, UK), with pumps, chilled autosampler and column oven. The HPLC column used was an ACE 3 C8 (100 × 2.1 mm, 3 mm) with guard column. The mobile phase A was water with 1 g/L ammonium acetate and 0.1% formic acid and mobile phase B was ACN with 1 g/L ammonium acetate and 0.1% formic acid pre-dissolved in 10% H_2O. The flow rate was 300 μL/min. The MS system used was a SCIEX 4000 QTrap triple quadrupole mass spectrometer (Sciex, Warrington, UK) operated in electrospray positive multiple reaction monitoring mode. Quantification was performed using Analyst 1.6 and identification of each compound in plasma was confirmed by LC retention times of each standard and precursor and product ion *m/z* ratios. The peak area of each analyte is compared to a known amount of standard to determine the amount of target compound present.

2-AG in these samples were below the limit of quantification with our methodology in the plasma samples and the data has not been reported.

Statistical Analysis

Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity. Correlations between plasma eCBs levels and hunger ratings, cardiovascular parameters or mood pre and post-activities were analyzed by linear regression. A quality control check was performed by a separate researcher on data entry.

RESULTS

All but one of the participants completed the study in full; one participant was unable to finish the cycling activity and did not have a final blood draw or complete the surveys. Thus nine participates were in the final comparison, except for the cycling activity where $n = 8$.

Haemodynamics

Thirty minutes of dancing significantly increased HR ($t_{(8)} = 4.894$, $P < 0.01$, **Figure 1A**) and decreased diastolic blood pressure ($t_{(8)} = 2.764$, $P < 0.05$, **Figure 1I**). Thirty minutes of reading caused a small but significant reduction in HR ($t_{(8)} = 3.736$, $P < 0.01$, **Figure 1B**). Thirty minutes of singing increased systolic blood pressure ($t_{(8)} = 5.66$, $P < 0.001$, **Figure 1G**). Thirty minutes of cycling significantly increased HR ($t_{(7)} = 7.314$, $P < 0.001$, **Figure 1D**) and decreased diastolic blood pressure ($t_{(7)} = 2.567$, $P < 0.05$, **Figure 1L**).

Hunger Scores

The only significant change in hunger and appetite scores were observed after 30 min of reading when volunteers reported a significantly higher desire to eat (**Figure 2N**). Volunteers tended to have reduced hunger ratings after dancing, singing and cycling (**Figure 2**), but this only reached near significance for

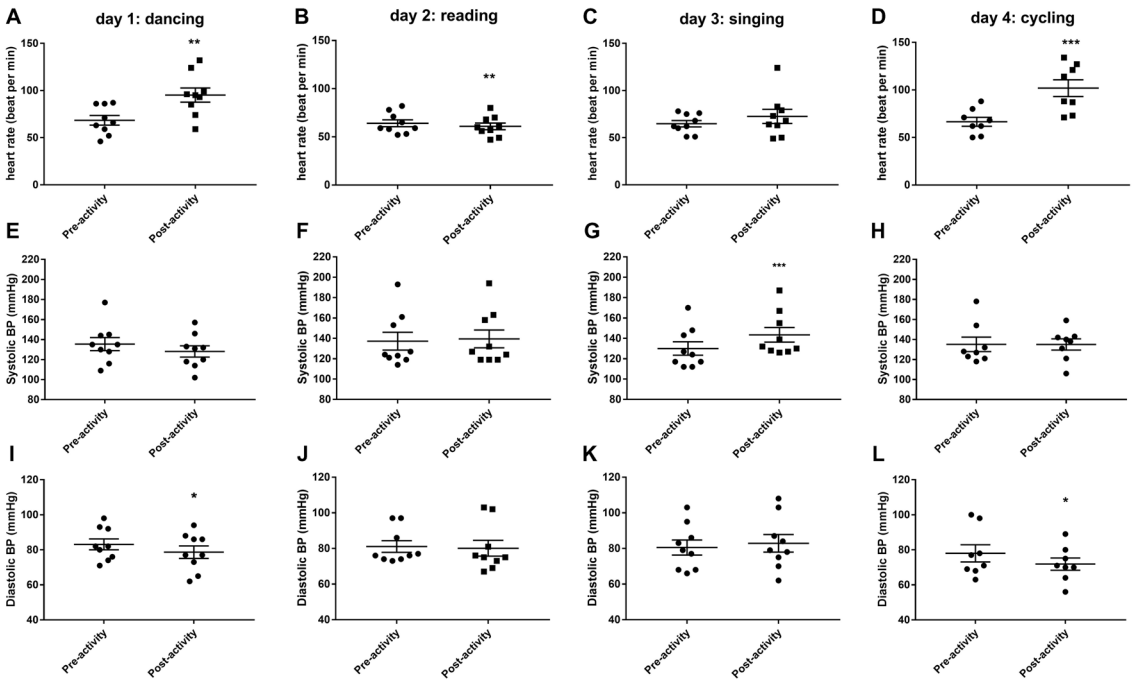

FIGURE 1 | Changes in cardiovascular parameters (heart rate, HR; **A–D**), systolic blood pressure **(E–H)** and diastolic blood pressure **(I–L)** before and after 30 min activity (dancing, reading, singing or cycling) in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$).

the question "how hungry do you feel?" immediate post-cycling ($t_{(7)} = 2.348$, $P = 0.0512$, **Figure 2D**).

Mood Scores

Dancing decreased negative mood and emotions ($t_{(8)} = 3.671$, $P < 0.01$, **Figure 3E**), while reading decreased positive mood and emotions ($t_{(8)} = 5.751$, $P < 0.001$, **Figure 3B**). Only singing was found to significantly improve positive mood and emotions ($t_{(8)} = 4.951$, $P < 0.01$, **Figure 3C**) and also tended to decrease negative mood and emotions (eight out of nine volunteers reported a lower NAS post-singing, **Figure 3E**). Cycling has no effect on mood ratings.

Plasma Levels of Endocannabinoids

Dancing had no effect on circulating levels of eCBs measured immediately the activity, although there was a trend for AEA and OEA levels to be increased (**Figures 4A,E**). Thirty minutes of reading significantly increased plasma OEA levels ($t_{(8)} = 4.586$, $P < 0.01$, **Figure 4F**) and tended to increase PEA levels ($t_{(8)} = 2.02$, $P = 0.078$, **Figure 4J**). Singing significantly increased the plasma levels of all eCBs measurable; AEA ($t_{(8)} = 3.049$, $P < 0.05$, **Figure 4C**), OEA ($t_{(8)} = 4.81$, $P < 0.01$, **Figure 4G**) and PEA ($t_{(8)} = 3.319$, $P < 0.05$, **Figure 4K**). OEA levels were also increased after 30 min cycling ($t_{(6)} = 3.594$, $P < 0.05$, **Figure 4H**).

At baseline (before activities started) across all 4 days, there was a significant positive correlation between plasma OEA levels and the rating for "how much food could you eat?" ($r^2 = 0.2226$, $F = 9.16$, $P < 0.01$) and positive mood and emotions ($r^2 = 0.1355$, $F = 5.172$, $P < 0.05$). Resting HR was positively correlated with both plasma AEA ($r^2 = 0.3363$, $F = 16.72$, $P < 0.001$) and PEA ($r^2 = 0.169$, $F = 6.711$, $P < 0.05$) levels.

Across all days and time points (pre- and post-activity), plasma AEA levels were positively correlated with the rating for "how full are you?" ($r^2 = 0.0626$, $F = 4.472$, $P < 0.05$, **Figure 5A**), and plasma OEA levels tended to be positively correlated with the rating for "how much food could you eat?" ($r^2 = 0.0404$, $F = 2.821$, $P = 0.097$, **Figure 5B**) and "how strong is your desire to eat?" ($r^2 = 0.04624$, $F = 3.248$, $P = 0.076$, **Figure 5C**) and with increased ratings for positive mood and emotion ($r^2 = 0.1269$, $F = 9.879$, $P < 0.01$, **Figure 5D**). All three ethanolamines were positively correlated with HR (AEA: $r^2 = 0.4394$, $F = 53.3$, $P < 0.0001$, **Figure 5E**; OEA: $r^2 = 0.2639$, $F = 24.37$, $P < 0.0001$, **Figure 5F** and PEA: $r^2 = 0.2093$, $F = 18$, $P < 0.0001$, **Figure 5G**).

DISCUSSION

It is well reported that running is correlated with mood elevation. These positive effects have been attributed to an evolutionary trait, where positive re-enforcement ultimately led to increased food foraging, survival and subsequent passing of relevant genes to offspring and have recently been attributed, at least in part, to increases in eCBs (Bramble and Lieberman, 2004; Raichlen et al., 2012). Our study aimed to examine whether activities other than running also increase eCBs and enhance mood. We have shown for the first time that singing significantly increases levels of AEA, OEA and PEA in healthy post-menopausal females and

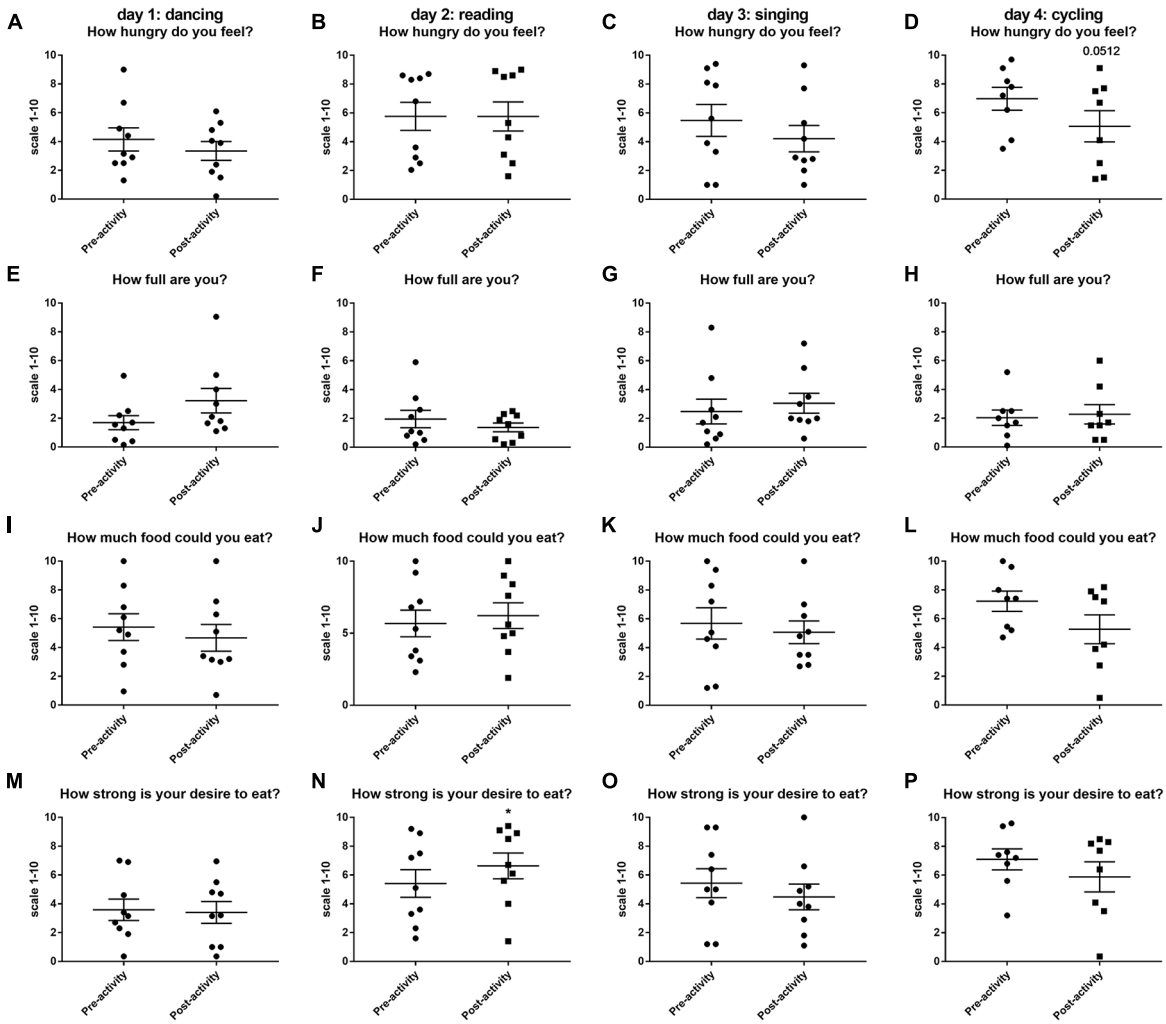

FIGURE 2 | Changes in hunger and appetite scores as assessed using a visual analog scale (VAS) (1–10) before and after 30 min activity (dancing **(A,E,L,M)**, reading **(B,F,J,N)**, singing **(C,G,K,O)** or cycling **(D,H,L,P)** in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (*$P < 0.05$).

enhanced mood. Dancing (on mood) and cycling (on eCBs) also had positive effects in this group. Although singing was the most beneficial activity in this study, this is likely to reflect the fact that the volunteers were recruited from local choirs and already find this an enjoyable activity. These data provide biochemical evidence of an increase in novel signaling messengers known to improve mood, reduce stress and anxiety, enhance memory, protect brain function and reduce pain.

Singing, in particular group singing, has been associated with an increase in positive mood and improved immune function in humans (Kreutz et al., 2004; Schladt et al., 2017). Choir singing also enables social interactions, exhibiting a greater benefit to mood than singing alone (Schladt et al., 2017). Our results also demonstrate that singing increases mood, and also for the first time that singing increasing circulating levels of AEA, OEA and PEA. As AEA is a partial agonist of CB_1 and has full agonist activity at TRPV1, an increase in the levels of AEA post activity could therefore facilitate increases in positive emotions, as well as anxiolytic and analgesic effects (Chapman et al., 2009; Starowicz et al., 2012). Levels of OEA post activity were correlated with a decrease in hunger and desire to eat. This supports previous data that OEA attenuates food consumption and increase lipolysis and energy expenditure (Lo Verme et al., 2005a,b). *In vivo* studies conducted in mice have also suggested beneficial neuroprotective effects of OEA, this protective effect could potentially be translated to humans and warrants further study (Galan-Rodriguez et al., 2009; Zhou et al., 2012; Yang et al., 2015). An abundance of evidence has supported PEA as a potential therapy for neurological and inflammatory disorders, particularly those associated with pain (Costa et al., 2008; Keppel Hesselink, 2012; Esposito and Cuzzocrea, 2013). PEA has also been taken into clinical trials, whereby 600 mg of PEA was shown to be effective in various pain states, without exhibiting any safety issues

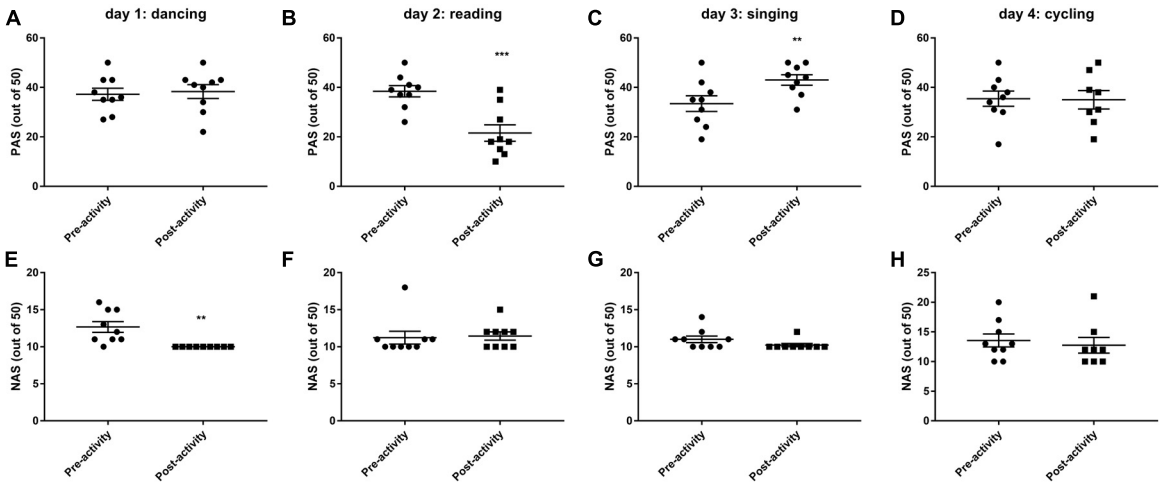

FIGURE 3 | Changes in postive (PAS, **A–D**) and negative (NAS, **E–H**) mood and emotions before and after 30 min activity (dancing, reading, singing or cycling) in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (**$P < 0.01$, ***$P < 0.001$).

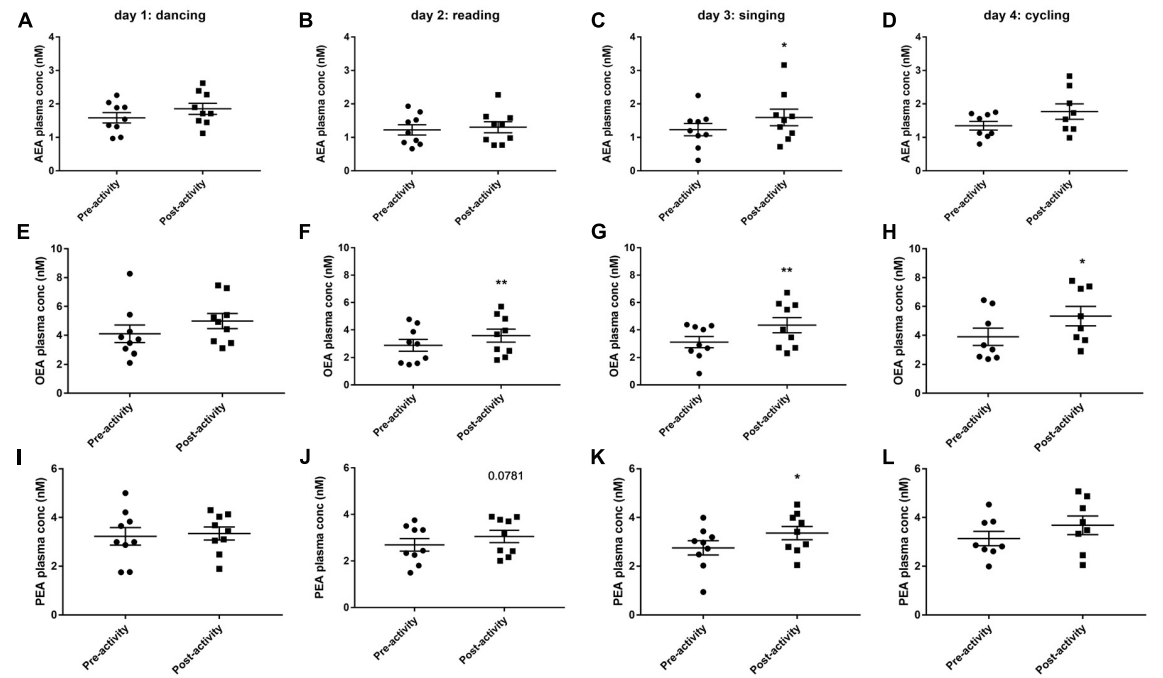

FIGURE 4 | Plasma endocannabinoid levels (AEA, anandamide, **A–D**; OEA, oleoylethanolamine, **E–H**; PEA, palmitoylethanolamine, **I–L**) before and after 30 min activity (dancing, reading, singing or cycling) in nine healthy female volunteers. Data is presented as a scatter plot with mean ± SEM. Data sets were compared by paired Student's *t*-test pre and post-activity (*$P < 0.05$, **$P < 0.01$).

(Hesselink and Hekker, 2012). Therefore, it could be beneficial to increase levels of PEA via activities such as singing, to promote neuroprotection, analgesia and reduce inflammation. It is also important to note that increasing OEA and PEA can indirectly increase AEA responses by the entourage effect by competitive inhibition of AEA degradation by fatty acid amide hydrolase (FAAH; Di Marzo et al., 2001; Costa et al., 2008; Ho et al., 2008). Overall, singing could be a valuable activity in patient populations that suffer with dysfunctions in psychological well-being and struggle to participate in aerobic/moderate intensity exercise.

Cycling resulted in a significant increase in OEA, and in a trend for increases in both AEA and PEA. These changes corresponded with a decrease in participants desire to eat and

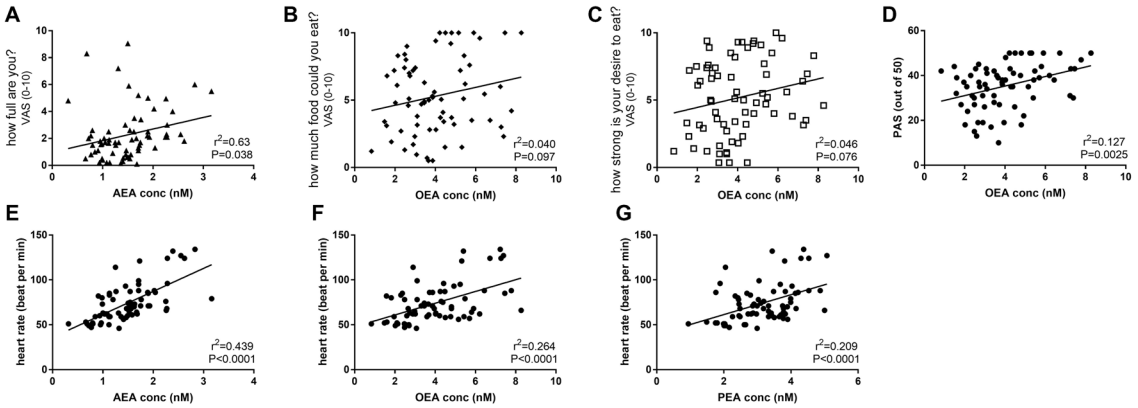

FIGURE 5 | Correlations between plasma eCBs levels and appetite **(A–C)**, mood **(D)** and HR **(E–G)** in healthy female volunteers when measured pre or post-activities and analyzed by linear regression.

how hungry they felt. These data are consistent with results from a previous study where plasma OEA levels were significantly increased after 30 min of cycling in 16 male non-smokers with a mean age of 22.9 years (Cedernaes et al., 2016). Our data did not show that cycling positively affected mood (no increase in PAS or decrease in NAS). Brellenthin et al. (2017) showed that acute aerobic exercise (both prescribed and preferred) resulted in positive mood outcomes in individuals capable of a range of levels of physical activity, as well as showing modulation of the eCB system. Interestingly, the group that undertook their "preferred exercise" had the best effect in reducing anxiety and improving mood. In the present study, singing by participants recruited from a choir support these observations, suggesting that the eCB system is not only responsible for the motivation for exercise (i.e., reward driven), but also the pleasure associated with an activity that an individual enjoys. It would also have been interesting if another group had been included (i.e. not recruited from a choir) to directly assess the concept of preferred vs. prescribed activity and to confirm that carrying out an activity that is "pleasurable" to an individual is an important factor in the psychological benefits of exercise and other related activities. Subjects were also not asked to rate how much they enjoyed each of the activities, this would have been an interesting endpoint to assess to what degree the participant's moods were influenced by how much they liked a particular activity and should be considered in future study. These factors would also have provided further evidence to why individuals in this study failed to experience positive mood changes or significant increases in AEA post cycling; compared with the study by Heyman et al. (2012) where increases in AEA were seen in well-trained cyclists, who presumably enjoy cycling.

Exercise intensity may be another factor explaining the lack of AEA increases in our participants. Brellenthin et al. (2017) showed that the greatest increases in 2-AG and AEA were seen in the higher intensity exercise group. Sparling et al. (2003) also showed significant increases in AEA when participants reached 70%–80% max HR. According to Gulati et al. (2010), maximum HR for women is calculated as 206−(0.88 × age of patient). As the average age of our participants was 61 years, their average maximum HR (max HR) is approximately 154 bpm, meaning their 70%–80% max HR should be 107–123 bpm. Cycling was the only activity that almost reached this (average 102 bpm immediately post exercise) and dancing resulted in an average HR of 95 bpm (immediately post exercise). This could suggest that our activities may not have been intense enough to elicit significant changes in circulating eCBs.

A number of studies have shown that dance is an effective therapy in improving mood (including mild depression), enhancing social interactions, boosting self-confidence, as well as improving physical activity (Akandere and Demir, 2011; Kiepe et al., 2012; Meekums et al., 2015). In one study, dancing caused an increase in plasma serotonin levels and a decrease in negative psychological symptoms in a group of 20 female adolescents with mild depression, compared to 20 control subjects (Jeong et al., 2005). We found post activity that there was a significant decrease in negative emotions following 30 min of dancing. It should be acknowledged that the decrease in negative emotions could also be because this was the activity undertaken on day 1 and participants had higher NAS scores before starting the study. Although there was a trend in increasing levels of AEA and OEA levels post-activity, this did not reach significance. Our results suggest that dancing did not effectively increase eCB levels or improve mood, however this could be because they were unfamiliar with the class, therefore not finding it as enjoyable as singing as this was more familiar to them, or that the class was not at a high enough intensity to produce changes in eCB levels. It should also be noted that our participants were older than those previously studied, and there could be an age-related decline in the eCB response to exercise.

Reading was used as a control activity to assess baseline eCB levels and mood. We found that reading was the only activity that increased participants desire to eat but had little impact on overall fullness or actual hunger and was correlated to increases in OEA post activity. Reading also decreased the ratings for positive mood and emotions. In hindsight, because subjects were unaware of the task, the activity set-up looked

like they were about to take an exam, which may have resulted in unforeseen heightened anxiety levels. Recent studies have implicated the eCB system as a possible mediator of hedonic vs. homeostatic eating response to the consumption of food (as a reward) as well as acute stress and anxiety (Matias et al., 2006; Monteleone et al., 2015, 2017). Dlugos et al. (2012) showed that AEA, PEA and OEA were all increased in serum in response to stress. They also found that higher levels of AEA at baseline, associated with decreased levels of anxiety. Furthermore, a common phenomenon is that typically negative emotions, particularly boredom, stress and depressive emotions increase our desire to eat in order to increase positive emotions (Koball et al., 2012; Yau and Potenza, 2013; Moynihan et al., 2015). These factors could explain the elevated levels of OEA post activity and lower PAS scores.

A limitation of our study is that participants already had very low negative affect scores and high positive affect scores. This suggests that the individuals that took part in the study were generally happy and positive and there was therefore little room for mood to be further improved. It would therefore be interesting in future work to see the effects of these same activities on individuals that exhibit depressive, or anxious behavior in order to see greater differences in negative emotional responses. Intensity of a physical activity has also been shown to influence exercise induced increases in eCB levels. Raichlen et al. (2013) built on previous work showing that eCBs follow a U-shaped curve, with moderate level activity resulting in the biggest increase in eCB levels (Berger and Motl, 2000). This trend in eCB levels is also correlated with mood as the positive emotional state post exercise is not experienced at very low or very high intensities (Berger and Motl, 2000). As all the participants were unfamiliar to the activities they carried out, a lot of their focus would have been on "mastering" the class rather than actually enjoying it in the moment.

It can also not be overlooked that this study only recruited healthy female volunteers. Evidence from animal studies has already shown distinct sexual dimorphism in the eCB system, particularly in CB_1 expression and activation (Reich et al., 2009; Mateos et al., 2011; Dias-Rocha et al., 2018). Limited preliminary evidence from human studies have also shown variations in the eCB system between males and females (Cupini et al., 2006; Hill et al., 2008). Thus future study should look to establish whether the effects observed in this study translate to male participants as well as females.

In conclusion, we found that activities other than running (singing, dancing and cycling) can increase plasma eCB levels. Singing was the only activity to increase plasma levels of AEA and improve positive mood outcomes, suggesting that singing in this group of volunteers was able to produce an endogenous "high." This is interesting as the participants were recruited from a choir, suggesting that the enjoyment of an activity may influence their feeling of reward and the eCB response. This preliminary evidence suggests that activities like singing could be recommended to individuals suffering from mood disorders such as anxiety and depression, as well as a potential therapy for neurological and inflammatory disorders. Future research should consider an individual's preference to a particular activity, as this could be an important factor in influencing the eCB system, as well as being a factor in deciding appropriate therapy.

AUTHOR CONTRIBUTIONS

NS and SO'S wrote the article with contributions from all the other authors. SO'S, SM and NS carried out cardiovascular measurements, surveys and blood processing. SO'S processed the study data and performed the statistical analysis. CO and DB performed the eCB analysis on the plasma samples. PH carried out the blood draws from the subjects. VM developed the study with SO'S.

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REFERENCES

Ahern, G. P. (2003). Activation of TRPV1 by the satiety factor oleoylethanolamide. *J. Biol. Chem.* 278, 30429–30434. doi: 10.1074/jbc.M305051200

Akandere, M., and Demir, B. (2011). The effect of dance over depression. *Coll. Antropol.* 35, 651–656.

Berger, B. G., and Motl, R. W. (2000). Exercise and mood: a selective review and synthesis of research employing the profile of mood states. *J. Appl. Sport Psychol.* 12, 69–92. doi: 10.1080/10413200008404214

Bramble, D. M., and Lieberman, D. E. (2004). Endurance running and the evolution of Homo. *Nature* 432, 345–352. doi: 10.1038/nature03052

Brellenthin, A. G., Crombie, K. M., Hillard, C. J., and Koltyn, K. F. (2017). Endocannabinoid and mood responses to exercise in adults with varying activity levels. *Med. Sci. Sports Exerc.* 49, 1688–1696. doi: 10.1249/MSS.0000000000001276

Cedernaes, J., Fanelli, F., Fazzini, A., Pagotto, U., Broman, J.-E., Vogel, H., et al. (2016). Sleep restriction alters plasma endocannabinoids concentrations before but not after exercise in humans. *Psychoneuroendocrinology* 74, 258–268. doi: 10.1016/j.psyneuen.2016.09.014

Chapman, V., Kendall, D., and Sagar, D. R. (2009). "Cannabinoid receptor mediated analgesia: novel targets for chronic pain states," in *Synaptic Plasticity in Pain*, ed. M. Malcangio (New York, NY: Springer), 337–351.

Costa, B., Comelli, F., Bettoni, I., Colleoni, M., and Giagnoni, G. (2008). The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine model of neuropathic pain: involvement of CB1, TRPV1 and PPARγ receptors and neurotrophic factors. *Pain* 139, 541–550. doi: 10.1016/j.pain.2008.06.003

Cota, D. (2007). CB1 receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. *Diabetes Metab. Res. Rev.* 23, 507–517. doi: 10.1002/dmrr.764

Cota, D., Marsicano, G., Tschöp, M., Grübler, Y., Flachskamm, C., Schubert, M., et al. (2003). The endogenous cennabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J. Clin. Invest.* 112, 423–431. doi: 10.1172/jci200317725

Crawford, J. R., and Henry, J. D. (2004). The positive and negative affect schedule (PANAS): construct validity, measurement propertiesand normative data in a large non-clinical sample. *Br. J. Clin. Psychol.* 43, 245–265. doi: 10.1348/0144665031752934

Cupini, L. M., Bari, M., Battista, N., Argirò, G., Finazzi-Agrò, A., Calabresi, P., et al. (2006). Biochemical changes in endocannabinoid system are expressed in platelets of female but not male migraineurs. *Cephalalgia* 26, 277–281. doi: 10.1111/j.1468-2982.2005.01031.x

De Petrocellis, L., and Di Marzo, V. (2009). An introduction to the endocannabinoid system: from the early to the latest concepts. *Best Pract. Res. Clin. Endocrinol. Metab.* 23, 1–15. doi: 10.1016/j.beem.2008.10.013

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., et al. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946–1949. doi: 10.1126/science.1470919

Di Marzo, V., Melck, D., Orlando, P., Bisogno, T., Zagoory, O., Bifulco, M., et al. (2001). Palmitoylethanolamide inhibits the expression of fatty acid amide hydrolase and enhances the anti-proliferative effect of anandamide in human breast cancer cells. *Biochem. J.* 358, 249–255. doi: 10.1042/0264-6021:3580249

Dias-Rocha, C. P., Almeida, M. M., Santana, E. M., Costa, J. C. B., Franco, J. G., Pazos-Moura, C. C., et al. (2018). Maternal high-fat diet induces sex-specific endocannabinoid system changes in newborn rats and programs adiposity, energy expenditure and food preference in adulthood. *J. Nutr. Biochem.* 51, 56–68. doi: 10.1016/j.jnutbio.2017.09.019

Dietrich, A., and McDaniel, W. F. (2004). Endocannabinoids and exercise. *Br. J. Sports Med.* 38, 536–541. doi: 10.1136/bjsm.2004.011718

Dlugos, A., Childs, E., Stuhr, K. L., Hillard, C. J., and de Wit, H. (2012). Acute stress increases circulating anandamide and other N-acylethanolamines in healthy humans. *Neuropsychopharmacology* 37, 2416–2427. doi: 10.1038/npp.2012.100

Esposito, E., and Cuzzocrea, S. (2013). Palmitoylethanolamide in homeostatic and traumatic central nervous system injuries. *CNS Neurol. Disord. Drug Targets* 12, 55–61. doi: 10.2174/1871527311312010010

Fu, J., Gaetani, S., Oveisi, F., Lo Verme, J., Serrano, A., Rodríguez De Fonseca, F., et al. (2003). Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. *Nature* 425, 90–93. doi: 10.1038/nature01921

Fuss, J., Bindila, L., Wiedemann, K., Auer, M. K., Briken, P., and Biedermann, S. V. (2017). Masturbation to orgasm stimulates the release of the endocannabinoid 2-arachidonoylglycerol in humans. *J. Sex. Med.* 14, 1372–1379. doi: 10.1016/j.jsxm.2017.09.016

Galan-Rodriguez, B., Suarez, J., Gonzalez-Aparicio, R., Bermudez-Silva, F. J., Maldonado, R., Robledo, P., et al. (2009). Oleoylethanolamide exerts partial and dose-dependent neuroprotection of substantia nigra dopamine neurons. *Neuropharmacology* 56, 653–664. doi: 10.1016/j.neuropharm.2008.11.006

Gulati, M., Shaw, L. J., Thisted, R. A., Black, H. R., Bairey Merz, C. N., and Arnsdorf, M. F. (2010). Heart rate response to exercise stress testing in asymptomatic women: the St. James women take heart project. *Circulation* 122, 130–137. doi: 10.1161/CIRCULATIONAHA.110.939249

Hahn, A. H., Merullo, D. P., Spool, J. A., Angyal, C. S., Stevenson, S. A., and Riters, L. V. (2017). Song-associated reward correlates with endocannabinoid-related gene expression in male European starlings (*Sturnus vulgaris*). *Neuroscience* 346, 255–266. doi: 10.1016/j.neuroscience.2017.01.028

Hansen, H. S., Moesgaard, B., Hansen, H. H., Petersen, G. (2000). *N*-Acylethanolamines and precursor phospholipids—relation to cell injury. *Chem. Phys. Lipids* 108, 135–150. doi: 10.1016/s0009-3084(00)00192-4

Hesselink, J. M., and Hekker, T. A. (2012). Therapeutic utility of palmitoylethanolamide in the treatment of neuropathic pain associated with various pathological conditions: a case series. *J. Pain Res.* 5, 437–442. doi: 10.2147/jpr.s32143

Heyman, E., Gamelin, F. X., Goekint, M., Piscitelli, F., Roelands, B., Leclair, E., et al. (2012). Intense exercise increases circulating endocannabinoid and BDNF levels in humans—possible implications for reward and depression. *Psychoneuroendocrinology* 37, 844–851. doi: 10.1016/j.psyneuen.2011.09.017

Hill, M. N., Miller, G. E., Ho, W. S., Gorzalka, B. B., and Hillard, C. J. (2008). Serum endocannabinoid content is altered in females with depressive disorders: a preliminary report. *Pharmacopsychiatry* 41, 48–53. doi: 10.1055/s-2007-993211

Ho, W.-SV., Barrett, D. A., and Randall, M. D. (2008). 'Entourage' effects of *N*-palmitoylethanolamide and *N*-oleoylethanolamide on vasorelaxation to anandamide occur through TRPV1 receptors. *Br. J. Pharmacol.* 155, 837–846. doi: 10.1038/bjp.2008.324

Jeong, Y.-J., Hong, S.-C., Lee, M. S., Park, M.-C., Kim, Y.-K., and Suh, C.-M. (2005). Dance movement therapy improves emotional responses and modulates neurohormones in adolescents with mild depression. *Int. J. Neurosci.* 115, 1711–1720. doi: 10.1080/00207450590958574

Kang, J., Scholp, A., and Jiang, J. J. (2017). A review of the physiological effects and mechanisms of singing. *J. Voice* 32, 390–395. doi: 10.1016/j.jvoice.2017.07.008

Karwad, M. A., Macpherson, T., Wang, B., Theophilidou, E., Sarmad, S., Barrett, D. A., et al. (2017). Oleoylethanolamine and palmitoylethanolamine modulate intestinal permeability *in vitro* via TRPV1 and PPARα. *FASEB J.* 31, 469–481. doi: 10.1096/fj.201500132

Keppel Hesselink, J. M. (2012). New targets in pain, non-neuronal cells, and the role of palmitoylethanolamide. *Open Pain J.* 5, 12–23. doi: 10.2174/1876386301205010012

Kiepe, M. S., Stöckigt, B., and Keil, T. (2012). Effects of dance therapy and ballroom dances on physical and mental illnesses: a systematic review. *Arts Psychother.* 39, 404–411. doi: 10.1016/j.aip.2012.06.001

Klein, C., Hill, M. N., Chang, S. C., Hillard, C. J., and Gorzalka, B. B. (2012). Circulating endocannabinoid concentrations and sexual arousal in women. *J. Sex. Med.* 9, 1588–1601. doi: 10.1111/j.1743-6109.2012.02708.x

Koball, A. M. Meers, M. R., Storfer-Isser, A., Domoff, S. E., and Musher-Eizenman, D. R. (2012). Eating when bored: revision of the emotional eating scale with a focus on boredom. *Health Psychol.* 31, 521–524. doi: 10.1037/a0025893

Kreutz, G., Bongard, S., Rohrmann, S., Hodapp, V., and Grebe, D. (2004). Effects of choir singing or listening on secretory immunoglobulin A, cortisol, and emotional state. *J. Behav. Med.* 27, 623–635. doi: 10.1007/s10865-004-0006-9

Lo Verme, J., Fu, J., Astarita, G., La Rana, G., Russo, R., Calignano, A., et al. (2005a). The nuclear receptor peroxisome proliferator-activated receptor-α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol. Pharmacol.* 67, 15–19. doi: 10.1124/mol.104.006353

Lo Verme, J., Gaetani, S., Fu, J., Oveisi, F., Burton, K., and Piomelli, D. (2005b). Regulation of food intake by oleoylethanolamide. *Cell. Mol. Life Sci.* 62, 708–716. doi: 10.1007/s00018-004-4494-0

Mateos, B., Borcel, E., Loriga, R., Luesu, W., Bini, V., Llorente, R., et al. (2011). Adolescent exposure to nicotine and/or the cannabinoid agonist CP 55,940 induces gender-dependent long-lasting memory impairments and changes in brain nicotinic and CB1cannabinoid receptors. *J. Psychopharmacol.* 25, 1676–1690. doi: 10.1177/0269881110370503

Matias, I., Gonthier, M.-P., Orlando, P., Martiadis, V., De Petrocellis, L., Cervino, C., et al. (2006). Regulation, function, and dysregulation of endocannabinoids in models of adipose and β-pancreatic cells and in obesity and hyperglycemia. *J. Clin. Endocrinol. Metab.* 91, 3171–3180. doi: 10.1210/jc.2005-2679

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., et al. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50, 83–90. doi: 10.1016/0006-2952(95)00109-d

Meekums, B., Karkou, V., and Nelson, E. A. (2015). Dance movement therapy for depression. *Cochrane Database Syst. Rev.* 2:CD009895. doi: 10.1002/14651858.CD009895

Monteleone, A. M., Di Marzo, V., Aveta, T., Piscitelli, F., Dalle Grave, R., Scognamiglio, P., et al. (2015). Deranged endocannabinoid responses to hedonic eating in underweight and recently weight-restored patients with anorexia nervosa. *Am. J. Clin. Nutr.* 101, 262–269. doi: 10.3945/ajcn.114.096164

Monteleone, A. M., Piscitelli, F., Dalle Grave, R., El Ghoch, M., Di Marzo, V., Maj, M., et al. (2017). Peripheral endocannabinoid responses to hedonic eating in binge-eating disorder. *Nutrients* 9:E1377. doi: 10.3390/nu9121377

Monteleone, P., Piscitelli, F., Scognamiglio, P., Monteleone, A. M., Canestrelli, B., Di Marzo, V., et al. (2012). Hedonic eating is associated with increased

peripheral levels of ghrelin and the endocannabinoid 2-arachidonoyl-glycerol in healthy humans: a pilot study. *J. Clin. Endocrinol. Metab.* 97, E917–E924. doi: 10.1210/jc.2011-3018

Moynihan, A. B., van Tilburg, W. A. P., Igou, E. R., Wisman, A., Donnelly, A. E., and Mulcaire, J. B. (2015). Eaten up by boredom: consuming food to escape awareness of the bored self. *Front. Psychol.* 6:369. doi: 10.3389/fpsyg.2015.00369

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T., and Ueda, N. (2004). Molecular characterization of a phospholipase D generating anandamide and its congeners. *J. Biol. Chem.* 279, 5298–5305. doi: 10.1074/jbc.m306642200

Pearce, E., Launay, J., Machin, A., and Dunbar, R. I. (2016). Is group singing special? Health, well-being and social bonds in community-based adult education classes. *J. Community Appl. Soc. Psychol.* 26, 518–533. doi: 10.1002/casp.2278

Raichlen, D. A., Foster, A. D., Gerdeman, G. L., Seillier, A., and Giuffrida, A. (2012). Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the 'runner's high'. *J. Exp. Biol.* 215, 1331–1336. doi: 10.1242/jeb.063677

Raichlen, D. A., Foster, A. D., Seillier, A., Giuffrida, A., and Gerdeman, G. L. (2013). Exercise-induced endocannabinoid signaling is modulated by intensity. *Eur. J. Appl. Physiol.* 113, 869–875. doi: 10.1007/s00421-012-2495-5

Reagon, C., Gale, N., Enright, S., Mann, M., and van Deursen, R. (2016). A mixed-method systematic review to investigate the effect of group singing on health related quality of life. *Complement. Ther. Med.* 27, 1–11. doi: 10.1016/j.ctim.2016.03.017

Reich, C. G., Taylor, M. E., and McCarthy, M. M. (2009). Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats. *Behav. Brain Res.* 203, 264–269. doi: 10.1016/j.bbr.2009.05.013

Richardson, D., Ortori, C. A., Chapman, V., Kendall, D. A., and Barrett, D. A. (2007). Quantitative profiling of endocannabinoids and related compounds in rat brain using liquid chromatography-tandem electrospray ionization mass spectrometry. *Anal. Biochem.* 360, 216–226. doi: 10.1016/j.ab.2006.10.039

Riters, L. V., Spool, J. A., Merullo, D. P., and Hahn, A. H. (2017). Song practice as a rewarding form of play in songbirds. *Behav. Processes* doi: 10.1016/j.beproc.2017.10.002 [Epub ahead of print].

Schladt, T. M., Nordmann, G. C., Emilius, R., Kudielka, B. M., de Jong, T. R., and Neumann, I. D. (2017). Choir versus solo singing: effects on mood, and salivary oxytocin and cortisol concentrations. *Front. Hum. Neurosci.* 11:430. doi: 10.3389/fnhum.2017.00430

Sparling, P. B., Giuffrida, A., Piomelli, D., Rosskopf, L., and Dietrich, A. (2003). Exercise activates the endocannabinoid system. *Neuroreport* 14, 2209–2211. doi: 10.1097/00001756-200312020-00015

Starowicz, K., Makuch, W., Osikowicz, M., Piscitelli, F., Petrosino, S., Di Marzo, V., et al. (2012). Spinal anandamide produces analgesia in neuropathic rats: possible CB_1- and TRPV1-mediated mechanisms. *Neuropharmacology* 62, 1746–1755. doi: 10.1016/j.neuropharm.2011.11.021

Tarr, B., Launay, J., and Dunbar, R. I. M. (2016). Silent disco: dancing in synchrony leads to elevated pain thresholds and social closeness. *Evol. Hum. Behav.* 37, 343–349. doi: 10.1016/j.evolhumbehav.2016.02.004

Tsatsoulis, A., and Fountoulakis, S. (2006). The protective role of exercise on stress system dysregulation and comorbidities. *Ann. N Y Acad. Sci.* 1083, 196–213. doi: 10.1196/annals.1367.020

Watson, D., Clark, L. A., and Tellegen, A. (1988). Development and validation of brief measures of positive and negative affect: the PANAS scales. *J. Pers. Soc. Psychol.* 54, 1063–1070. doi: 10.1037/0022-3514.54.6.1063

Yang, L.-C., Guo, H., Zhou, H., Suo, D.-Q., Li, W.-J., Zhou, Y., et al. (2015). Chronic oleoylethanolamide treatment improves spatial cognitive deficits through enhancing hippocampal neurogenesis after transient focal cerebral ischemia. *Biochem. Pharmacol.* 94, 270–281. doi: 10.1016/j.bcp.2015.02.012

Yau, Y. H., and Potenza, M. N. (2013). Stress and eating behaviors. *Minerva Endocrinol.* 38, 255–267.

Zajenkowski, M., Jankowski, K. S., and Koata, D. (2015). Let's dance—feel better! Mood changes following dancing in different situations. *Eur. J. Sport Sci.* 15, 640–646. doi: 10.1080/17461391.2014.969324

Zhou, Y., Yang, L., Ma, A., Zhang, X., Li, W., Yang, W., et al. (2012). Orally administered oleoylethanolamide protects mice from focal cerebral ischemic injury by activating peroxisome proliferator-activated receptor α. *Neuropharmacology* 63, 242–249. doi: 10.1016/j.neuropharm.2012.03.008

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8. General Discussion

In neuropathological conditions such as ischaemic stroke, Alzheimer's and Parkinson's, the BBB becomes compromised, exacerbating the detrimental effects these diseases have on CNS homeostasis (Rosenberg, 2012; Zhang et al., 2020). Key studies and comprehensive reviews have shown that CBD and Δ^9-THC display efficacy in a range of neurological disorders, including ischaemic stroke, as well as offering protection at the BBB (Cristino et al., 2020; Hayakawa et al., 2010). However, the neuroprotective properties of minor phytocannabinoids remain to be fully elucidated. In light of the results obtained from a systematic review conducted in Chapter 4 (Stone et al., 2020), we hypothesised that CBG and CBDV would also prove to be protective in ischaemic stroke. Experiments were performed to determine the effects of CBDV and CBG on cells of the NVU subjected to OGD (Chapter 5). CBDA has been found to exhibit similar biological properties to CBD and acts more potently than CBD as an antiemetic via the activation of 5-HT_{1A} (Rock and Parker, 2013). Given these data and that of previous work from our group (Hind et al., 2016), we hypothesised that CBDA may also reduce BBB permeability, perhaps by the same mechanism as CBD. In Chapter 6, experiments were performed to assess CBDAs ability to modulate BBB permeability post OGD. In addition to the central thesis aims, a clinical study was conducted to investigate endocannabinoids and their ability to influence mood and is presented as an additional chapter (Chapter 7). Altogether, the results presented in this thesis add to the growing body of evidence on the anti-inflammatory and neuroprotective properties of phytocannabinoids, as well as the importance of endocannabinoids in exercise and the regulation of mood.

To protect the brain, the BBB must remain impenetrable to harmful agents such as xenobiotics and peripheral immune cells, whilst enabling the selective transport of essential nutrients (Abbott et al., 2006, 2010). In ischaemic stroke, oxidative stress, inflammatory cytokine release and immune cell activation contribute to BBB disruption, which can lead to oedema and haemorrhagic transformation, altogether

exacerbating neuronal injury (reviewed in L and X, 2016). It is worth noting, however, that whilst BBB disruption is generally associated with a worse outcome for patients, it enables the possibility of greater drug penetration into the CNS (Borlongan and Emerich, 2003). Modelling the BBB *in vitro* is important tool to study the crosstalk between cells of the NVU under normal and pathological conditions. Following rigorous model development conducted in Chapter 3, we generated a model which incorporated four primary human cell types that constitute the NVU (Stone et al., 2019), which was based on a previous model developed by our group (Hind, 2014). Results showed that our OGD model was effective to stimulate cellular responses associated with *in vivo* ischaemia-reperfusion injury.

In Chapter 4 we conducted a systematic review to gather all available published data on minor phytocannabinoids (excluding Δ^9-THC and CBD) and their neuroprotective effects. Evidence presented in our review found that CBDV and CBG exhibited anti-inflammatory, antioxidant, anti-excitotoxic and anticonvulsant properties in various models of neurodegeneration (Stone et al., 2020). In agreement with this and supporting our hypothesis, in Chapter 5 we demonstrated that CBDV and CBG exhibited anti-inflammatory effects in astrocytes and HBMECs subjected to OGD. Specifically, both CBG and CBDV attenuated IL-6 secretion in astrocytes and to a lesser extent in HBMECs. These data are consistent with findings in other studies where CBG was found to decrease expression of IL-6 in an *in vivo* model of Huntington's disease (Valdeolivas et al., 2015), while CBDV decreased IL-6 in a mouse model of colitis (Pagano et al., 2019). Interestingly, while CBDV and CBG did not affect levels of IL-6 in pericytes, CBDA decreased IL-6 in pericytes post OGD, suggesting that these compounds may exhibit different mechanisms of action in different cell types.

IL-6 increases endothelial permeability, promotes TJ remodelling (Maruo et al., 1992) and is correlated with poor outcomes in ischaemic stroke patients (Hotter et al., 2019; Shaafi et al., 2014). As these compounds were found to attenuate IL-6 secretion, they may also offer protection against TJ disruption caused by ischaemia, however, the

direct effects of CBG, CBDV and CBDA on TJ expression and localisation have yet to be investigated.

To our knowledge, this is the first study to report that CBDV reduced levels of MCP-1 in HBMECs and attenuated levels of astrocyte derived VEGF post OGD. In addition, we found that both CBDA and CBD were effective in decreasing levels of MCP-1, however this was only conducted in one experiment and would need to be repeated to confirm the validity of these data. Our data corroborate findings the literature; in a viral model of multiple sclerosis (MS) CBD was found to mitigate MCP-1 secretion in astrocytes (Mecha et al., 2013) and recently CBDV was found to attenuate MCP-1 in a model of colitis (Pagano et al., 2019). As CBDV and CBDA exhibit a similar chemical structure to CBD, differing in just a shortened propyl side chain and a carboxylic acid group respectively, it is not surprising that they share similar biological and pharmacological properties. Interestingly, both CBDV and CBDA did not affect levels of VEGF in pericytes, but CBDV attenuated levels of VEGF in astrocytes. Therefore, it would be valuable to determine whether CBDA can also attenuate VEGF levels in astrocytes post OGD. Given that MCP-1 is associated with microglial activation and increases in endothelial permeability (Stamatovic et al., 2005, 2009; Yang et al., 2011), these preliminary data suggest that CBDV and CBDA may help prevent immune over-activation and endothelial permeability post OGD. As CBDV also reduced levels of VEGF and in another study was found to reduce intestinal permeability (Pagano et al., 2019) this compound may be particularly promising in preventing BBB disruption and future *in vivo* experiments should be undertaken to clarify this potential.

In Chapter 5, we also investigated the effects of CBDV and CBG on cellular and DNA damage in astrocytes. Both compounds reduced cell damage, which for CBDV appeared to be partially blocked by antagonists for GPR55 and GPR18, but CBGs effects were not inhibited by any of the antagonists tested. CBG and CBDV have displayed antioxidant capabilities in preclinical models of neurodegeneration (Di Giacomo et al., 2020; Schubert et al., 2019) and given that CBDVs and CBGs effects

were not clearly blocked by any of the antagonists tested, it was postulated that the non-specific antioxidant properties may be largely responsible for these effects. We next investigated whether CBG and CBDV would also protect against DNA damage in astrocytes. Interestingly, while CBG decreased levels of DDR proteins, including Chk1, H2AX and p53, elevated in astrocytes post OGD, CBDV increased levels of ATR, Chk1, H2AX, MDM2 and p53, but exhibited a trend for decreasing ATR and Chk1 at higher concentrations. In Chapter 6, CBDA and CBD were assessed for their ability to protect against DNA damage in neurons, but neither the compounds nor the OGD protocol altered levels of any of the measured DDR proteins. We concluded that this may be due to the DDR pathway being activated earlier in neurons compared to astrocytes because they are more susceptible to ischaemia associated damage (Becerra-Calixto and Cardona-Gómez, 2017; Chen et al., 1997; Ordy et al., 1993). Thus, it may be of interest to ascertain whether CBD or CBDA can influence the DDR pathway at earlier time points in neurons and in astrocytes.

CBDs acidic precursor, CBDA, has a similar chemical structure and studies have shown these compounds share pharmacological traits (reviewed in Formato et al., 2020). Using the BBB model we developed in Chapter 3, we demonstrated for the first time that CBDA pre-treatment prevented changes in permeability caused by OGD. Moreover, we found CBDAs ability to reduce IL-6 in pericytes was mediated by 5-HT_{1A} receptor activation (refer to Chapter 6). These data are similar to previous data generated by our group, showing CBD reduced permeability via PPARy and 5-HT_{1A} (Hind et al., 2016; Mishima et al., 2005). In light of the above, it is likely that CBDA, like CBD, exerts its effects on BBB permeability partly by acting as an agonist of 5-HT_{1A}, however unfortunately this was not confirmed in this study. Future studies should assess whether CBDA can also modulate inflammatory cytokine release and BBB permeability in *in vivo* models of ischaemic stroke, as well as clarify whether CBDAs neuroprotective properties are due to its action as a 5-HT_{1A} agonist.

In neuronal monocultures, we found that CBDA (100 nM) modulated levels of heat shock proteins, reducing HSP60, HSP90α and HSP27 whilst exhibiting a trend for increasing HSP27 total, HSP60 and 90 at higher concentrations. The multifaceted roles of HSPs in ischaemia-reperfusion injury have been increasingly recognised (Leak et al., 2013; Shi et al., 2017; E Zhang et al., 2020). Zhang and colleagues (2020) found that HSP protein expression and elevated levels of proinflammatory cytokines post ishcaemia-reperfusion injury were associated with downregulated phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamysin (mTOR) signalling. In an *in vivo* model of MS, Giacoppo and colleagues (2017) reported that CBD was able to restore the PI3K/AKT/mTOR pathway, increasing phosphorlyation of PI3K, Akt and mTOR and was associated with decreases in pro-inflammatory mediators, enhanced BDNF expression and upregulation of PPARy. Given that CBDA modulated levels of HSPs and reduced proinflammatory cytokine release post OGD, it is possible that like CBD, CBDAs mechanism of action may involve manipulation of the PI3K/Akt/mTOR signalling pathway, but this remains to be investigated.

Endocannabinoids have been implicated in neuronal plasticity and neuroprotection, as well as being elevated in neuropathological conditions (Hillard, 2008; Naccarato et al., 2010). Specifically, AEA was found to protect neurons in a model of CNS inflammation (Eljaschewitsch et al., 2006), OEA (10 μM) reduced BBB permeability induced by a 4 h OGD protocol in *vitro*, and *in vivo* reduced infarct volume and attenuated neurological dysfunction mediated by PPARα activation (Hind et al., 2015; Zhou et al., 2012). In addition, administration of PEA was found to be protective in an *in vivo* model of traumatic brain injury (TBI) (Ahmad et al., 2012). In Chapter 7, we found that singing increased levels of AEA, PEA and OEA as well as improving positive mood and emotions. Levels of OEA were also linked with participants desire to eat, overall hunger and mood (Stone et al., 2018). Heyman et al., (2012) found that cycling increased levels of AEA, which was positively correlated with BDNF and Brellenthin et al., (2017) found AEA,2-AG, OEA and PEA were increased after aerobic exercise and for AEA and 2-AG this was correlated with improvements in mood, including depression. Given these

data and that presented in our study, influencing levels of endocannabinoids through exercise and other activities such as signing could have significant benefits on neuronal plasticity, as well as potentially offer neuroprotection, but this remains to be fully investigated. Collectively, these data build on existing knowledge on the role of endocannabinoids in the regulation of appetite, mood and reward and highlight their therapeutic potential in neuroprotection and mental health.

8.1 Study Limitations and directions for future study

Despite the positive data on the neuroprotective properties of these compounds, this study is limited to their effects *in vitro*. Whilst primary human cells offer closer modelling to human physiology than animal derived cells or immortalised cell lines, they were likely taken from a mixture of donor sources namely surgical specimens, foetal tissues and post-mortem donors, which differ from healthy adult physiology. It is also important to recognise that ischaemic stroke occurs in elderly patients and it is well known that aged cells undergo marked changes in their phenotype (Cai et al., 2017). Therefore, whilst the cells used in our four-cell model and monoculture experiments are more representative of the human BBB than immortalised cell lines or animal cells, they are unlikely to provide full representation of the pathophysiological environment present in ischaemic stroke patients. Therefore, it is paramount that future studies assess the neuroprotective properties of these compounds in aged animals and animals with comorbidities to confirm their clinical potential.

Our method of measuring permeability, whilst producing stable readings, was not able to run continuously and therefore involved moving cultures in and out of incubators to measure TEER at specific time points. It would be interesting for future studies to possibly incorporate techniques such as electric cell-substrate impedance sensing (ECIS) to monitor changes in permeability post OGD and the effects of phytocannabinoids in real time (Anchan et al., 2019). The importance of cerebral blood flow and the effects of shear stress on endothelial cell physiology has been well established (Desai et al., 2002; Partyka et al., 2017). Our model did not incorporate

flow and therefore the effects of shear stress were not accounted for. Thus, it may be necessary for future studies to develop a method incorporating flow to be more representative of BBB physiology.

The influence of overactive microglia and peripheral immune cells in potentiating post stroke injury has been well characterised (Shigemoto-Mogami et al., 2018; Szalay et al., 2016; Taylor and Sansing, 2013). Microglia have also been shown to express cannabinoid receptors including CB_2 and GPR55 (Saliba et al., 2018). It is worth noting that CB_2 mRNA expression levels are elevated post ischaemia and CB_2 receptor activation has been shown to be protective, specifically reducing IL-6, LDH and glutamate-mediated neurodegeneration (Contartese et al., 2012; Wang et al., 2018; Yu et al., 2015). Our model did not incorporate microglia, nor did we assess the effects of CBDV, CBG or CBDA on microglial monocultures. Studies have shown CBG protected stimulated macrophages against oxidative stress via a CB_2 dependent mechanisms and protected motor neurons against medium taken from lipopolysaccharide (LPS) stimulated macrophages (Giacoppo, Gugliandolo, et al., 2017; Gugliandolo et al., 2018). Recently Zamberletti and co-authors (2019) found that CBDV restored CB_2 expression and reduced microglial activation in rats exposed to valproic acid (VPA). However, CBDA has not been investigated in the context of neuroinflammation or whether it can influence immune cell activation. Thus, it would be interesting to see if CBDV, CBG or CBDA modulate microglial activation in preclinical models of ischaemic stroke.

Phytocannabinoids exhibit a complex pharmacology and future experiments are needed to generate a more comprehensive picture of CBDVs, CBGs and CBDAs biological effects and their corresponding mechanisms of action. It is worth mentioning that Pagano et al., (2019) found CBDVs ability to reduce intestinal permeability and inflammatory cytokine release was mediated by TRPA1 receptors, which were not probed in our panel of antagonists. Pires and Earley, (2018) found that mitochondrial derived ROS stimulate TRPA1 dependent Ca^{2+} influx in cerebral arteries during hypoxia,

whilst Araújo and colleagues (2017) found that LDH release induced by OGD in chick retina was reversed by TRPA1 antagonist, HC-030031. These points support the role of TRPA1 receptors in hypoxia and ischaemia and it is possible that CBDVs protective effects and perhaps CBG and CBDA, in our model may be due to their interaction at TRPA1 receptors, which should be investigated in future studies.

Inflammation and oxidative stress facilitate the breakdown of TJs post ischaemia, significantly contributing to BBB breakdown. Future experiments could analyse changes in TJ expression post ischaemia by analysing mRNA levels in BBB insert lysates or by immunocytochemistry. The direct influence of phytocannabinoids on TJ expression and localisation could also be examined. Whilst we concluded that CBG and CBDVs protection against cell damage was likely to be due to their antioxidant effects, direct measurement of oxidative stress was not conducted and could be assessed in future study.

Looking forward, the pharmacokinetics (PK) of minor phytocannabinoids also need to be fully established before confirming clinical translatability (Stone et al., 2020). CBD has shown great promise as a neuroprotectant, partly due to its tolerability and safety in humans and its ability to cross the BBB (Millar et al., 2018). We found that despite evidence of CBDs efficacy in various disorders, there was an absence of PK data on CBD particularly regarding CBDs bioavailability in man (Millar et al., 2018, 2019). Likewise, it is paramount for PK data on minor phytocannabinoids, CBDA, CBG and CBDV to be generated in order for these compounds to be fully considered as therapeutic options in man. Limited PK data on these compounds in rodents presented in Deiana et al., (2012) and Anderson et al., (2019) demonstrate that CBDA, CBDV and CBG readily penetrate the brain, corroborating their potential as clinical neuroprotectants. Currently, CBDV is in clinical trials for autism spectrum disorder (phase 2, clinical trial identifier: NCT03849456) and was taken into clinical trials for epilepsy but did not meet the primary end point in controlling focal seizures (clinical trial identifier: NCT02365610). However, it is worth noting that the percentage of serious adverse

effects (SAEs) were low and reported adverse effects (AEs) were of mild to moderate severity, altogether suggesting CBDV was well tolerated in humans (dose-titrated up to 800 mg per day)(Morano et al., 2020). Considering the novel data presented in this thesis, together with evidence from the aforementioned studies, CBDA, CBDV and CBG are promising neuroprotectants and should be explored further in *in vivo* models of ischaemic stroke and perhaps other neurological disorders.

8.2 Overall conclusions

This thesis examined the protective effects of phytocannabinoids on cells of the NVU and successfully developed a four-cell BBB model. Using an OGD protocol to simulate ischaemic stroke *in vitro*, pre-treatment with CBG, CBDV and CBDA attenuated multiple aspects of OGD induced damage, including elevated levels of proinflammatory cytokines, adhesion molecules, heat shock proteins, as well as cellular and DNA damage. Furthermore, CBDA reduced BBB permeability and protected neurons in a four-cell BBB model subjected to OGD. In addition, manipulation of the endocannabinoid system by activities such as singing may pose a novel therapeutic avenue in various neurological disorders. The results presented in this study, together with the existing literature, suggest CBDA, CBG and CBDV may offer protection in ischaemic stroke, particularly against IL-6 and VEGF/MCP-1 induced increases in BBB permeability and cell damage. Overall, these novel data add to the growing body of evidence on the biological effects of phytocannabinoids and their potential as neuroprotective agents, particularly in conditions which implicate BBB dysfunction.

9. Appendix

Professional internship reflective statement

Note to examiners:
A mandatory 3-month placement for DTP PhD students was carried out between February and May 2019. A brief, reflective statement on the internship and the skills developed is included to enable readers to recognise the range of PhD training that was experienced as a BBSRC doctoral student. This professional internship was completely separate to the PhD studies and was undertaken to develop different skills that could be useful in future endeavours.

I accepted an internship at the scientific consultancy company Blossom Medical, under the supervision of a wonderful mentor, Dr Andy Yates. During the three months I learned a variety of new skills including, drug marketing, legislation, R&D and business development. I gained knowledge of the cannabidiol (CBD) commercial market and companies responsible for CBD's novel food legislation. I also learned about the centre of medicinal cannabis (CMC) which promotes the widespread access to cannabis-based medicines (CBMs), as well as the education of various regulatory bodies and medical professionals on medical cannabis use.

I wanted to establish whether I would pursue a career outside of lab-based research, either in scientific business development or legislation. I also wanted to build my corporate skills, such as interacting in business meetings, professional conduct and liaising with individuals with a range of skills and backgrounds. Shadowing Dr Andy Yates enabled me to partake in all of these and gain insight into the variety of projects he was involved with as a scientific consultant. Also, there was a range in the urgency of the tasks I was set meaning I had to be quick to delve into a piece of work if something was required promptly- sometimes within 1-2hrs. This helped me to develop my time management skills and ability to prioritise tasks in accordance with deadlines.

Whilst it was very insightful and invaluable experience, I did miss working in the lab and the practical side of science. I realised that I still wanted to increase my scientific skills and experience, possibly to pursue an academic career or a lab-based job in industry. However, I did appreciate the different career possibilities outside academia and the variety of job opportunities for someone with the breadth of skills I have developed over the course of my PhD. Overall, I thoroughly enjoyed my professional internship, it enabled me to be part of life outside PhD study and academic life whilst learning different intrapersonal skills and knowledge of scientific industry and the business sector.

A systematic review of cannabidiol dosing in clinical populations

The following chapter is presented in its final manuscript format and is published in British Journal of Clinical Pharmacology (Millar et al., 2019).

Statement of personal contribution: performed systematic search and retrieval of articles and data extraction which was conducted independently from SM. Helped in editing the manuscript, confirming findings and input in the discussion.

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SYSTEMATIC REVIEW AND META-ANALYSIS

BJCP BRITISH PHARMACOLOGICAL SOCIETY

A systematic review of cannabidiol dosing in clinical populations

S.A. Millar[1] | N.L. Stone[1] | Z.D. Bellman[1] | A.S. Yates[2] | T.J. England[1] | S.E. O'Sullivan[1]

[1] Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital, Derby, UK

[2] Artelo Biosciences, La Jolla, CA USA

Correspondence
Sophie A. Millar, Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital, Derby, UK.
Email: sophieannemillar@gmail.com; stxsamil@nottingham.ac.uk

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Aims: Cannabidiol (CBD) is a cannabis-derived medicinal product with potential application in a wide-variety of contexts; however, its effective dose in different disease states remains unclear. This review aimed to investigate what doses have been applied in clinical populations, in order to understand the active range of CBD in a variety of medical contexts.

Methods: Publications involving administration of CBD alone were collected by searching PubMed, EMBASE and ClinicalTrials.gov.

Results: A total of 1038 articles were retrieved, of which 35 studies met inclusion criteria covering 13 medical contexts. Twenty-three studies reported a significant improvement in primary outcomes (e.g. psychotic symptoms, anxiety, seizures), with doses ranging between <1 and 50 mg/kg/d. Plasma concentrations were not provided in any publication. CBD was reported as well tolerated and epilepsy was the most frequently studied medical condition, with all 11 studies demonstrating positive effects of CBD on reducing seizure frequency or severity (average 15 mg/kg/d within randomised controlled trials). There was no signal of positive activity of CBD in small randomised controlled trials (range n = 6–62) assessing diabetes, Crohn's disease, ocular hypertension, fatty liver disease or chronic pain. However, low doses (average 2.4 mg/kg/d) were used in these studies.

Conclusion: This review highlights that CBD has a potential wide range of activity in several pathologies. Pharmacokinetic studies as well as conclusive phase III trials to elucidate effective plasma concentrations within medical contexts are severely lacking and highly encouraged.

KEYWORDS

cannabidiol, cannabinoid, dose, dosing, therapeutics

1 | INTRODUCTION

Cannabidiol (CBD) is a non-intoxicating major constituent of the *Cannabis sativa* plant that has been increasing in interest due to its potentially diverse range of therapeutic properties and its favourable safety and tolerability profile.[1] Side effects are generally mild and infrequent, such as sleepiness, diarrhoea or increased temperature. It is also reported that clinically significant drug-interactions pose a

low risk.[2] There is no evidence for dependency or abuse potential with CBD use, as concluded by the World Health Organisation Expert Committee on Drug Dependence.[1] The purported effects of CBD include analgesic, anti-inflammatory, antioxidant, anxiolytic, anticonvulsant and cytotoxic effects, which are mediated through signalling mechanisms including the cannabinoid receptor 1 (weak agonist), the cannabinoid receptor 2 (inverse agonist), the serotonin 1a receptor ($5\text{-}HT_{1A}$), G protein-coupled receptor 55 (GPR55), G protein-coupled receptor 18 (GPR18) and the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptors, amongst others.[3]

Clinically, CBD is being investigated in multiple disease states including neurodegeneration, anxiety disorder, orphan childhood diseases with a prevalence of <5 in 10 000 individuals (e.g. tuberous sclerosis complex) and addiction (ongoing trials in cannabis and cocaine craving).[4-6] Epidiolex has recently become the first Food and Drug Administration-approved CBD medicine, indicated for use in Lennox–Gastaut or Dravet syndrome (childhood epilepsy) by oral administration. Sativex is an oromucosal spray containing both CBD and δ-9-tetrahydrocannibinol, which is licenced in the EU and Canada for the treatment of multiple sclerosis associated spasticity. At the time of writing, there are 49 clinical trials registered on clinicaltrials.gov investigating CBD alone (either not yet recruiting, recruiting or active) and there have been at least a further 100 clinical trials previously registered containing CBD, indicating a significant clinical interest with an ongoing need to ensure that human volunteers engaged in these trials are given doses that are optimised for efficacy and safety. Surprisingly, none of the 49 currently registered trials have explicitly included a study design to investigate the dose-ranging efficacy of CBD.

Hemp-derived CBD is commercially available and is currently used as a health and food supplement commonly for anxiety and pain relief. This market represents a flourishing industry expected to rise financially and globally.[7] However, the blurred lines between CBD as a licensed medicine and CBD as an over-the-counter remedy contribute to the overall lack of understanding of what dose of CBD may be considered *therapeutic*. This is further hampered by the lack of standardisation in over-the-counter CBD products and their unregulated labelled doses.

Despite the prevalence of CBD use and current hype, guidance on dose recommendations has not advanced and is not clear, additionally hampered by the striking lack of accessible pharmacokinetic and bioavailability data of CBD in humans.[8] No published study to date has reported the absolute oral bioavailability of CBD in humans.[8] Limited dose-determination studies have left a paucity in data surrounding desired plasma concentrations to achieve minimum effective doses. Additionally, the lack of information on the role of different formulations and routes of administration on absorption are also apparent. The aim of this review was to comprehensively collate all published data relating to CBD administration in clinical populations to describe the range of CBD doses assessed across different pathological states.

What is already known about this subject

- Due to its favourable toxicity and side effect profile, cannabidiol is under increasing investigation in the commercial and medical industry to treat many clinical indications.

What this study adds

- This study identifies the wide active dosing range of cannabidiol (<1 to 50 mg/kg/d) within a variety of medical conditions including epilepsy, anxiety and graft-*vs*-host disease.
- This review indicates that studies that used higher doses tended to have better therapeutic outcomes compared to lower doses overall.
- This study identifies a strong existing need for dose-ranging clinical studies to be conducted in which plasma concentrations can provide a better indication of the therapeutic range of cannabidiol.

2 | METHODS

2.1 | Search strategy

The systematic review was carried out in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. A systematic search of PubMed, EMBASE (including MEDLINE) and clinicaltrials.gov was conducted to retrieve all articles reporting CBD administration in clinical populations using 'CBD or Cannabidiol' as search terms. Searches were restricted to 'humans' and 'clinical trials and case reports' in PubMed and EMBASE, with no restrictions on clinicaltrials.gov. The searches were carried out by 8 August 2018 by 2 independent researchers.

2.2 | Eligibility criteria

The titles and abstracts of retrieved studies were examined by 2 independent researchers, and inappropriate articles were rejected. Inclusion criteria were as follows: an original, peer-reviewed published paper that involved administration of CBD to a clinical population, or reported on clinicaltrials.gov, and included an outcome measurement to assess the efficacy of CBD i.e. improvement in disease. Exclusion criteria were: administration in healthy participants only; CBD administered in combination with other cannabinoids such as with δ-9-tetrahydrocannibinol or as whole cannabis extracts; article not in English; no stated concentration of CBD used; or no statistical results reported. The reference lists of included studies were hand-searched for additional relevant studies.

2.3 | Data acquisition and analysis

The included articles were analysed, and the following data extracted: sample size, clinical population/medical context; study design and length; administration route of CBD; source of CBD; dose of CBD; side effects; and primary outcome results. All data entry was checked by an additional independent researcher. Risk of bias of the 15 randomised controlled trials was assessed using the 2011 Cochrane Collaboration's tool for assessing risk of bias.

As this review included studies of participants of all ages (from infants to adults), dosing is reported in mg/kg of body weight to allow for comparison. Where not available as mg/kg (24 studies), dose was converted for adults using an average adult body weight of 62 kg.[9] In only 1 publication, a case report on a child, an average child weight of 40 kg had to be used to convert reported mg/d dose into mg/kg/d.[10]

A positive effect of CBD was determined by the presence of a significant improvement in primary end points(s) or outcomes reported compared to placebo or baseline. A lack of positive effect was determined if no significant improvements were reported. Mixed findings were reported for example in case reports wherein some patients improved, others did not, or where a primary outcome was not specified (exploratory study) and in which some endpoints improved while others worsened (1 study) or remained unchanged.

3 | RESULTS

The initial search yielded 1038 records, from which 896 abstracts were reviewed, and 35 articles were included in the final analysis, comprising a total number of 1223 participants. A flow chart of article retrieval and selection is presented in Figure 1. Fifteen studies were randomised controlled trials (RCTs), 8 were clinical trials but not both randomised and controlled in design (for example open-label trials), and 12 articles were case reports/series. A description of each study is presented in tables 1–3 according to study design. Results of the risk of bias assessment of the RCTs are presented in Figure 2. A component of blinding was included in 74% of the RCTs . No study was reported with a high risk of selection bias, detection bias, or reporting bias. Overall, most information was from studies at low risk of bias. No study reported plasma concentrations of CBD. All studies reported oral administration of CBD, either as an oral solution (n = 11), capsules (n = 13), spray/sublingual (n = 4), or orally but unspecified (n = 6).

Of the 15 RCTs, the range of doses investigated varied from <1 mg/kg up to 20 mg/kg per day (average 9 mg/kg/d).[11-19,21-25] Seven RCTs reported CBD efficacy (average dose 14 mg/kg/d),[11-13,16,19,20,24] 7 studies describe neutral effects of CBD (average dose 5 mg/kg/d)[14,15,17,21-23,25] and 1 study showed both positive and negative outcomes.[18] In the remaining 8 clinical trials of various

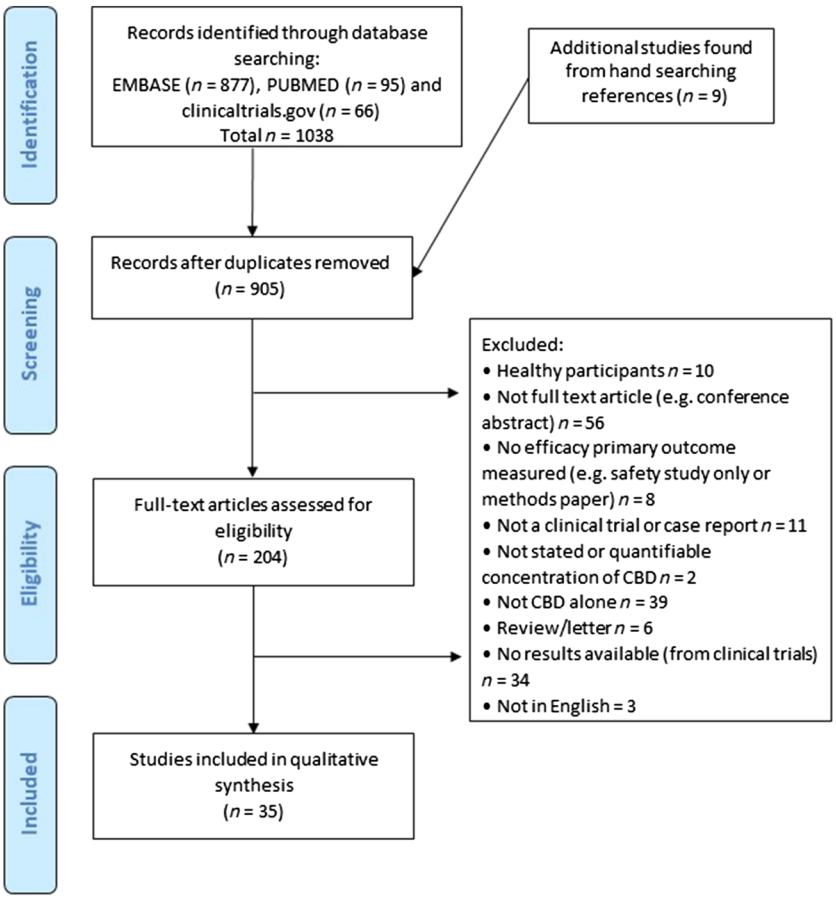

FIGURE 1 Flow chart of study retrieval and selection

TABLE 1 Summary of included studies: randomised controlled trials

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
McGuire, 2018 (NCT02006628)[11]	Schizophrenia, adults	88	Phase II exploratory double-blind, parallel-group, RCT. Add-on therapy to anti-psychotic drugs.	8 wk	1000 mg/d (16.7 mg/kg/d)	Oral solution	GW	Positive psychotic symptoms reduced. Negative, overall and general psychotic symptoms unchanged. Higher proportion of CBD treated patients rated as *improved*. No differences in functionality. No significant improvement in cognitive function except for motor speed. Overall reported as clinically significant improvements with CBD.	Yes	Rates of adverse events similar between CBD and placebo groups
Thiele, 2018 (NCT02224690)[12]	Seizures (Lennox–Gastaut syndrome), ages 2–55 y	171	Double-blind, phase III, RCT. Add-on therapy to AEDs.	14 wk	20 mg/kg/d	Oral solution	GW	Monthly frequency of drop seizures decreased by a median of 43.9% in the CBD group, significantly more than in the placebo group	Yes	Diarrhoea, somnolence, pyrexia, decreased appetite, vomiting
Devinsky, 2018 (NCT02224560)[13]	Lennox–Gastaut syndrome (epilepsy), ages 2–55 y	225	Phase III, double-blind, RCT. Add-on therapy to AEDs.	14 wk	10 or 20 mg/kg/d	Oral solution	GW	Significantly greater reduction in CBD groups in drop seizure frequency than in placebo	Yes	9% taking CBD had elevated liver aminotransferases. Somnolence, decreased appetite, diarrhoea, upper respiratory tract infection, pyrexia, vomiting.

(Continues)

TABLE 1 (Continued)

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Boggs, 2018 (NCT00588731)[14]	Schizophrenia, adults	36	Double-blind, parallel group, RCT. Add-on therapy to anti-psychotic drugs.	6 wk	600 mg/d (10 mg/kg/d)	Oral capsules	STI	No effect on cognition or symptoms	No	Similar rates between placebo and CBD, with exception of sedation which was higher in CBD group.
Naftali, 2017 (NCT01037322)[15]	Crohn's disease, adults	19	RCT	8 wk	20 mg/d (0.3 mg/kg/d)	Orally, sublingual	On-site	No difference in disease index	No	None observed
Devinsky, 2017 (NCT02091375)[16]	Treatment resistant Dravet syndrome (epilepsy), aged 2–18 y	120	Double-blind, RCT. Add-on therapy to AEDs.	14 wk	20 mg/kg/d	Oral solution	GW	Reduction in frequency of convulsive seizures compared to baseline, significantly greater reduction than with placebo	Yes	Diarrhoea, vomiting, fatigue, pyrexia, somnolence, abnormal results on liver-function: tests were higher in the CBD group than placebo
Jadoon, 2016 (NCT01217112)[17]	Type 2 diabetes patients, adults	62	Double-blind, RCT	13 wk	200 mg/d (3.3 mg/kg/d)	Oral	GW	No change in HDL-cholesterol concentrations or glycaemic control.	No	Well tolerated
Chagas, 2014[18]	Parkinson's disease, adults	21	Double-blind exploratory RCT. Add-on therapy to anti-Parkinson's drugs.	6 wk	75 or 300 mg/d (1.25 or 5 mg/kg/d)	Oral capsules	THC	No effect on motor and general symptoms; 300-mg dose improved well-being and quality of life scores.	Mixed	None reported
Leweke, 2012[19]	Schizophrenia, adults	42	Phase II, double-blind, parallel-group, RCT	4 wk	800 mg/d (max: 13.3 mg/kg/d)	NA	NA	Significant improvement of psychotic symptoms compared to baseline	Yes	Well tolerated
Bergamaschi, 2011[20]	Generalised SAD, adults	24	Double-blind, RCT	Acute	600 mg (10 mg/kg)	Oral capsule	STI and THC	Reduction in anxiety, cognitive impairment, discomfort in speech performance. Alert factors in anticipatory speech were also reduced.	Yes	None reported

(Continues)

TABLE 1 (Continued)

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Tomida, 2006[21]	Ocular hypertension, adults	6	Double-blind, 4-way cross-over, RCT	Acute	20 or 40 mg (0.3 or 0.7 mg/kg)	Oromucosal spray	GW	20 mg of CBD was ineffective, while 40 mg slightly increased intraocular pressure.	No	Mild—e.g. oral discomfort.
Notcutt, 2004[22]	Chronic pain, adults	24	Double-blind, 4-way cross-over, RCT. Add-on therapy to pain medication.	8 wk	Approx. 9 sprays/d, equivalent of 22.5 mg/d (0.4 mg/kg/d)	Sublingual spray	GW	Symptom control or sleep duration was not improved with CBD; however, sleep quality was.	No	Mid—drowsiness, dry mouth
Consroe, 1991[23]	Huntington's disease, adults	15	Double-blind, cross-over, RCT	6 wk	10 mg/kg/d	Oral capsules	US NIDA	CBD was ineffective	No	Similar between CBD and placebo
Cunha, 1980[24]	Epilepsy, adults	15	Double-blind, RCT study. Add-on therapy to AEDs.	Up to 4.5 months	200–300 mg/d (5 mg/kg/d)	Oral capsules	NA	All but 1 patient improved condition	Yes	Well tolerated
***NCT01284634**[25]	Fatty liver disease, adults	25	Partially-blinded, phase II, RCT	8 wk	200, 400 or 800 mg/d (3.3, 6.7, or 13.3 mg/kg/d)	Oral capsules	GW	No differences in liver triglyceride levels	No	Similar between CBD and placebo

[a]If not supplied, mg/kg/d was calculated based on average adult weight of 62 kg to enable comparisons.

[b]Significant compared to placebo/control ($P < .05$) unless stated otherwise.

*Registered clinical trial identifier: not published in any peer-reviewed journal but results available from clinicaltrials.gov.

AEDs, anti-epileptic drugs; CBD, cannabidiol; GW, GW Pharmaceuticals; HDL, high density lipoprotein; NA, not available; NIDA, National Institute on Drug Abuse; RCT, randomised controlled trial; SAD, social anxiety disorder; STI, STI Pharmaceuticals; THC, THC Pharm.

TABLE 2 Summary of included studies: clinical studies

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Rosenberg, 2017[26]	Epilepsy, 1–30 y	48	Open label clinical study	12 wk	2–5 mg/kg/d titrated up to 50 mg/kg/d or intolerance	Oral solution or by gastric tube	GW	Improvement in quality of life as well as some cognitive functions (memory and control)	Yes	Somnolence, drowsiness, fatigue
Devinsky, 2016[27]	Drug-resistant epilepsy, ages 1–30 y	137	Prospective, open-label trial	12 wk	2–5 mg/kg/d, up-titrated to 25 or 50 mg/kg/d	Oral solution or gastric tube	GW	Monthly motor seizures reduced by a median of 35.5% from baseline	Yes	Somnolence, fatigue, diarrhoea, decreased appetite, weight loss, status epilepticus (6%).
Hess, 2016[28]	Drug-resistant epilepsy in tuberous sclerosis complex, 2–31 y	18	Prospective study	6–12 months	5 mg/kg/d titrated up to 50 mg/kg/d if tolerated	Oral solution	GW	Decreased seizure frequency	Yes	Drowsiness, ataxia, diarrhoea
Yeshurun, 2015 (NCT01385124)[29]	Cell transplant, (GVHD), adults	48	Prospective, phase II clinical trial	37-day	300 mg/d (5 mg/kg/d)	Oral solution	STI	No patients developed acute GVHD. Significantly reduced risk ratio compared to historical case controls.	Yes	None reported
Crippa, 2011[5]	Generalised SAD, adults	10	Double-blind, placebo-controlled study	Acute	400 mg (6.7 mg/kg)	Oral capsule	THC	Reduced subjective anxiety	Yes	None reported
Hallak, 2010[30]	Schizophrenia, adults	28	Placebo-controlled study	Acute	300 or 600 mg (5 or 10 mg/kg)	Oral capsules	Gift	No beneficial effects on selective attention	No	None reported
Zuardi, 2009[31]	Psychosis in Parkinson's disease, adults	6	Open-label pilot study	4 wk	150 mg/d, increased by 150 mg each week to a total of 400 mg/d (6.7 mg/kg/d)	Oral capsule	THC	Decrease in psychotic symptoms and Parkinson's disease rating compared to baseline	Yes	None reported
Consroe, 1986[32]	Dystonic movement disorder, adults	5	Preliminary open pilot study	6 wk	100–600 mg/d, increased weekly (1.7–10 mg/kg/d)	Oral capsules	NA	Dose-related improvement in dystonia disability	Yes	Mild—drop in standing blood pressure

[a]If not supplied, mg/kg/d was calculated based on average adult weight of 62 kg to enable comparisons.

[b]Significant compared to placebo/control ($P < .05$) unless stated otherwise.

CBD, cannabidiol; GW, GW Pharmaceuticals; GVHD, graft-vs-host disease; STI, STI Pharmaceuticals; SAD, social anxiety disorder; THC, THC Pharm.

TABLE 3 Summary of included studies: case studies

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Kaplan, 2017[33]	Refractory seizures in Sturge–Weber syndrome, children	5	Case-series	14 wk	5–25 mg/kg/d	Oral solution	GW	Decreases in seizure frequency	Yes	Mild
Warren, 2017[34]	Brain tumour related epilepsy, aged 17–40 y	3	Case series	2–10 mo	10–50 mg/kg/d	Oral	GW	Improvement in seizure frequency (n = 2) and severity (*n* = 3)	Yes	Diarrhoea
Gofshteyn, 2017[35]	Febrile infection-related epilepsy syndrome, children	7	Open-label case series	Acute and up to 48 weeks	15–25 mg/kg/d	Oral solution	GW	Improvements in frequency and duration of seizures	Yes	Dizziness, decreased appetite, weight loss
Shannon, 2016[10]	Anxiety and insomnia in PTSD, child	1	Case report	5 mo	25 mg/d (0.6 mg/kg/d)	Oral capsule and spray	CannaVest Corp	Increased sleep quality and duration, and decreased anxiety secondary to PTSD	Yes	None observed
Saade, 2015[36]	Seizures, 10-month old infant	1	Case report	6 mo	25 mg/kg/d	Oral solution	GW	Substantial reductions in seizures	Yes	None reported
Chagas, 2014[37]	RBD in Parkinson's disease, adults	4	Case series	6 wk	75 mg/d (1.25 mg/kg/d)	NA	NA	Substantial reduction in RBD-associated events compared to baseline	Yes	None reported
Crippa, 2013[38]	Cannabis dependency, adult	1	Case report	10 d	300 mg/d increased to 600 mg/d (5–10 mg/kg/d)	Oral capsule	THC	Absence of withdrawal symptoms	Yes	None reported
Zuardi, 2010[39]	Bipolar disorder, adults	2	Case series	30 d	600 mg/d increased to 1200 mg/d (20 mg/kg/d)	Oral	STI and THC	CBD was ineffective for manic episode	No	None observed
Zuardi, 1995[40]	Schizophrenia, adult	1	Case report	4 wk	1500 mg/d (25 mg/kg/d)	Oral capsules	NA	Improvements in psychiatric ratings	Yes	Well tolerated; none reported
Zuardi, 2006[41]	Treatment-resistant schizophrenia, adults	3	Case series	30 d	40 mg/d, increased to 1280 mg/d (21.3 mg/kg/d)	Oral	GW	1 patient showed mild improvement to baseline and discontinuing treatment worsened symptoms	No	Well tolerated; none observed

(Continues)

TABLE 3 (Continued)

Study	Clinical population	Total *n*	Design	Trial length	CBD dose (mg) and approx. mg/kg/d[a]	Route of admin.	CBD source	Results[b]: Primary endpoint(s)	+ effect	Side effects
Snider, 1985[42]	Parkinson's disease, adult	1	Case report	4 wk	100–400 mg/d (3.3 mg/kg/d)	Oral	NA	Improvement of dyskinesia up to 200 mg/d, worsening of Parkinson disease symptoms with 300–400 mg/d	Mixed	Dizziness, drowsiness, increased Parkinson symptoms
Snider, 1984[43]	Meige syndrome, adult	1	Case report	Long-term	Initially 100 mg/d increased to 400 mg/d (6.6 mg/kg/d)	Oral	NA	50% improvement in spasm frequency and severity	Yes	Dry mouth, headache, sedation

[a]If not supplied, mg/kg/d was calculated based on average adult weight of 62 kg to enable comparisons.

[b]Significant compared to placebo/control ($P < .05$) unless stated otherwise.

CBD, cannabidiol; GW, GW Pharmaceuticals; PTSD, post-traumatic stress disorder; RBD, rapid eye movement sleep behaviour disorder; STI, STI Pharmaceuticals; THC, THC Pharm.

FIGURE 2 *Risk of bias* summary of the randomised controlled trials included in the systematic review. Green indicates low-risk bias, red indicates high-risk bias, and yellow indicates intermediate or unclear risk

study design, 7 studies reported CBD positively (average dosing 23 mg/kg/d)[5,26-29,31,32] and 1 study was neutral (8 mg/kg/d).[30] Within the 12 case studies and case series, 9 described positive effects of CBD (average dosing 16 mg/kg/d),[10,33-38,40,43] 2 were neutral (average dosing 21 mg/kg/d)[39,41] and 1 study described mixed results (3 mg/kg/d).[42]

Epilepsy was the most frequently studied medical condition, with all 11 studies describing beneficial effects of CBD in reducing the severity or frequency of seizures.[12,13,16,24,26-28,33-36] Within the 4 conducted RCTs (n = 531), an average dosing of 15 mg/kg/d was used where CBD was administered successfully as an add-on therapy to usual anti-epileptic drugs.[12,13,16,24] Significant improvements were observed compared to placebo as an add-on therapy. Within the other 3 clinical trials of prospective open-label design (n = 203), CBD was administered at an average dosing of 42 mg/kg/d and significant improvements in quality of life and seizure frequency compared to baseline were observed.[26-28] 3 case series and 1 case report (total n = 16) reported beneficial effects of CBD on seizure frequency, duration and severity with an average administered dose of 21 mg/kg/d.[33-36]

Seven studies were conducted in the context of schizophrenia and bipolar disorder. Within the RCTs, 2 conducted with an average dosing

of 15 mg/kg/d over 4 or 8 weeks reported positive reductions in psychotic or psychiatric symptoms and a better side effect profile (n = 130).[11,19] One of these compared CBD against an active control (amisulpride), and the other as an add-on therapy to usual medication compared to placebo as an add-on therapy. However, a third RCT employing CBD as an add-on therapy did not report any improvements in cognition or symptoms of schizophrenia after a lower average dose of 10 mg/kg/d over 6 weeks (n = 36).[14] An acute dose of 5 or 10 mg/kg/d did not improve selective attention in a placebo-controlled trial of 28 schizophrenia patients.[30] A number of case studies have also been conducted by Zuardi and colleagues in this medical context. In 2 patients with bipolar disease, 20 mg/kg/d was ineffective in treating manic episodes.[39] CBD was similarly unable to improve symptoms in 3 schizophrenia patients, although 1 patient described mild improvement.[41] Another case report described improvement in psychiatric ratings following an average dose of 25 mg/kg/d over 4 weeks.[40]

Results are mixed within Parkinson's disease studies. Within an RCT in 21 patients, 1.25 or 5 mg/kg/d CBD had no effect on motor and general symptoms. However, the 5 mg/kg/d dose improved well-being and quality of life scores.[18] The remaining studies are case studies in which CBD decreased psychotic symptoms and Parkinson's disease ratings (n = 6; 7 mg/kg/d),[31] improved rapid eye movement sleep behaviour disorder (n = 4; 1 mg/kg/d),[37] decreased dyskinesia with 2 to 3 mg/kg/d doses (n = 1), but exaggerated Parkinson's disease symptoms with 5 and 7 mg/kg/d doses.[42]

CBD did not change therapeutic outcome variables in a double-blind RCT in Huntington disease patients compared to placebo (n = 15; 10 mg/kg/d for 6 weeks),[23] but improved dystonia disability in an open pilot study (n = 5; 10 mg/kg/d for 6 weeks),[32] and improved spasm frequency and severity in a case report in 1 patient with Meige syndrome (7 mg/kg/d).[43]

Within the RCTs, CBD did not significantly change the primary outcomes in diabetes (n = 62), Crohn's disease (n = 19), ocular hypertension (n = 6), chronic pain (mostly neuropathic; n = 24), or fatty liver disease (n = 25).[15,17,21,22,25] However, an average dose of 2.4 mg/kg/d (range 0.3–13.3 mg/kg/d) was used in these studies, which is very low in the clinical and clinical trial setting compared to other studies. Low doses (10 mg/kg) did, however, produce positive responses in generalised social anxiety disorder (SAD) in a double-blind RCT in 24 patients.[20] Likewise, in another double-blind placebo-controlled study, a dose of 6.7 mg/kg reduced subjective anxiety in 10 adults with generalised SAD.[5] Additionally, in a case report in a child, 0.6 mg/kg/d increased sleep quality and duration, and decreased anxiety secondary to PTSD.[10]

Lastly, it was found that doses of 5 mg/kg/d prevented occurrence of graft-*vs*-host disease in a phase II clinical trial (n = 48) and 5–10 mg/kg/d doses have been shown in a case report to remove withdrawal symptoms from a patient with cannabis dependency.[29,38]

Within studies that compared CBD against a placebo or control (n = 17 publications), only 1 compared CBD against an active control (and a greater clinical improvement and side effect profile was observed with CBD against amisulpride), 8 compared CBD against a placebo (monotherapy), and 8 studies compared CBD as an add-on therapy (adjunctive to antipsychotic medication, antiepileptic medication, anti-Parkinson medication or pain medication) against placebo. Analysis of these data revealed that a greater proportion of studies reported a beneficial effect of CBD in the add-on therapy group compared to the monotherapy group (n = 6 and n = 2 respectively). However, higher doses were used overall within the add-on therapy group compared to the monotherapy group (average 11 and 6 mg/kg/d, respectively) and, due to such a small data set and heterogeneity of studies, we did not perform any further analysis.

4 | DISCUSSION

To our knowledge, this is the first study to compile and compare all publications in which CBD was administered to clinical populations. The aim of this systematic review was to better understand the range of doses of CBD used in clinical studies. In total, 13 medical contexts were included in this review amongst 35 studies including clinical trials and case reports. A positive effect of CBD was reported in 66% of studies, covering disorders including schizophrenia, SAD, epilepsy, cannabis dependency and graft-*vs*-host disease, with doses ranging between <1 and 50 mg/kg/d (i.e. <62–3100 mg/d for an adult). Although we acknowledge that these results mix widely heterogeneous studies, it appears well founded to highlight the differences in average dosing for positive effect studies against those without positive effects, which is confirmed when analysing studies per medical context within each study design format. This suggests that CBD potentially displays a wide therapeutic range, and variable minimum doses are required for effect depending on primary outcomes assessed and the population group. However, it is vital to note that no conclusions can be drawn on the efficacy of CBD as larger phase III and conclusive efficacy trials have not been conducted, with exception of epilepsy. A number of phase III clinical trials are registered on clinicaltrials.gov, which should provide more evidence in the coming years in the contexts of pain, anxiety, Crohn's disease, bipolar disorder, Fragile X syndrome, epilepsy and more.

CBD is increasingly popular, both as a food and health supplement and as a licensed medicine. Within this review, 51% of studies have been published in the last 5 years (since 2013); however, the included articles span over decades, with prominent publications first appearing in the 1980s and early 1990s.[24,40] Despite its long history of sole administration to patients, there is surprisingly little published about the pharmacokinetic properties of CBD, particularly its bioavailability, making it difficult to estimate true effective doses.[8] Historically, there is a striking lack of dose-ranging studies and, looking forward, there are no registered trials on clinicaltrials.gov including specific dose-ranging investigations in their study design. Ideally, this review would have compared plasma concentrations of CBD in order to more accurately estimate therapeutic concentrations, but, due to the lack of reporting, this was not possible.

Different effective plasma concentrations of CBD may be required for achieving different endpoints across clinical populations, which is a

recognised trait in a number of other drugs and diseases. For example, aspirin (acetylsalicylic acid) is used at low doses for antiplatelet therapy, and at higher doses as an analgesic agent.[44,45] With CBD, lower doses may be effective in anxiety relief, while higher doses may be required for effective reduction in epileptic seizures. In studies where there are good rationales for CBD use (e.g. Crohn's disease and chronic pain[46,47]), neutral results may be secondary to subtherapeutic dosing, and dose-escalation trials with embedded pharmacokinetic studies are the next logical step.[15,22] Studies in this review using higher doses concluded that CBD was generally well-tolerated with the most frequent side effects including drowsiness, nausea, somnolence, fatigue and vomiting.

Among the clinical trial records retrieved from clinicaltrials.gov, only 60% of completed trials had results uploaded and available. This may represent a significant publication bias and is suggestive of disregard for the priority of publication of negative results, which is a well-recognised problem.[48] Unfortunately, this may potentially skew the findings presented in this review and so should be interpreted with caution and is acknowledged as a limitation. We also acknowledge that despite all routes of administration being oral, there may be further bias introduced between studies as one dose cannot be directly compared to another due to lack of standardisation of formulations and pharmacokinetic activity, including differences in bioavailability between an oral spray and an oral capsule.

Future studies should also consider the safety of drug interactions with CBD. CBD is a known inhibitor of the cytochrome P450 (CYP) system[49] and can therefore increase plasma concentrations of medicines already in use, in particular antiepileptic drugs. Indeed, this has been reported in a number of publications investigating concomitant use of CBD and antiepileptic drugs.[50] Similarly, CYP inhibitors are predicted to increase CBD plasma concentrations which should be equally monitored. Where possible, further well designed trials with CBD may disentangle whether CBD offers unique therapeutic potential in addition to benefits seen when used as an add-on treatment.

5 | CONCLUSION

Although larger confirmatory and efficacy clinical trials examining dosing in more detail for each medical context is required, this review summarises that CBD appears to offer a wide-range of activity between 1 and 50 mg/kg/d, and there was a tendency of studies with positive outcomes to have used higher doses of CBD. We recommend pharmacokinetic dosing schedules in subsequent trials to consider this range along with safety data and individual patient requirements. Finally, we implore all completed trial results to be made readily available so the research community can progress and learn from equally important positive and negative outcomes for the ultimate benefit of patients.

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COMPETING INTERESTS

A.S.Y. and S.E.O. are paid consultants for Artelo Biosciences and the UK Centre for Medicinal Cannabis. All other authors declare no competing interests.

CONTRIBUTORS

S.E.O. and S.A.M.: substantial contributions to the conception or design of the work. S.M.: writing of the manuscript. S.A.M., Z.D.B. and N.L.S.: database searching and data extraction. All authors: analysis and interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ORCID

S.A. Millar https://orcid.org/0000-0003-1318-7671

REFERENCES

1. WHO. Cannabidiol (CBD): World Health Organisation Expert Committee on Drug Dependence Thirty-ninth Meeting. 2017. Available from: https://www.who.int/medicines/access/controlled-substances/5.2_CBD.pdf. Accessed July 12, 2019.
2. Stout SM, Cimino NM. Exogenous cannabinoids as substrates, inhibitors, and inducers of human drug metabolizing enzymes: a systematic review. *Drug Metab Rev*. 2014;46(1):86-95. https://doi.org/10.3109/03602532.2013.849268
3. McPartland JM, Duncan M, Di Marzo V, Pertwee RG. Are cannabidiol and Delta(9) -tetrahydrocannabivarin negative modulators of the endocannabinoid system? A systematic review. *Br J Pharmacol*. 2015;172(3):737-753. https://doi.org/10.1111/bph.12944
4. Campos AC, Fogaca MV, Sonego AB, Guimaraes FS. Cannabidiol, neuroprotection and neuropsychiatric disorders. *Pharmacol Res*. 2016;112:119-127. https://doi.org/10.1016/j.phrs.2016.01.033
5. Crippa JA, Derenusson GN, Ferrari TB, et al. Neural basis of anxiolytic effects of cannabidiol (CBD) in generalized social anxiety disorder: a preliminary report. *J Psychopharmacol (Oxford, England)*. 2011;25(1):121-130. https://doi.org/10.1177/0269881110379283
6. Morgan CJ, Das RK, Joye A, Curran HV, Kamboj SK. Cannabidiol reduces cigarette consumption in tobacco smokers: preliminary findings. *Addict Behav*. 2013;38(9):2433-2436. https://doi.org/10.1016/j.addbeh.2013.03.011
7. Hazekamp A. The trouble with CBD oil. *Med Cannabis Cannabinoids*. 2018;1(1):65-72. https://doi.org/10.1159/000489287
8. Millar SA, Stone NL, Yates AS, O'Sullivan SE. A systematic review on the pharmacokinetics of Cannabidiol in humans. *Front Pharmacol*. 2018;9:1365. https://doi.org/10.3389/fphar.2018.01365
9. Walpole SC, Prieto-Merino D, Edwards P, Cleland J, Stevens G, Roberts I. The weight of nations: an estimation of adult human biomass. *BMC Public Health*. 2012;12(1):439. https://doi.org/10.1186/1471-2458-12-439
10. Shannon S, Opila-Lehman J. Effectiveness of Cannabidiol oil for pediatric anxiety and insomnia as part of posttraumatic stress disorder: a case report. *Perm J*. 2016;20(4):108-111. https://doi.org/10.7812/tpp/16-005

11. McGuire P, Robson P, Cubala WJ, et al. Cannabidiol (CBD) as an adjunctive therapy in schizophrenia: a multicenter randomized controlled trial. *Am J Psychiatry*. 2018;175(3):225-231. https://doi.org/10.1176/appi.ajp.2017.17030325
12. Thiele EA, Marsh ED, French JA, et al. Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet*. 2018. https://doi.org/10.1016/s0140-6736(18)30136-3
13. Devinsky O, Patel AD, Cross JH, et al. Effect of Cannabidiol on drop seizures in the Lennox-Gastaut syndrome. *N Engl J Med*. 2018;378(20):1888-1897. https://doi.org/10.1056/NEJMoa1714631
14. Boggs DL, Surti T, Gupta A, et al. The effects of cannabidiol (CBD) on cognition and symptoms in outpatients with chronic schizophrenia a randomized placebo controlled trial. *Psychopharmacology (Berl)*. 2018;235(7):1923-1932. https://doi.org/10.1007/s00213-018-4885-9
15. Naftali T, Mechulam R, Marii A, et al. Low-dose Cannabidiol is safe but not effective in the treatment for Crohn's disease, a randomized controlled trial. *Dig Dis Sci*. 2017;62(6):1615-1620. https://doi.org/10.1007/s10620-017-4540-z
16. Devinsky O, Cross JH, Wright S. Trial of Cannabidiol for drug-resistant seizures in the Dravet syndrome. *N Engl J Med*. 2017;377(7):699-700. https://doi.org/10.1056/NEJMc1708349
17. Jadoon KA, Ratcliffe SH, Barrett DA, et al. Efficacy and safety of Cannabidiol and Tetrahydrocannabivarin on glycemic and lipid parameters in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled, parallel group pilot study. *Diabetes Care*. 2016;39(10):1777-1786. https://doi.org/10.2337/dc16-0650
18. Chagas MH, Zuardi AW, Tumas V, et al. Effects of cannabidiol in the treatment of patients with Parkinson's disease: an exploratory double-blind trial. *J Psychopharmacol (Oxford, England)*. 2014;28(11):1088-1098. https://doi.org/10.1177/0269881114550355
19. Leweke FM, Piomelli D, Pahlisch F, et al. Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl Psychiatry*. 2012;2(3):e94. https://doi.org/10.1038/tp.2012.15
20. Bergamaschi MM, Queiroz RH, Chagas MH, et al. Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naive social phobia patients. *Neuropsychopharmacology*. 2011;36(6):1219-1226. https://doi.org/10.1038/npp.2011.6
21. Tomida I, Azuara-Blanco A, House H, Flint M, Pertwee RG, Robson PJ. Effect of sublingual application of cannabinoids on intraocular pressure: a pilot study. *J Glaucoma*. 2006;15(5):349-353. https://doi.org/10.1097/01.ijg.0000212260.04488.60
22. Notcutt W, Price M, Miller R, et al. Initial experiences with medicinal extracts of cannabis for chronic pain: results from 34 'N of 1′ studies. *Anaesthesia*. 2004;59(5):440-452. https://doi.org/10.1111/j.1365-2044.2004.03674.x
23. Consroe P, Laguna J, Allender J, et al. Controlled clinical trial of cannabidiol in Huntington's disease. *Pharmacol Biochem Behav*. 1991;40(3):701-708.
24. Cunha JM, Carlini EA, Pereira AE, et al. Chronic administration of cannabidiol to healthy volunteers and epileptic patients. *Pharmacology*. 1980;21(3):175-185.
25. clinicaltrials.gov. Study to Assess the Effect of Cannabidiol on Liver Fat Levels in Subjects With Fatty Liver Disease. Available from: https://clinicaltrials.gov/ct2/show/results/NCT01284634. Accessed July 12, 2019.
26. Rosenberg EC, Louik J, Conway E, Devinsky O, Friedman D. Quality of life in childhood epilepsy in pediatric patients enrolled in a prospective, open-label clinical study with cannabidiol. *Epilepsia*. 2017;58(8):e96-e100. https://doi.org/10.1111/epi.13815
27. Devinsky O, Marsh E, Friedman D, et al. Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial. *The Lancet Neurology*. 2016;15(3):270-278. https://doi.org/10.1016/s1474-4422(15)00379-8
28. Hess EJ, Moody KA, Geffrey AL, et al. Cannabidiol as a new treatment for drug-resistant epilepsy in tuberous sclerosis complex. *Epilepsia*. 2016;57(10):1617-1624. https://doi.org/10.1111/epi.13499
29. Yeshurun M, Shpilberg O, Herscovici C, et al. Cannabidiol for the prevention of graft-versus-host-disease after allogeneic hematopoietic cell transplantation: results of a phase II study. *Biol Blood Marrow Transplant*. 2015;21(10):1770-1775. https://doi.org/10.1016/j.bbmt.2015.05.018
30. Hallak JE, Machado-de-Sousa JP, Crippa JA, et al. Performance of schizophrenic patients in the Stroop color word test and electrodermal responsiveness after acute administration of cannabidiol (CBD). *Revista Brasileira de Psiquiatria (Sao Paulo, Brazil: 1999)*. 2010;32(1):56-61.
31. Zuardi AW, Crippa JA, Hallak JE, et al. Cannabidiol for the treatment of psychosis in Parkinson's disease. *J Psychopharmacol (Oxford, England)*. 2009;23(8):979-983. https://doi.org/10.1177/0269881108096519
32. Consroe P, Sandyk R, Snider SR. Open label evaluation of cannabidiol in dystonic movement disorders. *Int J Neurosci*. 1986;30(4):277-282.
33. Kaplan EH, Offermann EA, Sievers JW, Comi AM. Cannabidiol treatment for refractory seizures in Sturge-weber syndrome. *Pediatr Neurol*. 2017;71:18-23.e2. https://doi.org/10.1016/j.pediatrneurol.2017.02.009
34. Warren PP, Bebin EM, Nabors LB, Szaflarski JP. The use of cannabidiol for seizure management in patients with brain tumor-related epilepsy. *Neurocase*. 2017;23(5–6):287-291. https://doi.org/10.1080/13554794.2017.1391294
35. Gofshteyn JS, Wilfong A, Devinsky O, et al. Cannabidiol as a potential treatment for febrile infection-related epilepsy syndrome (FIRES) in the acute and chronic phases. *J Child Neurol*. 2017;32(1):35-40. https://doi.org/10.1177/0883073816669450
36. Saade D, Joshi C. Pure cannabidiol in the treatment of malignant migrating partial seizures in infancy: a case report. *Pediatr Neurol*. 2015;52(5):544-547. https://doi.org/10.1016/j.pediatrneurol.2015.02.008
37. Chagas MH, Eckeli AL, Zuardi AW, et al. Cannabidiol can improve complex sleep-related behaviours associated with rapid eye movement sleep behaviour disorder in Parkinson's disease patients: a case series. *J Clin Pharm Ther*. 2014;39(5):564-566. https://doi.org/10.1111/jcpt.12179
38. Crippa JA, Hallak JE, Machado-de-Sousa JP, et al. Cannabidiol for the treatment of cannabis withdrawal syndrome: a case report. *J Clin Pharm Ther*. 2013;38(2):162-164. https://doi.org/10.1111/jcpt.12018
39. Zuardi A, Crippa J, Dursun S, et al. Cannabidiol was ineffective for manic episode of bipolar affective disorder. *J Psychopharmacol (Oxford, England)*. 2010;24(1):135-137. https://doi.org/10.1177/0269881108096521
40. Zuardi AW, Morais SL, Guimaraes FS, Mechoulam R. Antipsychotic effect of cannabidiol. *J Clin Psychiatry*. 1995;56(10):485-486.
41. Zuardi AW, Hallak JE, Dursun SM, et al. Cannabidiol monotherapy for treatment-resistant schizophrenia. *J Psychopharmacol (Oxford, England)*. 2006;20(5):683-686. https://doi.org/10.1177/0269881106060967
42. Snider SR, Consroe P. Beneficial and adverse effects of cannabidiol in a Parkinson patient with sinemet-induced dystonic dyskinesia. *Neurology*. 1985;35:201.

43. Snider SR, Consroe P. Treatment of Meige's syndrome with cannabidiol. *Neurology*. 1984;34:147.

44. Paez Espinosa EV, Murad JP, Khasawneh FT. Aspirin: pharmacology and clinical applications. *Thrombosis*. 2012;2012:173124. https://doi.org/10.1155/2012/173124

45. Derry S, Moore RA. Single dose oral aspirin for acute postoperative pain in adults. *Cochrane Database Syst Rev*. 2012;(4):Cd002067. https://doi.org/10.1002/14651858.CD002067.pub2

46. Couch DG, Tasker C, Theophilidou E, Lund JN, O'Sullivan SE. Cannabidiol and palmitoylethanolamide are anti-inflammatory in the acutely inflamed human colon. *Clin Sci (London, England: 1979)*. 2017;131(21):2611-2626. https://doi.org/10.1042/cs20171288

47. Russo EB. Cannabinoids in the management of difficult to treat pain. *Ther Clin Risk Manag*. 2008;4(1):245-259.

48. Easterbrook PJ, Berlin JA, Gopalan R, Matthews DR. Publication bias in clinical research. *Lancet*. 1991;337(8746):867-872.

49. Zendulka O, Dovrtelova G, Noskova K, et al. Cannabinoids and cytochrome P450 interactions. *Curr Drug Metab*. 2016;17(3):206-226.

50. Gaston TE, Bebin EM, Cutter GR, Liu Y, Szaflarski JP. Interactions between cannabidiol and commonly used antiepileptic drugs. *Epilepsia*. 2017;58(9):1586-1592. https://doi.org/10.1111/epi.13852

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A systematic review on the pharmacokinetics of cannabidiol in humans

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Statement of personal contribution: performed systematic search and retrieval of articles and data extraction which was conducted independently from SM. Helped in editing the manuscript, confirming findings and input in the discussion.

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A Systematic Review on the Pharmacokinetics of Cannabidiol in Humans

Sophie A. Millar[1], Nicole L. Stone[1], Andrew S. Yates[2] and Saoirse E. O'Sullivan[1]*

[1] Division of Medical Sciences and Graduate Entry Medicine, School of Medicine, University of Nottingham, Royal Derby Hospital, Derby, United Kingdom, [2] Artelo Biosciences, San Diego, CA, United States

Background: Cannabidiol is being pursued as a therapeutic treatment for multiple conditions, usually by oral delivery. Animal studies suggest oral bioavailability is low, but literature in humans is not sufficient. The aim of this review was to collate published data in this area.

Methods: A systematic search of PubMed and EMBASE (including MEDLINE) was conducted to retrieve all articles reporting pharmacokinetic data of CBD in humans.

Results: Of 792 articles retireved, 24 included pharmacokinetic parameters in humans. The half-life of cannabidiol was reported between 1.4 and 10.9 h after oromucosal spray, 2–5 days after chronic oral administration, 24 h after i.v., and 31 h after smoking. Bioavailability following smoking was 31% however no other studies attempted to report the absolute bioavailability of CBD following other routes in humans, despite i.v formulations being available. The area-under-the-curve and C_{max} increase in dose-dependent manners and are reached quicker following smoking/inhalation compared to oral/oromucosal routes. C_{max} is increased during fed states and in lipid formulations. T_{max} is reached between 0 and 4 h.

Conclusions: This review highlights the paucity in data and some discrepancy in the pharmacokinetics of cannabidiol, despite its widespread use in humans. Analysis and understanding of properties such as bioavailability and half-life is critical to future therapeutic success, and robust data from a variety of formulations is required.

Keywords: pharmacokinetics, endocannabinoid system, bioavailability, CMAX, TMAX, half life, plasma clearance, volume of distribution

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****Correspondence:***
Sophie A. Millar
stxsamil@nottingham.ac.uk

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INTRODUCTION

The *Cannabis sativa* plant contains more than a hundred phytocannabinoid compounds, including the non-psychotomimetic compound cannabidiol (CBD) (Izzo et al., 2009). CBD has attracted significant interest due to its anti-inflammatory, anti-oxidative and anti-necrotic protective effects, as well as displaying a favorable safety and tolerability profile in humans (Bergamaschi et al., 2011), making it a promising candidate in many therapeutic avenues including epilepsy, Alzheimer's disease, Parkinson's disease, and multiple sclerosis. GW pharmaceuticals have developed an oral solution of pure CBD (Epidiolex®) for the treatment of severe, orphan, early-onset, treatment-resistant epilepsy syndromes, showing significant reductions in seizure frequency compared to placebo in several trials (Devinsky et al., 2017, 2018a; Thiele et al., 2018). Epidiolex® has recently

received US Food and Drug Administration (FDA) approval (GW Pharmaceuticals, 2018). CBD is also being pursued in clinical trials in Parkinson's disease, Crohn's disease, society anxiety disorder, and schizophrenia (Crippa et al., 2011; Leweke et al., 2012; Chagas et al., 2014; Naftali et al., 2017), showing promise in these areas. Additionally, CBD is widely used as a popular food supplement in a variety of formats for a range of complaints. It is estimated that the CBD market will grow to $2.1 billion in the US market in consumer sales by 2020 (Hemp Business, 2017).

From previous investigations including animal studies, the oral bioavailability of CBD has been shown to be very low (13–19%) (Mechoulam et al., 2002). It undergoes extensive first pass metabolism and its metabolites are mostly excreted via the kidneys (Huestis, 2007). Plasma and brain concentrations are dose-dependent in animals, and bioavailability is increased with various lipid formulations (Zgair et al., 2016). However, despite the breadth of use of CBD in humans, there is little data on its pharmacokinetics (PK). Analysis and understanding of the PK properties of CBD is critical to its future use as a therapeutic compound in a wide range of clinical settings, particularly regarding dosing regimens and routes of administration. Therefore, the aim of this systematic review was to collate and analyse all available CBD PK data recorded in humans and to highlight gaps in the literature.

METHODS

Search Strategy

The systematic review was carried out in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Moher et al., 2009). A systematic search of PubMed and EMBASE (including MEDLINE) was conducted to retrieve all articles reporting pharmacokinetic data of CBD in humans. Search terms included: CBD, cannabidiol, Epidiolex, pharmacokinetics, C_{max}, plasma concentrations, plasma levels, half-life, peak concentrations, absorption, bioavailability, AUC, T_{max}, C_{min}, and apparent volume of distribution. No restrictions were applied to type of study, publication year, or language. The searches were carried out by 14 March 2018 by two independent researchers.

Eligibility Criteria

The titles and abstracts of retrieved studies were examined by two independent researchers, and inappropriate articles were rejected. Inclusion criteria were as follows: an original, peer-reviewed paper that involved administration of CBD to humans, and included at least one pharmacokinetic measurement as listed in the search strategy.

Data Acquisition

The included articles were analyzed, and the following data extracted: sample size, gender, administration route of CBD, source of CBD, dose of CBD, and any pharmacokinetic details. Where available, plasma mean or median C_{max} (ng/mL) were plotted against CBD dose (mg). Similarly, mean or median T_{max} and range, and mean or median area under the curve (AUC_{0-t}) and SD were plotted against CBD dose (mg). The source/supplier of the CBD was also recorded. No further statistical analysis was possible due to sparsity of data and heterogeneity of populations used. All studies were assessed for quality using an amended version of the National Institute for Health (NIH), National Heart, Lung and Blood Institute, Quality Assessment Tool for Before-After (Pre-Post) Studies with No Control Group (National Institute for Health, 2014). A sample size of $\leq$10 was considered poor, between 11 and 19 was considered fair, and $\geq$20 was considered good (Ogungbenro et al., 2006).

Definitions of PK Parameters

T_{max}: Time to the maximum measured plasma concentration.
C_{max}: Maximum measured plasma concentration over the time span specified.
$t_{1/2}$: Final time taken for the plasma concentration to be reduced by half.
AUC_{0-t}: The area under the plasma concentration vs. time curve, from time zero to "t."
AUC_{0-inf}: The area under the plasma concentration vs. time curve from zero to t calculated as AUC_{0-t} plus the extrapolated amount from time t to infinity.
K_{el}: The first-order final elimination rate constant.

RESULTS

In total, 792 records were retrieved from the database searching, 24 of which met the eligibility criteria (**Figure 1**). **Table 1** summarizes each included study. Routes of administration included intravenous (i.v.) ($n = 1$), oromucosal spray ($n = 21$), oral capsules ($n = 13$), oral drops ($n = 2$), oral solutions ($n = 1$), nebuliser ($n = 1$), aerosol ($n = 1$), vaporization ($n = 1$), and smoking ($n = 8$). CBD was administered on its own in 9 publications, and in combination with THC or within a cannabis extract in the remainder. One study was conducted in children with Dravet syndrome, while the remainder were conducted in healthy adult volunteers (Devinsky et al., 2018b). Overall, the included studies were of good quality (**Supplementary Table 1**). However, many studies had small sample sizes. Additionally, not all studies included both males and females, and frequent cannabis smokers were included in a number of studies. Thus, interpretation and extrapolation of these results should be done with caution.

C_{max}, T_{max}, and Area Under the Curve

Within the 25 included studies, C_{max} was reported on 58 occasions (for example within different volunteer groups or doses in a single study), T_{max} on 56 occasions and area under the curve (AUC_{0-t}) on 45 occasions. These data from plasma/blood are presented in **Figures 2A–C**. The AUC_{0-t} and C_{max} of CBD is dose-dependent, and T_{max} occurs between 0 and 5 h, but does not appear to be dose-dependent.

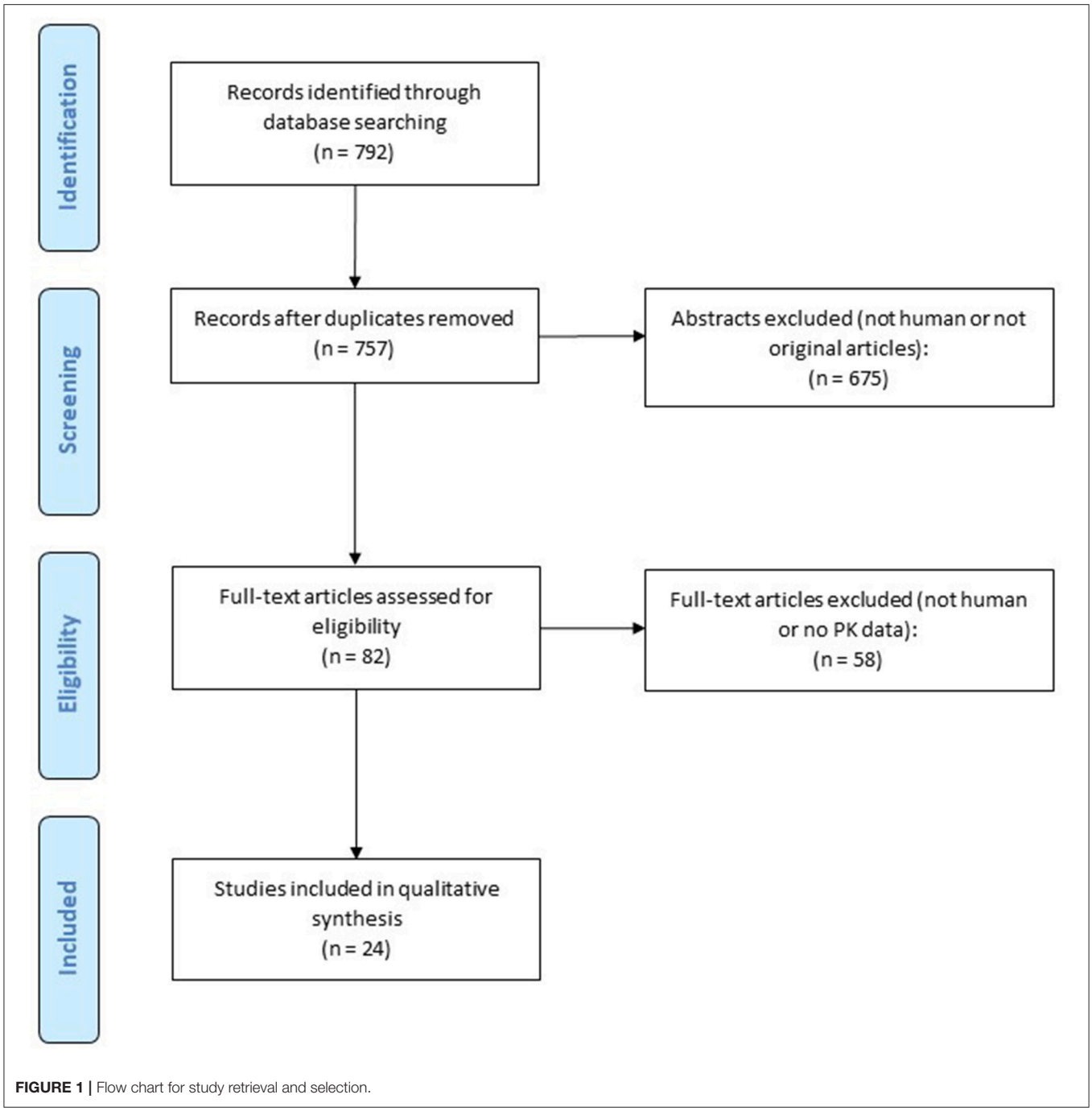

FIGURE 1 | Flow chart for study retrieval and selection.

Oromucosal Drops/Spray

A number of trials in humans were conducted by Guy and colleagues to explore administration route efficiency of sprays, an aerosol, and a nebuliser containing CBD or CBD and THC (CBD dose 10 or 20 mg) (Guy and Flint, 2004; Guy and Robson, 2004a,b). Oromucosal spray, either buccal, sublingual, or oropharyngeal administration, resulted in mean C_{max} between 2.5 and 3.3 ng/mL and mean T_{max} between 1.64 and 4.2 h. Sublingual drops resulted in similar C_{max} of 2.05 and 2.58 ng/mL and T_{max} of 2.17 and 1.67 h, respectively. Other oromucosal single dose studies reported C_{max} and T_{max} values within similar ranges (Karschner et al., 2011; Atsmon et al., 2017b).

Minimal evidence of plasma accumulation has been reported by chronic dosing studies over 5–9 days (Sellers et al., 2013; Stott et al., 2013a). C_{max} appears to be dose-dependent. A dose of 20 mg/day resulted in a mean C_{max} of 1.5 ng/mL and mean AUC_{0-t} of 6.1 h × ng/mL while 60 mg/day equated to a mean C_{max} of 4.8 ng/mL and AUC_{0-t} was 38.9 h × ng/mL (Sellers et al., 2013). In another study, C_{max} increased dose-dependently

TABLE 1 | Human studies reporting pharmacokinetic (PK) parameters for cannabidiol (CBD).

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
Ohlsson et al., 1986	5, M, infrequent to frequent cannabis smokers	i.v.	In lab	20 mg		686 (239)	ng/ml min × 10^{-3} = 16.67 (3.23)			24 (6)	74.4 (14.4)	Distribution volume: 32.7 (8.6) l/kg.
"		Smoking	In lab	19.2 ± 0.3mg		110 (55)	ng/ml min × 10^{-3} = 4.85 (1.72)			31 (4)		Estimated systemic availability (%) from smoking: 31 (13)
Consroe et al., 1991	15, M/F	Oral capsules	NIDA	10 mg/kg/day daily for 6 weeks						2–5 days		
Guy and Robson, 2004b	12, M/F	Oromucosal spray sublingual (CBD and THC)	GW	10 mg	1.63 (SD 0.68)	2.5 (1.83)	6.81 (4.33)	7.12 (4.31)		1.44 (0.79)		
		Oromucosal spray buccal (CBD and THC)	GW	10 mg	2.79 (SD 1.31)	3.02 (3.15)	6.4 (4.62)	6.8 (4.46)		1.81 (2.05)		
		Oromucosal spray oro-pharyngeal (CBD and THC)	GW	10 mg	2.04 (SD 1.13)	2.61 (1.91)	7.81 (5.13)	8.28 (5.32)		1.76 (0.8)		
		CBME oral capsule (CBD and THC)	GW	10 mg	1.27 (SD 0.84)	2.47 (2.23)	5.76 (4.94)	6.03 (4.97)		1.09 (0.46)		
Guy and Robson, 2004a	24, M	Oromucosal spray sublingual (CBD and THC)	GW	10 mg	4.22	3.33	11.34	11.97		1.81		
Guy and Flint, 2004	6 M/F	Nebuliser (CBD and THC)	GW	20 mg	0.6 (0.08–1)	9.49 (8.01)	9.41 (10.8)	12.11 (10.83)	0.98 (0.58)	1.1 (0.97)		
		Aerosol (with THC)	GW	20 mg	2.35 (0.75–6)	2.6 (1.38)	5.43 (5.88)	13.53 (3.64)	0.43 (0.26)	2.4 (2.02)		
		Sublingual drops (CBD)	GW	20 mg	2.17 (1–4)	2.05 (0.92)	2.60 (3.45)					
		Sublingual drops (CBD and THC)	GW	20 mg	1.67 (1–3)	2.58 (0.68)	3.49 (2.65)	9.65 (4.02)	0.37 (0.114)	1.97 (0.62)		
Nadulski et al., 2005a	24, M/F	Oral capsule (CBD and THC)	Scherer GmbH & Co. KG, Eberbach, Germany	5.4mg once a week for 3 weeks	Mean 0.99 (0.5–2)	0.93 (range 0–2.6)	Mean 4.35, range (2.7–5.6)					

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
	12, M/F	Oral capsule (CBD and THC) and breakfast consumed 1 hour after	Scherer GmbH & Co. KG, Eberbach, Germany	5.4mg once a week for 3 weeks	Mean 1.07 (0.5–2)	1.13 (range 0.39–1.9)	Mean 4.4 (range 2.5–5.3)					
Nadulski et al., 2005b	24, M/F	Cannabis extract	Sigma	5.4 mg	Mean 1.0 (0.5–2.0)	0.95 (range 0.3–2.57)						
Karschner et al., 2011	9, M/F cannabis smokers	Oromucosal spray (Sativex: CBD and THC)	GW	5 mg	3.6 (1.0–5.5)	Mean (SE): 1.6 (0.4)	4.5 (SE 0.6)					
				15 mg	4.6 (1.2–5.6)	Mean (SE): 6.7 (2.0)	18.1 (SE 3.6)					
Schwope et al., 2011	10, M/F, usual infrequent cannabis smokers	Cannabis cigarette	NIDA	2 mg	0.25 (0.25–0.50 h) whole blood/plasma	Median (range): plasma 2 (<LOQ−3.4)						
Eichler et al., 2012	9, M	Oral capsules (CBD and THC)	Cannapharm AG	Heated CBD (27.8 mg CBD: 0.8 mg CBDA)	0.83 (SD 0.17)	pmol/mL: 0.94 (0.22)	pmol h/moL 3.68 (1.34)					
				Unheated 14.8 mg CBD:10.8 mg CBDA)	1.17 (SD 0.39)	3.95 (0.92) pmoL/mL	pmol h/mol 7.67 (2.06)					
Lee et al., 2012	10, M/F, cannabis smokers	Cannabis cigarette	NIDA	2 mg	Median 0.25 (oral fluid)	0.03 (oral fluid)						
Sellers et al., 2013	60, M/F	Oromucosal spray (CBD and THC)	GW	20 mg, 5 days	1.4 (0, 8.45)	1.5 (0.78)	6.1 (5.76)	14.8 (7.87)				
	51, M/F			90 mg – 60 mg, 5 days	1.5 (0–6.45)	4.8 (3.4)	38.9 (33.75)	60.3 (37.71)				
Stott et al., 2013b	12, M	Oromucosal spray (CBD and THC)	GW	10 mg (fed state)	4.00 (3.02–9.02);	3.66 (2.28)	23.13 (9.29)	20.21 (8.43)	0.155 (0.089)	5.49 (2.17)	533 (318)	
Stott et al., 2013a	24, M	Oromucosal spray (CBD and THC)	GW	5 mg single dose	Mean 1.00 (0.75–1.50)	0.39 (0.08)	0.82 (0.33)	1.66 (0.51)	0.173 (0.084)	5.28 (3.28)	3,252 (1,002)	
				10 mg single dose	Mean 1.39 (0.75–2.25)	1.15 (0.74)	4.53 (3.53)	5.64 (4.09)	0.148 (0.079)	6.39 (4.48)	2,546 (1,333)	
				20 mg single dose	Mean 1.00 (0.75–1.75)	2.17 (1.23)	9.94 (9.02)	13.28 (12.86)	0.123 (0.097)	9.36 (6.81)	3,783 (4,299)	

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
				5 mg, 9 days	Mean 1.64 (1.00–4.02)	0.49 (0.21)	2.52 (0.73)					
				10 mg, 9 days	Mean 1.27 (0.75–2.52)	1.14 (0.86)	6.66 (3.10)					
				20 mg 9 days	Mean 2.00 (1.02–6.00)	3.22 (1.90)	20.34 (7.29)					
Stott et al., 2013c	36, M	Oromucosal spray (CBD and THC)	GW	10 mg (3 groups)	1.00 (0.50–4.00); 1.38 (0.75–6.00); 1.15 (0.50–3.02)	1.03 (0.81); 0.66 (0.37); 0.63 (0.43)	3.23 (2.13); 1.82 (1.03); 1.83 (1.19)	5.10 (3.06); 3.54 (0.80); 3.00 (1.43)	0.148(0.108); 0.122 (0.111); 0.224 (0.158)	10.86(12.71); 7.81 (3.00); 5.22 (4.51)	2817 (1913); 2998 (896); 4,741 (3,835)	Varea/F (L): 28312 (19355); 31994 (12794); 26298 (14532)
				15 mg	4.5 (1.2–5.6)	Mean (SE): 6.7 (2.0)						
Newmeyer et al., 2014	24, M/F, frequent or occasional cannabis smokers	Cannabis cigarette (frequent smokers)	NIDA	2 ± 0.6 mg	0.5 (0.5–1)	Median (range): 14.8 (1.4–162)	Median (range): 29 (4.7–211)					
		Cannabis cigarette (occasional smokers)		2 ± 0.6 mg	1 (0.5–2)	Median (range): 7 (1.9–111)	Median (range): 11.6 (4.1–185)					
Desrosiers et al., 2014	21, M/F frequent and occasional smokers	Cannabis cigarette (frequent smokers)	NIDA	2 mg	0.5 (0.0–1.1)	1.1 (0.0–1.6)						
		Cannabis cigarette (occasional smokers)		2 mg	0 (0–500)	0 (0–1300)						
Manini et al., 2015	17, M/F	Oral capsules Co-administered with i.v. fentanyl	GW	400 mg	3 and 1.5 (plasma) and 6 and 2 (urine)	Plasma: 181.2 (39.8) and 114.2 (9.5); Urine: 4600 and 2900	704 (283) and 482 (314) mcg*hr/dL					
				800 mg	3 and 4 (plasma) and 4 and 6 (urine)	Plasma: 221 (35.6) and 157.1 (49.0); Urine: 3700 and 2800	867 (304) and 722 (443) mcg*hr/dL					

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details							Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	AUC_{0-t} (mean, SD)[b] h × ng/mL	AUC_{0-inf} (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
Haney et al., 2016	8, M/F cannabis smokers	Oral capsules	STI pharmaceuticals	800 mg	Mean 3 (2–6)	77.9 (range 1.6–271.9)						
Cherniakov et al., 2017a	9, M	Oral capsules with piperine pro-nanolipospeheres (CBD and THC)	STI pharmaceuticals	10 mg	1 (0.5–1.5)	2.1 (0.4)	6.9 (1.3)					
		Oromucosal spray (CBD and THC; Sativex®)		10 mg	3 (1–5)	0.5 (0.1)	3.1 (0.4)					
Swortwood et al., 2017	20, M/F Cannabis smokers	Cannabis cigarettes – frequent smokers	NIDA	1.5 mg	Mean 0.29 (0.17–1.5) (oral fluid)	93.3 (range 0.65–350) (oral fluid)						
		Cannabis cigarettes – occasional smokers	NIDA	1.5 mg	Mean 0.17 (oral fluid)	55.9 (range 2.5–291) (oral fluid)						
		Cannabis containing brownie – frequent smokers	NIDA	1.5 mg	Mean 0.53 (0.17–1.5) (oral fluid)	8.0 (range 0.48–26.3) (oral fluid)						
		Cannabis containing brownie – occasional smokers	NIDA	1.5 mg	Mean 0.47 (0.17–1.5) (oral fluid)	5.9 (range 2.1–11.4) (oral fluid)						
		Vaporization – frequent smokers	Volcano® Medic, Storz & Bickel, Tuttlingen, Germany	1.5 mg	Mean 0.29 (0.17–1.5) (oral fluid)	76.3 (range 2.3–339) (oral fluid)						
		Vaporization – occasional smokers	Volcano® Medic, Storz & Bickel, Tuttlingen, Germany	1.5 mg	Mean 0.17 (oral fluid)	28.2 (range 0.23–167) (oral fluid)						
Atsmon et al., 2017b	15, M	CBD extract >93% in a PTL101 formulation (oral gelatin matrix pellet technology) Sublingual/buccal	AiFame-AiLab GmbH (CBD), Gelpell AG (capsules)	10 mg	3.0 (2.0–4.0)	3.22 (1.28)	9.64 (3.99)	10.31 (4.14)		2.95 (2.58)		
				100 mg	3.5 (1.5–5.0)	47.44 (20.14)	149.54 (34.34)	153.04 (34.7)		3.59 (0.26)		

(Continued)

TABLE 1 | Continued

References	Total n, sex	Administration	Source	CBD dose	Plasma[a] PK details						Other
					T_{max} (median, range)[b] hrs	C_{max} (mean, SD)[b] ng/mL	$AUC_{0\text{-}t}$ (mean, SD)[b] h × ng/mL	$AUC_{0\text{-}inf}$ (mean, SD) h × ng/mL	K_{el} (mean, SD) 1/h	$t_{1/2}$ (mean, SD)[b] h	CL/F (mean, SD) L/h	Mean (SD)
		Oromucosal spray (CBD and THC)	GW	10 mg	3.5 (1.0–5.0)	2.05 (1.1)	7.3 (2.86)	7.81 (2.81)	0.33 (0.09)	2.31 (0.72)		
Atsmon et al., 2017a	15, M	CBD and THC in a PTL401 capsule (self-emulsifying oral drug delivery system)	STI pharmaceuticals	10 mg	1.25 (0.5–4.0)	2.94 (0.73)	9.85 (4.47)	10.52 (4.53)	0.29 (0.17)	3.21 (1.62)		
Devinsky et al., 2018b	34, children	Oral solution	GW	2.5 mg			70.23 (mean from 3 groups)					
				5 mg/kg/day			241					
				10 mg/kg/day			722					
				20 mg/kg/day			963					

[a,b] *Unless otherwise stated. PK, pharmacokinetics; CBD, cannabidiol; THC, Tetrahydrocannabinol; M, male; F, female; AUC, area under the curve; Conc., concentration; GW, GW pharmaceuticals; NIDA, US national institute on drug abuse; LOQ, limit of quantification; IV, intravenous; CBME, cannabis based medicine extract; Min(s), min(s).*

from 0.4 to 1.2 and 2.2 ng/mL following 5, 10, and 20 mg single doses, respectively, and from 0.5 to 1.1 and 3.2 ng/mL, respectively following chronic dosing over 9 consecutive days (Stott et al., 2013a). There was a significant increase in time-dependent exposure during the chronic treatment. Mean AUC_{0-t} for the single doses were 0.8, 4.5, 9.9, and 2.5, 6.7, and 20.3 for the chronic dosing schedule, respectively. T_{max} does not appear to be dose-dependent, nor affected by acute or chronic dosing schedules.

Stott et al. reported an increase in CBD bioavailability under fed vs. fasted states in 12 men after a single 10 mg dose of CBD administered through an oromucosal spray which also contained THC (Stott et al., 2013a,b). Mean AUC and C_{max} were 5- and 3-fold higher during fed conditions compared to fasted (AUC_{0-t} 23.1 vs. 4.5; C_{max} 3.7 vs. 1.2 ng/mL). T_{max} was also delayed under the fed state (4.0 vs. 1.4 h).

In children, Devinsky et al. reported mean AUC as 70, 241, 722, and 963 h × ng/mL in groups receiving 2.5, 5, 10, and 20 mg/Kg/day of CBD in oral solution (Devinsky et al., 2018b).

Oral Intake

C_{max} and AUC following oral administration also appears to be dose dependent. A dose of 10 mg CBD resulted in mean C_{max} of 2.47 ng/mL at 1.27 h, and a dose of 400 or 800 mg co-administered with i.v. fentanyl (a highly potent opioid) to examine its safety resulted in a mean C_{max} of 181 ng/mL (at 3.0 h) and 114 ng/mL (at 1.5 h) for 400 mg, and 221 ng/mL (at 3.0 h) and 157 ng/mL (at 4.0 h) for 800 mg, in 2 sessions, respectively (Guy and Robson, 2004b; Manini et al., 2015). A dose of 800 mg oral CBD in a study involving 8 male and female cannabis smokers, reported a mean C_{max} of 77.9 ng/mL and mean T_{max} of 3.0 h (Haney et al., 2016). Although, an increase in dose corresponds with an increase in C_{max}, the C_{max} between the higher doses of CBD does not greatly differ, suggesting a saturation effect (e.g., between 400 and 800 mg).

One hour after oral capsule administration containing 5.4 mg CBD in males and females, mean C_{max} was reported as 0.93 ng/mL (higher for female participants than male) (Nadulski et al., 2005a). A subset ($n = 12$) consumed a standard breakfast meal 1 h after the capsules, which slightly increased mean C_{max} to 1.13 ng/mL. CBD remained detectable for 3–4 h after administration (Nadulski et al., 2005b).

Cherniakov et al. examined the pharmacokinetic differences between an oromucosal spray and an oral capsule with piperine pro-nanolipospheres (PNL) (both 10 mg CBD) in 9 men. The piperine-PNL oral formulation had a 4-fold increase in C_{max} (2.1 ng/mL vs. 0.5 ng/mL), and a 2.2-fold increase in AUC_{0-t} (6.9 vs. 3.1 h × ng/mL), while T_{max} was decreased (1.0 vs. 3.0 h) compared to the oromucosal spray (Cherniakov et al., 2017a). This group further developed self-emulsifying formulations and reported again an increased bioavailability and increased C_{max} within a shorter time compared to a reference spray (Atsmon et al., 2017a,b).

Intravenous Administration

The highest plasma concentrations of CBD were reported by Ohlsson et al. following i.v. administration of 20 mg of

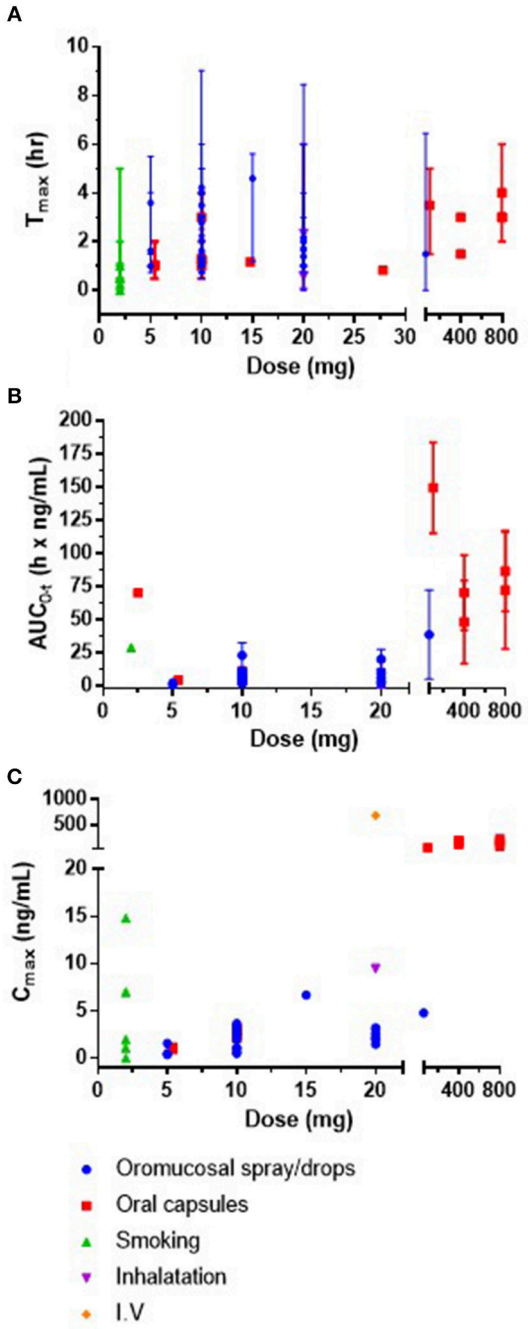

FIGURE 2 | (A) Mean or median Tmax (h) and range against CBD dose (mg) **(B)** mean or median area under the curve (AUC0-t) (h × ng/mL) and SD against CBD dose (mg) and **(C)** plasma mean or median concentration max (Cmax; ng/mL) against CBD dose (mg). It was not possible to present error bars for Cmax as SD and SEM were both reported in the data. IV, intravenous; SD, standard deviation; SEM, standard error of the mean.

deuterium-labeled CBD. Mean plasma CBD concentrations were reported at 686 ng/mL (3 min post-administration), which dropped to 48 ng/mL at 1 h.

Controlled Smoking and Inhalation

After smoking a cigarette containing 19.2 mg of deuterium-labeled CBD, highest plasma concentrations were reported as 110 ng/mL, 3 min post dose, which dropped to 10.2 ng/ml 1 h later (Ohlsson et al., 1986). Average bioavailability by the smoked route was 31% (Ohlsson et al., 1986). A nebuliser resulted in a C_{max} of 9.49 ng/mL which occurred at 0.6 h, whereas aerosol administration produced C_{max} (2.6 ng/mL) at 2.35 h (Guy and Flint, 2004). In 10 male and female usual, infrequent cannabis smokers, C_{max} was 2.0 ng/mL at 0.25 h after smoking a cigarette containing 2 mg of CBD (Schwope et al., 2011). CBD was detected in 60% of whole blood samples and in 80% of plasma samples at observed C_{max}, and no longer detected after 1.0 h. A study in 14 male and female cannabis smokers reported 15.4% detection in frequent smokers with no CBD detected in occasional smokers in whole blood analysis (Desrosiers et al., 2014). In plasma however, there was a 53.8 and 9.1% detection in the frequent and occasional groups, with corresponding C_{max} of 1.1 ng/mL in the frequent group, and below limits of detection in the occasional group.

Half-Life

The mean half-life ($t_{1/2}$) of CBD was reported as 1.1 and 2.4 h following nebuliser and aerosol administration (20 mg) (Guy and Flint, 2004), 1.09 and 1.97 h following single oral administration (10 and 20 mg) (Guy and Flint, 2004; Guy and Robson, 2004b), 2.95 and 3.21 h following 10 mg oral lipid capsules (Atsmon et al., 2017a,b), between 1.44 and 10.86 h after oromucosal spray administration (5–20 mg) (Guy and Robson, 2004b; Sellers et al., 2013; Stott et al., 2013a,b; Atsmon et al., 2017b), 24 h after i.v. infusion, 31 h after smoking (Ohlsson et al., 1986), and 2–5 days after chronic oral administration (Consroe et al., 1991).

Elimination Rate

Mean elimination rate constant (K_{el} [1/h]) has been reported as 0.148 in fasted state, and 0.155 in fed state after 10 mg CBD was administered in an oromucosal spray also containing THC (Stott et al., 2013a,b). After single doses of 5 and 20 mg CBD, mean K_{el} (1/h) was reported as 0.173 and 0.123 (Stott et al., 2013a). Following 20 mg CBD administration through a nebuliser and pressurized aerosol, mean K_{el} was reported as 0.98 and 0.43, respectively, while 20 mg CBD administered as sublingual drops was reported as 0.37 (Guy and Flint, 2004).

Plasma Clearance

Plasma apparent clearance, CL/F (L/h) has been reported to range from 2,546 to 4,741 in a fasted stated following 10 mg CBD administered via oromucosal spray (Stott et al., 2013a,c). This value decreases to 533 following the same concentration in a fed state (Stott et al., 2013b). A plasma apparent clearance of 3,252 and 3,783 was reported following 5 and 20 mg single doses of CBD via oromucosal spray (Stott et al., 2013a). Ohlsson et al. reported plasma apparent clearance as 74.4 L/h following i.v. injection (Ohlsson et al., 1986).

Volume of Distribution

Mean apparent volume of distribution (V/F [L]) was reported as 2,520 L following i.v. administration (Ohlsson et al., 1986). Following single acute doses through oromucosal spray administration, apparent volume of distribution was reported as 26,298, 31,994, and 28,312 L (Stott et al., 2013a).

DISCUSSION

The aim of this study was to review and analyse all available PK data on CBD in humans. Only 8 publications reported PK parameters after administering CBD on its own, and the others were in combination with THC/cannabis. Only 1 study reported the bioavailability of CBD in humans (31% following smoking). From the analysis of these papers, the following observations were made; peak plasma concentrations and area under the curve (AUC) are dose-dependent and show minimal accumulation; C_{max} is increased and reached faster following i.v., smoking or inhalation; C_{max} is increased and reached faster after oral administration in a fed state or in a pro-nanoliposphere formulation; T_{max} does not appear to be dose-dependent; and half-life depends on dose and route of administration. Overall, considerable variation was observed between studies, although they were very heterogeneous, and further work is warranted.

Human studies administering CBD showed that the AUC_{0-t} and C_{max} are dose-dependent, and T_{max} mostly occurred between 1 and 4 h. Animal studies in piglets, mice, and rats also all demonstrate a dose-dependent relationship between CBD and both plasma and brain concentrations (Long et al., 2012; Hammell et al., 2016; Garberg et al., 2017), suggesting that human brain concentrations will also be dose-dependent. Ten publications in this review reported the half-life of CBD which ranged from 1 h to 5 days and varies depending on the dose and route of administration. Very limited data was available for detailed analysis on the elimination rate, apparent clearance or distribution of CBD in humans.

Plasma levels of CBD were increased when CBD was administered with food or in a fed state, or when a meal is consumed post-administration. Oral capsules with piperine pro-nanolipospheres also increased AUC and C_{max}. This is also demonstrated in animal studies; co-administration of lipids with oral CBD increased systemic availability by almost 3-fold in rats (Zgair et al., 2016) and a pro-nanoliposphere formulation increased oral bioavailability by about 6-fold (Cherniakov et al., 2017b). As CBD is a highly lipophilic molecule, it is logical that CBD may dissolve in the fat content of food, increasing its solubility, and absorption and therefore bioavailability as demonstrated by numerous pharmacological drugs (Winter et al., 2013). Thus, it may be advisable to administer CBD orally in a fed state to allow for optimal absorption.

Only one study used intravenous administration of CBD and reported PK details, which could be a beneficial route of administration in some acute indications. Results from other routes such as rectal, transdermal, or intraperitoneal have also not been published in humans, although transdermal CBD gel and topical creams have been demonstrated to be successful in animal studies (Giacoppo et al., 2015; Hammell et al., 2016). Interestingly, intraperitoneal (i.p.) injection of CBD corresponded to higher plasma and brain concentrations than oral administration in mice, however in rats, similar concentrations were observed for both administration routes, and brain concentrations were in fact higher following oral compared to i.p. route (Deiana et al., 2012). No published data exists on the tissue distribution of CBD in humans. Although plasma levels of CBD do not show accumulation with repeated dosing, it is possible that there may be tissue accumulation.

Only one study in this review was conducted in children ($n = 34$) (Devinsky et al., 2018b). Children (4–10 years) with Dravet syndrome were administered an oral solution of CBD and AUC was reported to increase dose-dependently. It is important to emphasize the statement that children are not small adults, and there are many differences in their pharmacokinetic and pharmacodynamic profiles. Absorption, excretion, metabolism, and plasma protein binding are generally reduced in children compared to adults, and apparent volume of distribution is generally increased (Fernandez et al., 2011). These parameters need to be explored fully for CBD in order to understand and advise dose adjustments.

Within the adult studies, inter- and intra-subject variability was observed in studies, and it remains to be seen whether i.v. and other routes of administration that by-pass initial metabolism will alleviate this issue. Interestingly, although each of the subject's weight was taken into account, none of the studies addressed subject fat content as a factor in their exclusion criteria; as muscle can weigh more than the same proportion of fat. It is well-known that cannabinoids are highly lipophilic compounds and accumulate in fatty tissue which can then be released gradually (Gunasekaran et al., 2009). It may be of benefit in future study to either put in place more stringent exclusion criteria and measure subject fat content or assess the possible accumulation of CBD in fatty tissue. Differences in metabolism, distribution and accumulation in fat, and in biliary and renal elimination may be responsible for prolonged elimination half-life and variable pharmacokinetic outcomes. CBD use is widespread and has been recommended for use by the FDA in childhood-onset epilepsy. CBD also displays therapeutic promise in other disorders such as schizophrenia and post-traumatic stress disorder. If we are to understand the actions of CBD in those disorders and increase the success rate for treatment, these groups of patients and their distinct characteristics must be assessed as they may not be comparable to a healthy volunteer population.

A systematic review in 2014 concluded that CBD generally has a low risk of clinically significant drug-interactions (Stout and Cimino, 2014). A few studies in the current review included examination of drug-drug interactions with CBD. GW Pharmaceuticals performed a clinical trial investigating the pharmacokinetic interaction between CBD/THC spray (sativex) and rifampicin (cytochrome P450 inducer), ketoconazole, and omeprazole (cytochrome P450 inhibitors) (Stott et al., 2013c). Authors concluded overall that CBD in combination with the drugs were well-tolerated, but consideration should be noted when co-administering with other drugs using the CYP3A4

pathway. Caution is also advised with concomitant use of CBD and substrates of UDP-glucuronosyltransferases UGT1A9 and UGT2B7, and other drugs metabolized by the CYP2C19 enzyme (Al Saabi et al., 2013; Jiang et al., 2013). Manini et al. co-administered CBD with i.v. fentanyl (a high potency opioid) which was reported as safe and well-tolerated (Manini et al., 2015). In a number of trials with CBD in children with severe epilepsy, clobazam concentrations increased when CBD was co-administered and dosage of clobazam had to be reduced in some patients in one study (Geffrey et al., 2015; Devinsky et al., 2018b). Gaston and colleagues performed a safety study in adults and children in which CBD was administered with commonly-used anti-epileptic drugs (AEDs) (Gaston et al., 2017). Most changes in AED concentrations were within acceptable ranges but abnormal liver function tests were reported in those taking valproate and authors emphasized the importance of continued monitoring of AED concentrations and liver function during treatment with CBD.

Limitations of this review should be acknowledged. Different population types including healthy and patient populations and cannabis naïve or not were all grouped together which may impede generalizability. The proportions of men and women in each study were also not uniform, and it is still being elucidated whether men and women have distinct pharmacokinetic profiles with regards to cannabinoids (Fattore and Fratta, 2010). One study suggested that the PK of CBD was different in their female volunteers (Nadulski et al., 2005a). It should also be mentioned that CBD is currently not an approved product with a pharmacopeia entry so using different sources of CBD that are subject to different polymeric forms, different particle sizes, and different purities may also affect the PK profiles observed. It is important for future work that researchers record the source of the CBD material used so that results have the highest chance of being replicated. Despite a thorough search of the two databases chosen, the addition of more databases may have widened the search to increase the number of results and hence improve the reliability and validity of the findings. However, the review was carried out by two independent reviewers, and searches generated were analyzed separately and then compared.

In conclusion, this review demonstrates the lack of research in this area, particularly in routes of administration other than oral. An absence of studies has led to failure in addressing the bioavailability of CBD despite intravenous formulations being available. This is of critical importance due to the popularity of CBD products and will help interpret other PK values. Standardized and robust formulations of CBD and their PK data are required for both genders, with consideration of other factors such as adiposity, genetic factors that might influence absorption and metabolism, and the effects of disease states.

AUTHOR CONTRIBUTIONS

SM, SO, and AY: substantial contributions to the conception or design of the work. SM: writing of the manuscript. SM and NS: database searching and data extraction. All authors: the analysis and interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01365/full#supplementary-material

REFERENCES

Al Saabi, A., Allorge, D., Sauvage, F. L., Tournel, G., Gaulier, J. M., Marquet, P., et al. (2013). Involvement of UDP-glucuronosyltransferases UGT1A9 and UGT2B7 in ethanol glucuronidation, and interactions with common drugs of abuse. *Drug Metab. Dispos.* 41, 568–574. doi: 10.1124/dmd.112.047878

Atsmon, J., Cherniakov, I., Izgelov, D., Hoffman, A., Domb, A. J., Deutsch, L., et al. (2017a). PTL401, a new formulation based on pro-nano dispersion technology, improves oral cannabinoids bioavailability in healthy volunteers. *J. Pharm. Sci.* 107, 1423–1429. doi: 10.1016/j.xphs.2017.12.020

Atsmon, J., Heffetz, D., Deutsch, L., Deutsch, F., and Sacks, H. (2017b). Single-Dose pharmacokinetics of oral cannabidiol following administration of PTL101: a new formulation based on gelatin matrix pellets technology. *Clin. Pharmacol. Drug Dev.* 7:751–758. doi: 10.1002/cpdd.408

Bergamaschi, M. M., Queiroz, R. H., Zuardi, A. W., and Crippa, J. A. (2011). Safety and side effects of cannabidiol, a *Cannabis sativa* constituent. *Curr. Drug Saf.* 6, 237–249. doi: 10.2174/157488611798280924

Chagas, M. H., Zuardi, A. W., Tumas, V., Pena-Pereira, M. A., Sobreira, E. T., Bergamaschi, M. M., et al. (2014). Effects of cannabidiol in the treatment of patients with Parkinson's disease: an exploratory double-blind trial. *J. Psychopharmacol.* 28, 1088–1098. doi: 10.1177/0269881114550355

Cherniakov, I., Izgelov, D., Barasch, D., Davidson, E., Domb, A. J., and Hoffman, A. (2017a). Piperine-pro-nanolipospheres as a novel oral delivery system of cannabinoids: pharmacokinetic evaluation in healthy volunteers in comparison to buccal spray administration. *J. Control. Release* 266, 1–7. doi: 10.1016/j.jconrel.2017.09.011

Cherniakov, I., Izgelov, D., Domb, A. J., and Hoffman, A. (2017b). The effect of Pro NanoLipospheres (PNL) formulation containing natural absorption enhancers on the oral bioavailability of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) in a rat model. *Eur. J. Pharm. Sci.* 109, 21–30. doi: 10.1016/j.ejps.2017.07.003

Consroe, P., Kennedy, K., and Schram, K. (1991). Assay of plasma cannabidiol by capillary gas chromatography/ion trap mass spectroscopy following high-dose repeated daily oral administration in humans. *Pharmacol. Biochem. Behav.* 40, 517–522. doi: 10.1016/0091-3057(91)90357-8

Crippa, J. A., Derenusson, G. N., Ferrari, T. B., Wichert-Ana, L., Duran, F. L., Martin-Santos, R., et al. (2011). Neural basis of anxiolytic effects of cannabidiol (CBD) in generalized social anxiety disorder: a preliminary report. *J. Psychopharmacol.* 25, 121–130. doi: 10.1177/0269881110379283

Deiana, S., Watanabe, A., Yamasaki, Y., Amada, N., Arthur, M., Fleming, S., et al. (2012)Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Delta(9)-tetrahydrocannabivarin (THCV) and

cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behaviour. *Psychopharmacology* 219, 859–873. doi: 10.1007/s00213-011-2415-0

Desrosiers, N. A., Himes, S. K., Scheidweiler, K. B., Concheiro-Guisan, M., Gorelick, D. A., and Huestis, M. A. (2014). Phase I and II cannabinoid disposition in blood and plasma of occasional and frequent smokers following controlled smoked cannabis. *Clin. Chem.* 60, 631–643. doi: 10.1373/clinchem.2013.216507

Devinsky, O., Cross, J. H., and Wright, S. (2017). Trial of Cannabidiol for drug-resistant seizures in the Dravet Syndrome. *N. Engl. J. Med.* 377, 699–700. doi: 10.1056/NEJMc1708349

Devinsky, O., Patel, A. D., Cross, J. H., Villanueva, V., Wirrell, E. C., Privitera, M., et al. (2018a). Effect of Cannabidiol on drop seizures in the Lennox-Gastaut Syndrome. *N. Engl. J. Med.* 378, 1888–1897. doi: 10.1056/NEJMoa1714631

Devinsky, O., Patel, A. D., Thiele, E. A., Wong, M. H., Appleton, R., Harden, C. L., et al. (2018b). Randomized, dose-ranging safety trial of cannabidiol in Dravet syndrome. *Neurology* 90, e1204–e1211. doi: 10.1212/WNL.0000000000005254

Eichler, M., Spinedi, L., Unfer-Grauwiler, S., Bodmer, M., Surber, C., Luedi, M., et al. (2012). Heat exposure of *Cannabis sativa* extracts affects the pharmacokinetic and metabolic profile in healthy male subjects. *Planta Med.* 78, 686–691. doi: 10.1055/s-0031-1298334

Fattore, L., and Fratta, W. (2010). How important are sex differences in cannabinoid action? *Br. J. Pharmacol.* 160, 544–548. doi: 10.1111/j.1476-5381.2010.00776.x

Fernandez, E., Perez, R., Hernandez, A., Tejada, P., Arteta, M., and Ramos, J. T. (2011). Factors and mechanisms for pharmacokinetic differences between pediatric population and adults. *Pharmaceutics* 3, 53–72. doi: 10.3390/pharmaceutics3010053

Garberg, H. T., Solberg, R., Barlinn, J., Martinez-Orgado, J., Loberg, E. M., and Saugstad, O. D. (2017). High-dose cannabidiol induced hypotension after global hypoxia-ischemia in piglets. *Neonatology* 112, 143–149. doi: 10.1159/000471786

Gaston, T. E., Bebin, E. M., Cutter, G. R., Liu, Y., and Szaflarski, J. P. (2017). Interactions between cannabidiol and commonly used antiepileptic drugs. *Epilepsia* 58, 1586–1592. doi: 10.1111/epi.13852

Geffrey, A. L., Pollack, S. F., Bruno, P. L., and Thiele, E. A. (2015). Drug-drug interaction between clobazam and cannabidiol in children with refractory epilepsy. *Epilepsia* 56, 1246–1251. doi: 10.1111/epi.13060

Giacoppo, S., Galuppo, M., Pollastro, F., Grassi, G., Bramanti, P., and Mazzon, E. (2015). A new formulation of cannabidiol in cream shows therapeutic effects in a mouse model of experimental autoimmune encephalomyelitis. *Daru* 23:48. doi: 10.1186/s40199-015-0131-8

Gunasekaran, N., Long, L., Dawson, B., Hansen, G., Richardson, D., Li, K., et al. (2009). Reintoxication: the release of fat-stored Δ9-tetrahydrocannabinol (THC) into blood is enhanced by food deprivation or ACTH exposure. *Br. J. Pharmacol.* 158, 1330–1337. doi: 10.1111/j.1476-5381.2009.00399.x

Guy, G. W., and Flint, M. E. (2004). A single centre, placebo-controlled, four period, crossover, tolerability study assessing, pharmacodynamic effects, pharmacokinetic characteristics and cognitive profiles of a single dose of three formulations of Cannabis Based Medicine Extracts (CBMEs) (GWPD9901), plus a two period tolerability study comparing pharmacodynamic effects and pharmacokinetic characteristics of a single dose of a cannabis based medicine extract given via two administration routes (GWPD9901 EXT). *J. Cannabis Ther.* 3, 35–77. doi: 10.1300/J175v03n03_03

Guy, G. W., and Robson, P. J. (2004a). A phase I, double blind, three-way crossover study to assess the pharmacokinetic profile of Cannabis Based Medicine Extract (CBME) administered sublingually in variant cannabinoid ratios in normal healthy male volunteers. *J. Cannabis Ther.* 3, 121–152. doi: 10.1300/J175v03n04_02

Guy, G. W., and Robson, P. J. (2004b). A phase I, open label, four-way crossover study to compare the pharmacokinetic profiles of a single dose of 20 mg of a Cannabis Based Medicine Extract (CBME) Administered on 3 different areas of the buccal mucosa and to investigate the pharmacokinetics of CBME *per Oral* in Healthy Male and Female Volunteers (GWPK0112). *J. Cannabis Ther.* 3, 79–120. doi: 10.1300/J175v03n04_01

GW Pharmaceuticals (2018). *Subsidiary Greenwich Biosciences Announce FDA Approval of EPIDIOLEX® (Cannabidiol) Oral Solution–The First Plant-Derived Cannabinoid Prescription Medicine.*

Hammell, D. C., Zhang, L. P., Ma, F., Abshire, S. M., McIlwrath, S. L., Stinchcomb, A. L., et al. (2016). Transdermal cannabidiol reduces inflammation and pain-related behaviours in a rat model of arthritis. *Eur. J. Pain* 20, 936–948. doi: 10.1002/ejp.818

Haney, M., Malcolm, R. J., Babalonis, S., Nuzzo, P. A., Cooper, Z. D., Bedi, G., et al. (2016). Oral cannabidiol does not alter the subjective, reinforcing or cardiovascular effects of smoked cannabis. *Neuropsychopharmacology* 41, 1974–1982. doi: 10.1038/npp.2015.367

Hemp Business (2017). *Hemp Business Journal*. State of Hemp Market Report.

Huestis, M. A. (2007). Human cannabinoid pharmacokinetics. *Chem. Biodivers.* 4, 1770–1804. doi: 10.1002/cbdv.200790152

Izzo, A. A., Borrelli, F., Capasso, R., Di Marzo, V., and Mechoulam, R. (2009). Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends Pharmacol. Sci.* 30, 515–527. doi: 10.1016/j.tips.2009.07.006

Jiang, R., Yamaori, S., Okamoto, Y., Yamamoto, I., and Watanabe, K. (2013). Cannabidiol is a potent inhibitor of the catalytic activity of cytochrome P450 2C19. *Drug Metab. Pharmacokinet.* 28, 332–338. doi: 10.2133/dmpk.DMPK-12-RG-129

Karschner, E. L., Darwin, W. D., Goodwin, R. S., Wright, S., and Huestis, M. A. (2011). Plasma cannabinoid pharmacokinetics following controlled oral Δ9-tetrahydrocannabinol and oromucosal cannabis extract administration. *Clin. Chem.* 57, 66–75. doi: 10.1373/clinchem.2010.152439

Lee, D., Schwope, D. M., Milman, G., Barnes, A. J., Gorelick, D. A., and Huestis, M. A. (2012). Cannabinoid disposition in oral fluid after controlled smoked cannabis. *Clin. Chem.* 58, 748–756. doi: 10.1373/clinchem.2011.177881

Leweke, F. M., Piomelli, D., Pahlisch, F., Muhl, D., Gerth, C. W., Hoyer, C., et al. (2012). Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl. Psychiatry* 2:e94. doi: 10.1038/tp.2012.15

Long, L. E., Chesworth, R., Huang, X. F., Wong, A., Spiro, A., McGregor, I. S., et al. (2012). Distinct neurobehavioural effects of cannabidiol in transmembrane domain neuregulin 1 mutant mice. *PLoS ONE* 7:e34129. doi: 10.1371/journal.pone.0034129

Manini, A. F., Yiannoulos, G., Bergamaschi, M. M., Hernandez, S., Olmedo, R., Barnes, A. J., et al. (2015). Safety and pharmacokinetics of oral cannabidiol when administered concomitantly with intravenous fentanyl in humans. *J. Addict. Med.* 9, 204–210. doi: 10.1097/ADM.0000000000000118

Mechoulam, R., Parker, L. A., and Gallily, R. (2002). Cannabidiol: an overview of some pharmacological aspects. *J. Clin. Pharmacol.* 42, 11S−19S. doi: 10.1002/j.1552-4604.2002.tb05998.x

Moher, D., Liberati, A., Tetzlaff, J., and Altman, D. G. (2009). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Ann. Int. Med.* 151, 264–269, w64. doi: 10.7326/0003-4819-151-4-200908180-00135

Nadulski, T., Pragst, F., Weinberg, G., Roser, P., Schnelle, M., Schnelle, M., Fronk, E. M., et al. (2005a). Randomized, double-blind, placebo-controlled study about the effects of Cannabidiol (CBD) on the pharmacokinetics of D9-Tetrahydrocannabinol (THC) after oral application of THC verses standardized cannabis extract. *Ther. Drug Monit.* 27, 799–810. doi: 10.1097/01.ftd.0000177223.19294.5c

Nadulski, T., Sporkert, F., Schnelle, M., Stadelmann, A. M., Roser, P., Schefter, T., et al. (2005b). Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract. *J. Anal. Toxicol.* 29, 782–789. doi: 10.1093/jat/29.8.782

Naftali, T., Mechulam, R., Marii, A., Gabay, G., Stein, A., Bronshtain, M., et al. (2017). Low-dose cannabidiol is safe but not effective in the treatment for crohn's disease, a randomized controlled trial. *Dig. Dis. Sci.* 62, 1615–1620. doi: 10.1007/s10620-017-4540-z

National Institute for Health (2014). *National Lung and Blood Institute Quality Assessment Tool for Before-After (Pre-Post) Studies with No Control Group.* Available online at: https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools

Newmeyer, M. N., Desrosiers, N. A., Lee, D., Mendu, D. R., Barnes, A. J., Gorelick, D. A., et al. (2014). Cannabinoid disposition in oral fluid after controlled cannabis smoking in frequent and occasional smokers. *Drug Test. Anal.* 6, 1002–1010. doi: 10.1002/dta.1632

Ogungbenro, K., Aarons, L., and Graham, G. (2006). Sample size calculations based on generalized estimating equations for population pharmacokinetic experiments. *J. Biopharm. Stat.* 16, 135–150. doi: 10.1080/10543400500508705

Ohlsson, A., Lindgren, J. E., Andersson, S., Agurell, S., Gillespie, H., and Hollister, L. E. (1986). Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration. *Biomed. Environ. Mass Spectrom.* 13, 77–83. doi: 10.1002/bms.1200130206

Schwope, D. M., Karschner, E. L., Gorelick, D. A., and Huestis, M. A. (2011). Identification of recent cannabis use: whole-blood and plasma free and glucuronidated cannabinoid pharmacokinetics following controlled smoked cannabis administration. *Clin. Chem.* 57, 1406–1414. doi: 10.1373/clinchem.2011.171777

Sellers, E. M., Schoedel, K., Bartlett, C., Romach, M., Russo, E. B., Stott, C. G., et al. (2013). A multiple-dose, randomized, double-blind, placebo-controlled, parallel-group QT/QTc study to evaluate the electrophysiologic effects of THC/CBD spray. *Clin. Pharmacol. Drug Dev.*2, 285–294. doi: 10.1002/cpdd.36

Stott, C., White, L., Wright, S., Wilbraham, D., and Guy, G. (2013c). A Phase I, open-label, randomized, crossover study in three parallel groups to evaluate the effect of Rifampicin, Ketoconazole, and Omeprazole on the pharmacokinetics of THC/CBD oromucosal spray in healthy volunteers. *Springerplus* 2:236. doi: 10.1186/2193-1801-2-236

Stott, C. G., White, L., Wright, S., Wilbraham, D., and Guy, G. W. (2013a). A phase I study to assess the single and multiple dose pharmacokinetics of THC/CBD oromucosal spray. *Eur. J. Clin. Pharmacol.* 69, 1135–1147. doi: 10.1007/s00228-012-1441-0

Stott, C. G., White, L., Wright, S., Wilbraham, D., and Guy, G. W. (2013b). A phase I study to assess the effect of food on the single dose bioavailability of the THC/CBD oromucosal spray. *Eur. J. Clin. Pharmacol.* 69, 825–834. doi: 10.1007/s00228-012-1393-4

Stout, S. M., and Cimino, N. M. (2014). Exogenous cannabinoids as substrates, inhibitors, and inducers of human drug metabolizing enzymes: a systematic review. *Drug Metab. Rev.* 46, 86–95. doi: 10.3109/03602532.2013.849268

Swortwood, M. J., Newmeyer, M. N., Andersson, M., Abulseoud, O. A., Scheidweiler, K. B., and Huestis, M. A. (2017). Cannabinoid disposition in oral fluid after controlled smoked, vaporized, and oral cannabis administration. *Drug Test. Anal.* 9, 905–915. doi: 10.1002/dta.2092

Thiele, E. A., Marsh, E. D., French, J. A., Mazurkiewicz-Beldzinska, M., Benbadis, S. R., Joshi, C., et al. (2018). Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 391, 1085–1096. doi: 10.1016/S0140-6736(18)30136-3

Winter, H., Ginsberg, A., Egizi, E., Erondu, N., Whitney, K., Pauli, E., et al. (2013). Effect of a high-calorie, high-fat meal on the bioavailability and pharmacokinetics of PA-824 in healthy adult subjects. *Antimicrob. Agents Chemother.* 57, 5516–5520. doi: 10.1128/AAC.00798-13

Zgair, A., Wong, J. C., Lee, J. B., Mistry, J., Sivak, O., Wasan, K. M., et al. (2016). Dietary fats and pharmaceutical lipid excipients increase systemic exposure to orally administered cannabis and cannabis-based medicines. *Am. J. Transl. Res.* 8, 3448–3459.

Conflict of Interest Statement: AY was employed by company Artelo Biosciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

10. General references

Listed below are the references that correspond to those in the introduction (Chapter 1), general methods (Chapter 2) and general discussion (Chapter 8), while each manuscript contains its own references thererin.

Abbott NJ, Rönnbäck L and Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. *Nature Reviews Neuroscience* 7(1): 41–53. DOI: 10.1038/nrn1824.

Abbott NJ, Patabendige AAK, Dolman DEM, et al. (2010) Structure and function of the blood-brain barrier. *Neurobiology of Disease* 37(1). Elsevier Inc.: 13–25. DOI: 10.1016/j.nbd.2009.07.030.

Ahern GP (2003) Activation of TRPV1 by the satiety factor oleoylethanolamide. *Journal of Biological Chemistry*: 30429–30434. DOI: 10.1074/jbc.M305051200.

Ahmad A, Crupi R, Impellizzeri D, et al. (2012) Administration of palmitoylethanolamide (PEA) protects the neurovascular unit and reduces secondary injury after traumatic brain injury in mice. *Brain, Behavior, and Immunity* 26(8): 1310–1321. DOI: 10.1016/j.bbi.2012.07.021.

Alliot F, Godin I and Pessac B (1999) Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Developmental Brain Research* 117(2): 145–152. DOI: 10.1016/S0165-3806(99)00113-3.

Allt G and Lawrenson JG (2001) Pericytes: Cell biology and pathology. *Cells Tissues Organs*. DOI: 10.1159/000047855.

Alvarez FJ, Lafuente H, Rey-santano MC, et al. (2008) Neuroprotective Effects of the Nonpsychoactive Cannabinoid Cannabidiol in Hypoxic-Ischemic Newborn Piglets. 64(6): 653–658.

Amada N, Yamasaki Y, Williams CM, et al. (2013) Cannabidivarin (CBDV) suppresses pentylenetetrazole (PTZ)-induced increases in epilepsy-related gene expression. *PeerJ* 1(e214). DOI: 10.7717/peerj.214.

Anchan A, Kalogirou-Baldwin P, Johnson R, et al. (2019) Real-time measurement of melanoma cell-mediated human brain endothelial barrier disruption using electric cell-substrate impedance sensing technology. *Biosensors* 9(2). DOI: 10.3390/bios9020056.

Anderson LL, Low IK, Banister SD, et al. (2019) Pharmacokinetics of Phytocannabinoid Acids and Anticonvulsant Effect of Cannabidiolic Acid in a Mouse Model of Dravet Syndrome. *Journal of Natural Products* 82(11): 3047–3055. DOI: 10.1021/acs.jnatprod.9b00600.

Antonic A, Sena ES, Donnan GA, et al. (2012) Human In Vitro Models of Ischaemic Stroke: A Test Bed for Translation. *Translational Stroke Research* 3(3): 306–309. DOI: 10.1007/s12975-012-0201-x.

Araújo DSM, Miya-Coreixas VS, Pandolfo P, et al. (2017) Cannabinoid receptors and TRPA1 on neuroprotection in a model of retinal ischemia. *Experimental Eye Research* 154: 116–125. DOI: 10.1016/j.exer.2016.11.015.

Armulik A, Abramsson A and Betsholtz C (2005) Endothelial/pericyte interactions. *Circulation Research*. DOI: 10.1161/01.RES.0000182903.16652.d7.

Armulik A, Genové G, Mäe M, et al. (2010) Pericytes regulate the blood-brain barrier. *Nature*. DOI: 10.1038/nature09522.

Baggiolini M, Dewald B and Moser B (1993) lnterleukin-8 and Related Chemotactic Cytokines—CXC and CC Chemokines. *Advances in Immunology* 55(C): 97–179. DOI: 10.1016/S0065-2776(08)60509-X.

Bai J and Lyden PD (2015) Revisiting cerebral postischemic reperfusion injury: New insights in understanding reperfusion failure, hemorrhage, and edema. *International Journal of Stroke* 10(2): 143–152. DOI: 10.1111/ijs.12434.

Bai Y, Zhu X, Chao J, et al. (2015) Pericytes contribute to the disruption of the cerebral endothelial barrier via increasing VEGF expression: Implications for stroke. *PLoS ONE* 10(4): 1–20. DOI: 10.1371/journal.pone.0124362.

Baker D, Pryce G, Ludovic Croxford J, et al. (2000) Cannabinoids control spasticity and tremor in a multiple sclerosis model. *Nature*. DOI: 10.1038/35003583.

Banati RB, Gehrmann J, Schubert P, et al. (1993) Cytotoxicity of microglia. *Glia* 7(1): 111–118. DOI: 10.1002/glia.440070117.

Bandell M, Story GM, Hwang SW, et al. (2004) Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* 41(6): 849–857. DOI: 10.1016/S0896-6273(04)00150-3.

Baraldi PG, Preti D, Materazzi S, et al. (2010) Transient receptor potential ankyrin 1 (TRPA1) channel as emerging target for novel analgesics and anti-inflammatory agents. *Journal of Medicinal Chemistry*. DOI: 10.1021/jm100062h.

Barar J, Rafi MA, Pourseif MM, et al. (2016) Blood-brain barrier transport machineries and targeted therapy of brain diseases. *BioImpacts* 6(4): 225–248. DOI: 10.15171/bi.2016.30.

Barreto GE, Sun X, Xu L, et al. (2011) Astrocyte proliferation following stroke in the mouse depends on distance from the infarct. *PLoS ONE* 6(11). DOI: 10.1371/journal.pone.0027881.

Becerra-Calixto A and Cardona-Gómez GP (2017) The role of astrocytes in neuroprotection after brain stroke: Potential in cell therapy. *Frontiers in Molecular Neuroscience* 10. DOI: 10.3389/FNMOL.2017.00088.

Bell RD, Winkler EA, Sagare AP, et al. (2010) Pericytes Control Key Neurovascular Functions and Neuronal Phenotype in the Adult Brain and during Brain Aging. *Neuron*. DOI: 10.1016/j.neuron.2010.09.043.

Benarroch EE (2013) Microglia: Multiple roles in surveillance, circuit shaping, and response to injury. *Neurology* 81(12): 1079–1088. DOI: 10.1212/WNL.0b013e3182a4a577.

Benfenati V, Amiry-Moghaddam M, Caprini M, et al. (2007) Expression and functional characterization of transient receptor potential vanilloid-related channel 4 (TRPV4) in rat cortical astrocytes. *Neuroscience* 148(4): 876–892. DOI: 10.1016/j.neuroscience.2007.06.039.

Berciano MT, Andres MA, Calle E, et al. (1995) Age-induced hypertrophy of astrocytes in rat supraoptic nucleus: A cytological, morphometric, and immunocytochemical study. *The Anatomical Record* 243(1): 129–144. DOI: 10.1002/ar.1092430115.

Berger J and Moller DE (2002) The Mechanisms of Action of PPARs. *Annual Review of Medicine*. DOI: 10.1146/annurev.med.53.082901.104018.

Bevers MB and Neumar RW (2008) Mechanistic role of calpains in postischemic neurodegeneration. *Journal of Cerebral Blood Flow and Metabolism* 28(4): 655–673. DOI: 10.1038/sj.jcbfm.9600595.

Blanchette M and Daneman R (2015) Formation and maintenance of the BBB. *Mechanisms of Development* 138: 8–16. DOI: 10.1016/j.mod.2015.07.007.

Bolognini D, Rock EM, Cluny NL, et al. (2013) Cannabidiolic acid prevents vomiting in Suncus murinus and nausea-induced behaviour in rats by enhancing 5-HT1A receptor activation. *British Journal of Pharmacology* 168(6): 1456–1470. DOI: 10.1111/bph.12043.

Borlongan C V. and Emerich DF (2003) Facilitation of drug entry into the CNS via transient permeation of blood brain barrier: Laboratory and preliminary clinical evidence from bradykinin receptor agonist, Cereport. *Brain Research Bulletin* 60(3): 297–306. DOI: 10.1016/S0361-9230(03)00043-1.

Brellenthin AG, Crombie KM, Hillard CJ, et al. (2017) Endocannabinoid and mood responses to exercise in adults with varying activity levels. *Medicine and Science in Sports and Exercise* 49(8): 1688–1696. DOI: 10.1249/MSS.0000000000001276.

Brzozowska N, Li KM, Wang XS, et al. (2016) ABC transporters P-gp and Bcrp do not limit the brain uptake of the novel antipsychotic and anticonvulsant drug cannabidiol in mice. *PeerJ* 2016(5). DOI: 10.7717/peerj.2081.

Burstein SH, McQuain CA, Ross AH, et al. (2011) Resolution of inflammation by N-arachidonoylglycine. *Journal of Cellular Biochemistry*. DOI: 10.1002/jcb.23245.

Bush TG, Puvanachandra N, Horner CH, et al. (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 23(2): 297–308. DOI: 10.1016/S0896-6273(00)80781-3.

Cai W, Zhang K, Li P, et al. (2017) Dysfunction of the neurovascular unit in ischemic stroke and neurodegenerative diseases: An aging effect. *Ageing Research Reviews* 34: 77–87. DOI: 10.1016/j.arr.2016.09.006.

Cao G, Xing J, Xiao X, et al. (2007) Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *Journal of Neuroscience* 27(35): 9278–9293. DOI: 10.1523/JNEUROSCI.2826-07.2007.

Carrillo-Salinas FJ, Navarrete C, Mecha M, et al. (2014) A cannabigerol derivative suppresses immune responses and protects mice from experimental autoimmune encephalomyelitis. *PLoS ONE* 9(4). DOI: 10.1371/journal.pone.0094733.

Cascio MG, Gauson LA, Stevenson LA, et al. (2010) Evidence that the plant cannabinoid cannabigerol is a highly potent α 2-adrenoceptor agonist and moderately potent 5HT 1A receptor antagonist. *British Journal of Pharmacology* 159(1). DOI: 10.1111/j.1476-5381.2009.00515.x.

Castillo A, Tolón MR, Fernández-Ruiz J, et al. (2010) The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB2 and adenosine receptors. *Neurobiology of Disease* 37(2): 434–440. DOI: 10.1016/j.nbd.2009.10.023.

Ceprián M, Jiménez-Sánchez L, Vargas C, et al. (2017) Cannabidiol reduces brain

damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. *Neuropharmacology* 116: 151–159. DOI: 10.1016/j.neuropharm.2016.12.017.

Chagas MHN, Zuardi AW, Tumas V, et al. (2014) Effects of cannabidiol in the treatment of patients with Parkinson's disease: An exploratory double-blind trial. *Journal of Psychopharmacology*. DOI: 10.1177/0269881114550355.

Chen J, Jin K, Chen M, et al. (1997) Early detection of DNA strand breaks in the brain after transient focal ischemia: Implications for the role of DNA damage in apoptosis and neuronal cell death. *Journal of Neurochemistry* 69(1): 232–245. DOI: 10.1046/j.1471-4159.1997.69010232.x.

Chen Y, Vartiainen NE, Ying W, et al. (2001) Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. *Journal of Neurochemistry* 77(6): 1601–1610. DOI: 10.1046/j.1471-4159.2001.00374.x.

Chiang CS, Stalder A, Samimi A, et al. (1994) Reactive gliosis as a consequence of lnterleukin-6 expression in the brain: Studies in transgenic mice. *Developmental Neuroscience* 16(3–4): 212–221. DOI: 10.1159/000112109.

Contartese A, Valoti M, Corelli F, et al. (2012) A novel CB2 agonist, COR167, potently protects rat brain cortical slices against OGD and reperfusion injury. *Pharmacological Research* 66(6): 555–563. DOI: 10.1016/j.phrs.2012.08.003.

Cristino L, Bisogno T and Di Marzo V (2020) Cannabinoids and the expanded endocannabinoid system in neurological disorders. *Nature Reviews Neurology* 16(1): 9–29. DOI: 10.1038/s41582-019-0284-z.

da Fonseca ACC, Matias D, Garcia C, et al. (2014) The impact of microglial activation on blood-brain barrier in brain diseases. *Frontiers in Cellular Neuroscience* 8(November): 1–13. DOI: 10.3389/fncel.2014.00362.

De Petrocellis L and Di Marzo V (2010) Non-CB1, Non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: Focus on G-protein-coupled receptors and transient receptor potential channels. *Journal of Neuroimmune Pharmacology*. DOI: 10.1007/s11481-009-9177-z.

De Petrocellis L, Starowicz K, Moriello AS, et al. (2007) Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): Effect of cAMP, cannabinoid CB1 receptors and endovanilloids. *Experimental Cell Research* 313(9): 1911–1920. DOI: 10.1016/j.yexcr.2007.01.008.

De Petrocellis L, Vellani V, Schiano-Moriello A, et al. (2008) Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *Journal of Pharmacology and Experimental Therapeutics* 325(3): 1007–15. DOI: 10.1124/jpet.107.134809.

De Petrocellis L, Ligresti A, Moriello AS, et al. (2011) Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. *British Journal of Pharmacology* 163(7): 1479–1494. DOI: 10.1111/j.1476-5381.2010.01166.x.

De Petrocellis L, Orlando P, Moriello AS, et al. (2012) Cannabinoid actions at TRPV channels: Effects on TRPV3 and TRPV4 and their potential relevance to gastrointestinal inflammation. *Acta Physiologica*. DOI: 10.1111/j.1748-1716.2011.02338.x.

Deiana S, Watanabe A, Yamasaki Y, et al. (2012) Plasma and Brain Pharmacokinetic Profile of Cannabidiol (CBD), Cannabidivarine (CBDV), δ^9-Tetrahydrocannabivarin (THCV) and Cannabigerol (CBG) in Rats and Mice Following Oral and Intraperitoneal Administration and CBD Action on Obsessive-Compulsive Behavi. *Psychopharmacology* 219(3): 859–73. DOI: 10.1007/s00213-011-2415-0.

Del Zoppo GJ and Mabuchi T (2003) Cerebral microvessel responses to focal ischemia. *Journal of Cerebral Blood Flow and Metabolism*. DOI: 10.1097/01.WCB.0000078322.96027.78.

Desai SY, Marroni M, Cucullo L, et al. (2002) Mechanisms of endothelial survival under shear stress. *Endothelium: Journal of Endothelial Cell Research*. DOI: 10.1080/10623320212004.

Devane WA, Hanuš L, Breuer A, et al. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. DOI: 10.1126/science.1470919.

Di Giacomo V, Chiavaroli A, Recinella L, et al. (2020) Antioxidant and neuroprotective effects induced by cannabidiol and cannabigerol in rat CTX-TNA2 astrocytes and isolated cortexes. *International Journal of Molecular Sciences* 21(10). DOI: 10.3390/ijms21103575.

Di Marzo V, Bisogno T and De Petrocellis L (2005) The biosynthesis, fate and pharmacological properties of endocannabinoids. *Handbook of Experimental Pharmacology*. DOI: 10.1007/3-540-26573-2-5.

Dore-Duffy P (2008) Pericytes: pluripotent cells of the blood brain barrier. *Current Pharmaceutical Design* 14(16): 1581–1593. DOI: 10.2174/138161208784705469.

Douglas DR, Luoma V and Reddy U (2020) Acute management of ischaemic stroke. *Anaesthesia and Intensive Care Medicine* 21(1). Elsevier Ltd: 1–7. DOI: 10.1016/j.mpaic.2019.10.013.

Doyle KP, Simon RP and Stenzel-Poore MP (2008) Mechanisms of ischemic brain damage. *Neuropharmacology* 55(3): 310–318. DOI: 10.1016/j.neuropharm.2008.01.005.

Dudvarski Stankovic N, Teodorczyk M, Ploen R, et al. (2016) Microglia–blood vessel interactions: a double-edged sword in brain pathologies. *Acta Neuropathologica* 131(3): 347–363. DOI: 10.1007/s00401-015-1524-y.

Echeverry C, Prunell G, Narbondo C, et al. (2020) A Comparative In Vitro Study of the Neuroprotective Effect Induced by Cannabidiol, Cannabigerol, and Their Respective Acid Forms: Relevance of the 5-HT1A Receptors. *Neurotoxicity Research*. DOI: 10.1007/s12640-020-00277-y.

Eljaschewitsch E, Witting A, Mawrin C, et al. (2006) The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. *Neuron* 49(1): 67–79. DOI: 10.1016/j.neuron.2005.11.027.

Escudero C, Acurio J, Lopez E, et al. (2020) Vascular endothelial growth factor and poor-prognosis after ischemic stroke. *European Journal of Neurology*: 0–1. DOI: 10.1111/ENE.14641.

Esposito G, De Filippis D, Carnuccio R, et al. (2006) The marijuana component cannabidiol inhibits β-amyloid-induced tau protein hyperphosphorylation through Wnt/β-catenin pathway rescue in PC12 cells. *Journal of Molecular Medicine* 84(3):

253–258. DOI: 10.1007/s00109-005-0025-1.
Esposito G, Scuderi C, Savani C, et al. (2007) Cannabidiol in vivo blunts β-amyloid induced neuroinflammation by suppressing IL-1β and iNOS expression. *British Journal of Pharmacology* 151(8): 1272–1279. DOI: 10.1038/sj.bjp.0707337.
Feigin VL, Nichols E, Alam T, et al. (2019) Global, regional, and national burden of neurological disorders, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Neurology*. DOI: 10.1016/S1474-4422(18)30499-X.
Fenstermacher J, GROSS P, SPOSITO N, et al. (1988) Structural and Functional Variations in Capillary Systems within the Brain. *Annals of the New York Academy of Sciences* 529(1): 21–30. DOI: 10.1111/j.1749-6632.1988.tb51416.x.
Fernández-Ruiz J, Sagredo O, Pazos MR, et al. (2013) Cannabidiol for neurodegenerative disorders: Important new clinical applications for this phytocannabinoid? *British Journal of Clinical Pharmacology* 75(2): 323–33. DOI: 10.1111/j.1365-2125.2012.04341.x.
Ferrer I (2017) Diversity of astroglial responses across human neurodegenerative disorders and brain aging. *Brain Pathology* 27(5): 645–674. DOI: 10.1111/bpa.12538.
Formato M, Crescente G, Scognamiglio M, et al. (2020) (–)-Cannabidiolic Acid, a Still Overlooked Bioactive Compound: An Introductory Review and Preliminary Research. *Molecules (Basel, Switzerland)*. DOI: 10.3390/molecules25112638.
Freeman RM, Adekanmi O, Waterfield MR, et al. (2006) The effect of cannabis on urge incontinence in patients with multiple sclerosis: A multicentre, randomised placebo-controlled trial (CAMS-LUTS). *International Urogynecology Journal*. DOI: 10.1007/s00192-006-0086-x.
Gantz I, Muraoka A, Yang YK, et al. (1997) Cloning and chromosomal localization of a gene (GPR18) encoding a novel seven transmembrane receptor highly expressed in spleen and testis. *Genomics*. DOI: 10.1006/geno.1997.4752.
Gaoni Y and Mechoulam R (1964) Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *Journal of the American Chemical Society* 86: 1646–1647. DOI: 10.1021/ja01062a046.
Gees M, Colsoul B and Nilius B (2010) The role of transient receptor potential cation channels in Ca2+ signaling. *Cold Spring Harbor perspectives in biology* 2(10). DOI: 10.1101/cshperspect.a003962.
Gervois P, Torra IP, Fruchart JC, et al. (2000) Regulation of lipid and lipoprotein metabolism by PPAR activators. *Clinical Chemistry and Laboratory Medicine*. DOI: 10.1515/CCLM.2000.002.
Giacoppo S, Gugliandolo A, Trubiani O, et al. (2017) Cannabinoid CB2 receptors are involved in the protection of RAW264.7 macrophages against the oxidative stress: An in vitro study. *European Journal of Histochemistry* 61(1): 1–13. DOI: 10.4081/ejh.2017.2749.
Giacoppo S, Pollastro F, Grassi G, et al. (2017) Target regulation of PI3K/Akt/mTOR pathway by cannabidiol in treatment of experimental multiple sclerosis. *Fitoterapia*. DOI: 10.1016/j.fitote.2016.11.010.
Gidday JM, Gasche YG, Copin JC, et al. (2005) Leukocyte-derived matrix metalloproteinase-9 mediates blood-brain barrier breakdown and is

proinflammatory after transient focal cerebral ischemia. *American Journal of Physiology - Heart and Circulatory Physiology* 289(2 58-2). DOI: 10.1152/ajpheart.01275.2004.

Gorelick PB (2019) The global burden of stroke: persistent and disabling. *The Lancet Neurology* 18(5): 417–418. DOI: 10.1016/S1474-4422(19)30030-4.

Granger DN (1988) Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *American Journal of Physiology - Heart and Circulatory Physiology*. DOI: 10.1152/ajpheart.1988.255.6.h1269.

Granja AG, Carrillo-Salinas F, Pagani A, et al. (2012) A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis. *Journal of Neuroimmune Pharmacology* 7(4). DOI: 10.1007/s11481-012-9399-3.

Gray RA and Whalley BJ (2020) The proposed mechanisms of action of CBD in epilepsy. *Epileptic Disorders* 22(S1): S10–S15. DOI: 10.1684/epd.2020.1135.

Gugliandolo A, Pollastro F, Grassi G, et al. (2018) In vitro model of neuroinflammation: Efficacy of cannabigerol, a non-psychoactive cannabinoid. *International Journal of Molecular Sciences* 19(7). DOI: 10.3390/ijms19071992.

Hamakubo T, Kannagi R, Murachi T, et al. (1986) Distribution of calpains I and II in rat brain. *Journal of Neuroscience* 6(11): 3103–3111. DOI: 10.1523/jneurosci.06-11-03103.1986.

Haorah J, Ramirez SH, Schall K, et al. (2007) Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *Journal of Neurochemistry* 101(2): 566–576. DOI: 10.1111/j.1471-4159.2006.04393.x.

Hayakawa K, Mishima K and Fujiwara M (2010) Therapeutic potential of non-psychotropic cannabidiol in ischemic stroke. *Pharmaceuticals* 3(7): 2197–2212. DOI: 10.3390/ph3072197.

Herkenham M, Lynn AB, Little MD, et al. (1990) Cannabinoid receptor localization in brain. *Proceedings of the National Academy of Sciences of the United States of America*. DOI: 10.1073/pnas.87.5.1932.

Herx LM and Yong VW (2001) Interleukin-1 beta is required for the early evolution of reactive astrogliosis following CNS lesion. *Journal of neuropathology and experimental neurology* 60(10): 961–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11589427.

Heyman E, Gamelin FX, Goekint M, et al. (2012) Intense exercise increases circulating endocannabinoid and BDNF levels in humans––– Possible implications for reward and depression. *Psychoneuroendocrinology* 37(6). Elsevier Ltd: 844–851. DOI: 10.1016/j.psyneuen.2011.09.017.

Hill AJ, Weston SE, Jones NA, et al. (2010) 9-Tetrahydrocannabivarin suppresses in vitro epileptiform and in vivo seizure activity in adult rats. *Epilepsia* 51(8): 1522–1532. DOI: 10.1111/j.1528-1167.2010.02523.x.

Hill JD, Zuluaga-Ramirez V, Gajghate S, et al. (2018) Activation of GPR55 increases neural stem cell proliferation and promotes early adult hippocampal neurogenesis. *British Journal of Pharmacology* 175(16): 3407–3421. DOI: 10.1111/bph.14387.

Hill TDM, Cascio MG, Romano B, et al. (2013) Cannabidivarin-rich cannabis extracts are

anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism. *British Journal of Pharmacology* 170(3): 679–92. DOI: 10.1111/bph.12321.
Hillard C (2008) Role of Cannabinoids and Endocannabinoids in Cerebral Ischemia. *Current Pharmaceutical Design* 14(23): 2347–2361. DOI: 10.2174/138161208785740054.
Hind WH (2014) *The Effects of Cannabinoids on Blood-Brain Barrier Permeability in vitro*. University of Nottingham.
Hind WH, Tufarelli C, Neophytou M, et al. (2015) Endocannabinoids modulate human blood-brain barrier permeability in vitro. *British Journal of Pharmacology* 172(12): 3015–3027. DOI: 10.1111/bph.13106.
Hind WH, England TJ and O'Sullivan SE (2016) Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARγ and 5-HT1A receptors. *British Journal of Pharmacology* 173(5): 815–825. DOI: 10.1111/bph.13368.
Hoang M V., Smith LEH and Senger DR (2011) Calpain inhibitors reduce retinal hypoxia in ischemic retinopathy by improving neovascular architecture and functional perfusion. *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1812(4): 549–557. DOI: 10.1016/j.bbadis.2010.08.008.
Holloway PM and Gavins FNE (2016) Modeling Ischemic Stroke in Vitro: Status Quo and Future Perspectives. *Stroke* 47(2): 561–569. DOI: 10.1161/STROKEAHA.115.011932.
Hotter B, Hoffmann S, Ulm L, et al. (2019) IL-6 Plasma levels correlate with cerebral perfusion deficits and infarct sizes in stroke patients without associated infections. *Frontiers in Neurology* 10(FEB). DOI: 10.3389/fneur.2019.00083.
Howard RS (2016) The management of ischaemic stroke. *Anaesthesia and Intensive Care Medicine* 17(12). Elsevier Ltd: 591–595. DOI: 10.1016/j.mpaic.2016.09.009.
Howarth C, Gleeson P and Attwell D (2012) Updated energy budgets for neural computation in the neocortex and cerebellum. *Journal of Cerebral Blood Flow and Metabolism*. DOI: 10.1038/jcbfm.2012.35.
Howlett AC, Barth F, Bonner TI, et al. (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacological Reviews*. DOI: 10.1124/pr.54.2.161.
Huang J, Choudhri TF, Winfree CJ, et al. (2000) Postischemic cerebrovascular E-selectin expression mediates tissue injury in murine stroke. *Stroke*. DOI: 10.1161/01.STR.31.12.3047.
Huang J, Upadhyay UM and Tamargo RJ (2006) Inflammation in stroke and focal cerebral ischemia. *Surgical Neurology* 66(3): 232–245. DOI: 10.1016/j.surneu.2005.12.028.
Huang Q, Wang X, Lin X, et al. (2020) The Role of Transient Receptor Potential Channels in Blood-Brain Barrier Dysfunction after Ischemic Stroke. *Biomedicine and Pharmacotherapy* 131. Elsevier Masson SAS: 110647. DOI: 10.1016/j.biopha.2020.110647.
Hughes S, Gardiner T, Hu P, et al. (2006) Altered pericyte-endothelial relations in the rat retina during aging: Implications for vessel stability. *Neurobiology of Aging*. DOI: 10.1016/j.neurobiolaging.2005.10.021.

Iannotti FA, Hill CL, Leo A, et al. (2014) Nonpsychotropic plant cannabinoids, Cannabidivarin (CBDV) and Cannabidiol (CBD), activate and desensitize Transient Receptor Potential Vanilloid 1 (TRPV1) channels in vitro: Potential for the treatment of neuronal hyperexcitability. *ACS Chemical Neuroscience* 5(11): 1131–1141. DOI: 10.1021/cn5000524.

Iuvone T, Esposito G, Esposito R, et al. (2004) Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on β-amyloid-induced toxicity in PC12 cells. *Journal of Neurochemistry* 89(1): 134–141. DOI: 10.1111/j.1471-4159.2003.02327.x.

Jo AO, Ryskamp DA, Phuong TTT, et al. (2015) TRPV4 and AQP4 channels synergistically regulate cell volume and calcium homeostasis in retinal müller glia. *Journal of Neuroscience* 35(39): 13525–13537. DOI: 10.1523/JNEUROSCI.1987-15.2015.

Jolivel V, Bicker F, Binamé F, et al. (2015) Perivascular microglia promote blood vessel disintegration in the ischemic penumbra. *Acta Neuropathologica* 129(2): 279–295. DOI: 10.1007/s00401-014-1372-1.

Kajihara H, Tsutsumi E, Kinoshita A, et al. (2001) Activated astrocytes with glycogen accumulation in ischemic penumbra during the early stage of brain infarction: Immunohistochemical and electron microscopic studies. *Brain Research*. DOI: 10.1016/S0006-8993(01)02640-3.

Kaplan JS, Stella N, Catterall WA, et al. (2017) Cannabidiol attenuates seizures and social deficits in a mouse model of Dravet syndrome. *Proceedings of the National Academy of Sciences of the United States of America* 114(42): 11229–11234. DOI: 10.1073/pnas.1711351114.

Kettenmann H, Hanisch U-K, Noda M, et al. (2011) Physiology of Microglia. *Physiological Reviews* 91(2): 461–553. DOI: 10.1152/physrev.00011.2010.

Khaksar S and Bigdeli MR (2017) Correlation between cannabidiol-induced reduction of infarct volume and inflammatory factors expression in ischemic stroke model. *Basic and Clinical Neuroscience* 8(2): 139–146. DOI: 10.18869/nirp.bcn.8.2.139.

Kozela E, Pietr M, Juknat A, et al. (2010) Cannabinoids Δ9-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-κB and interferon-β/STAT proinflammatory pathways in BV-2 microglial cells. *Journal of Biological Chemistry* 285(3): 1616–1626. DOI: 10.1074/jbc.M109.069294.

Krause DN and Edvinsson L (2002) *Cerebral Blood Flow and Metabolism*. 2nd Editio. Lippincott Williams.

Krey G, Braissant O, L'Horset F, et al. (1997) Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Molecular Endocrinology*. DOI: 10.1210/mend.11.6.0007.

Kwiatkowska M, Parker LA, Burton P, et al. (2004) A comparative analysis of the potential of cannabinoids and ondansetron to suppress cisplatin-induced emesis in the Suncus murinus (house musk shrew). *Psychopharmacology*. DOI: 10.1007/s00213-003-1739-9.

L L and X W (2016) Ischemia-reperfusion Injury in the Brain: Mechanisms and Potential Therapeutic Strategies. *Biochemistry & Pharmacology: Open Access* 5(4). DOI: 10.4172/2167-0501.1000213.

Lähteenvuo J and Rosenzweig A (2012) Effects of aging on angiogenesis. *Circulation Research*. DOI: 10.1161/CIRCRESAHA.111.246116.
Leak RK, Zhang L, Stetler RA, et al. (2013) HSP27 Protects the Blood-Brain Barrier Against Ischemia-Induced Loss of Integrity. *CNS & Neurological Disorders - Drug Targets* 12(3): 325–337. DOI: 10.2174/18715273113120300006.
Lewandowski C and Barsan W (2001) Treatment of acute ischemic stroke. *Annals of Emergency Medicine* 37(2): 202–216. DOI: 10.1067/mem.2001.111573.
Li L, Lundkvist A, Andersson D, et al. (2008) Protective role of reactive astrocytes in brain ischemia. *Journal of Cerebral Blood Flow and Metabolism* 28(3): 468–481. DOI: 10.1038/sj.jcbfm.9600546.
Li P, Stetler RA, Leak RK, et al. (2018) Oxidative stress and DNA damage after cerebral ischemia: Potential therapeutic targets to repair the genome and improve stroke recovery. *Neuropharmacology* 134: 208–217. DOI: 10.1016/j.neuropharm.2017.11.011.
Li Y and Chopp M (1999) Temporal profile of nestin expression after focal cerebral ischemia in adult rat. *Brain Research* 838(1–2): 1–10. DOI: 10.1016/S0006-8993(99)01502-4.
Li YN, Pan R, Qin XJ, et al. (2014) Ischemic neurons activate astrocytes to disrupt endothelial barrier via increasing VEGF expression. *Journal of Neurochemistry* 129(1): 120–129. DOI: 10.1111/jnc.12611.
Ligresti A, Moriello AS, Starowicz K, et al. (2006) Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *Journal of Pharmacology and Experimental Therapeutics* 318(3): 1375–1387. DOI: 10.1124/jpet.106.105247.
Liu Z and Chopp M (2016) Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke. *Progress in Neurobiology*. DOI: 10.1016/j.pneurobio.2015.09.008.
Louw DF, Yang FW and Sutherland GR (2000) The effect of δ-9-tetrahydrocannabinol on forebrain ischemia in rat. *Brain Research* 857(1–2): 183–187. DOI: 10.1016/S0006-8993(99)02422-1.
Ma YL, Weston SE, Whalley BJ, et al. (2008) The phytocannabinoid Δ 9-tetrahydrocannabivarin modulates inhibitory neurotransmission in the cerebellum. *British Journal of Pharmacology* 154(1): 204–215. DOI: 10.1038/bjp.2008.57.
Maréchal A and Zou L (2013) DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harbor Perspectives in Biology* 5(9). DOI: 10.1101/cshperspect.a012716.
Marion-Letellier R, Savoye G and Ghosh S (2016) Fatty acids, eicosanoids and PPAR gamma. *European Journal of Pharmacology*. DOI: 10.1016/j.ejphar.2015.11.004.
Markovà J, Essner U, Akmaz B, et al. (2019) Sativex ® as add-on therapy vs. further optimized first-line ANTispastics (SAVANT) in resistant multiple sclerosis spasticity: a double-blind, placebo-controlled randomised clinical trial. *International Journal of Neuroscience* 129(2): 119–128. DOI: 10.1080/00207454.2018.1481066.
Maroon J and Bost J (2018) Review of the neurological benefits of phytocannabinoids. *Surgical Neurology International* 9(1): 91. DOI: 10.4103/sni.sni_45_18.
Maruo N, Morita I, Shirao M, et al. (1992) IL-6 increases endothelial permeability in

vitro. *Endocrinology* 131(2): 710–714. DOI: 10.1210/endo.131.2.1639018.
McConnell HL, Kersch CN, Woltjer RL, et al. (2017) The translational significance of the neurovascular unit. *Journal of Biological Chemistry*. DOI: 10.1074/jbc.R116.760215.
McHugh D (2012) GPR18 in microglia: Implications for the CNS and endocannabinoid system signalling. *British Journal of Pharmacology* 167(8): 1575–1582. DOI: 10.1111/j.1476-5381.2012.02019.x.
Mecha M, Feliú A, Iñigo PM, et al. (2013) Cannabidiol provides long-lasting protection against the deleterious effects of inflammation in a viral model of multiple sclerosis: A role for A2A receptors. *Neurobiology of Disease* 59: 141–150. DOI: 10.1016/j.nbd.2013.06.016.
Mechoulam R and Shvo Y (1963) Hashish—I. The structure of cannabidiol. *Tetrahedron* 19(12): 2073–2078. DOI: 10.1016/0040-4020(63)85022-X.
Mechoulam R, Braun P and Gaoni Y (1967) A Stereospecific Synthesis of (—)-Δ1- and (—)-Δ11(6)-Tetrahydrocannabinols. *Journal of the American Chemical Society*. DOI: 10.1021/ja00993a072.
Mechoulam R, Ben-Shabat S, Hanus L, et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical Pharmacology*. DOI: 10.1016/0006-2952(95)00109-D.
Menon B, Ramalingam K and Kumar R (2020) Evaluating the Role of Oxidative Stress in Acute Ischemic Stroke. *Journal of Neurosciences in Rural Practice* 11(1): 156–159. DOI: 10.1055/s-0039-3402675.
Milanlioglu A, Aslan M, Ozkol H, et al. (2016) Serum antioxidant enzymes activities and oxidative stress levels in patients with acute ischemic stroke: influence on neurological status and outcome. *Wiener Klinische Wochenschrift*. DOI: 10.1007/s00508-015-0742-6.
Millar SA, Stone NL, Yates AS, et al. (2018) A systematic review on the pharmacokinetics of cannabidiol in humans. *Frontiers in Pharmacology* 26(9): 1365. DOI: 10.3389/fphar.2018.01365.
Millar SA, Stone NL, Bellman ZD, et al. (2019) A systematic review of cannabidiol dosing in clinical populations. *British Journal of Clinical Pharmacology* 85(9): 1888–1900. DOI: 10.1111/bcp.14038.
Mishima K, Hayakawa K, Abe K, et al. (2005) Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. *Stroke* 36(5): 1071–1076. DOI: 10.1161/01.STR.0000163083.59201.34.
Miyanohara J, Shirakawa H, Sanpei K, et al. (2015) A pathophysiological role of TRPV1 in ischemic injury after transient focal cerebral ischemia in mice. *Biochemical and Biophysical Research Communications* 467(3): 478–483. DOI: 10.1016/j.bbrc.2015.10.027.
Momeni HR (2011) Role of calpain in apoptosis. *Cell Journal*.
Morano A, Fanella M, Albini M, et al. (2020) Cannabinoids in the treatment of epilepsy: Current status and future prospects. *Neuropsychiatric Disease and Treatment* 16: 381–396. DOI: 10.2147/NDT.S203782.
Moretti A, Ferrari F and Villa RF (2015) Pharmacological therapy of acute ischaemic stroke: Achievements and problems. *Pharmacology & Therapeutics* 153. Elsevier

Inc.: 79–89. DOI: 10.1016/j.pharmthera.2015.06.004.
Morrison HW and Filosa JA (2013) A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion. *Journal of Neuroinflammation* 10. DOI: 10.1186/1742-2094-10-4.
Mottillo EP, Bloch AE, Leffs T, et al. (2012) Lipolytic products activate peroxisome proliferator-activated receptor (PPAR) α and δ in brown adipocytes to match fatty acid oxidation with supply. *Journal of Biological Chemistry*. DOI: 10.1074/jbc.M112.374041.
Mozaffarian D, Benjamin EJ, Go AS, et al. (2016) Executive Summary: Heart Disease and Stroke Statistics—2016 Update. *Circulation* 133(4): 447–454. DOI: 10.1161/CIR.0000000000000366.
Muller C, Morales P and Reggio PH (2019) Cannabinoid ligands targeting TRP channels. *Frontiers in Molecular Neuroscience* 11. DOI: 10.3389/fnmol.2018.00487.
Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. DOI: 10.1038/365061a0.
Naccarato M, Pizzuti D, Petrosino S, et al. (2010) Possible anandamide and palmitoylethanolamide involvement in human stroke. *Lipids in Health and Disease* 9(1). DOI: 10.1186/1476-511X-9-47.
Nadal X, del Río C, Casano S, et al. (2017) Tetrahydrocannabinolic acid is a potent PPARγ agonist with neuroprotective activity. *British Journal of Pharmacology* 174(23): 4263–4276. DOI: 10.1111/bph.14019.
Nayak D, Roth TL and McGavern DB (2014) Microglia development and function. *Annual Review of Immunology* 32: 367–402. DOI: 10.1146/annurev-immunol-032713-120240.
Nichols NR, Day JR, Laping NJ, et al. (1993) GFAP mRNA increases with age in rat and human brain. *Neurobiology of Aging* 14(5): 421–429. DOI: 10.1016/0197-4580(93)90100-P.
Nilius B, Vennekens R and Owsianik G (2008) Vanilloid Transient Receptor Potential Cation Channels: An Overview. *Current Pharmaceutical Design* 14(1): 18–31. DOI: 10.2174/138161208783330763.
Nishizawa Y (2001) Glutamate release and neuronal damage in ischemia. *Life Sciences* 69(4): 369–381. DOI: 10.1016/S0024-3205(01)01142-0.
O'Collins VE, Macleod MR, Donnan GA, et al. (2006) 1,026 Experimental treatments in acute stroke. *Annals of Neurology*. DOI: 10.1002/ana.20741.
O'Sullivan SE (2016) An update on PPAR activation by cannabinoids. *British Journal of Pharmacology* 173(12): 1899–1910. DOI: 10.1111/bph.13497.
O'Sullivan SE, Tarling EJ, Bennett AJ, et al. (2005) Novel time-dependent vascular actions of Δ9- tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma. *Biochemical and Biophysical Research Communications*. DOI: 10.1016/j.bbrc.2005.09.121.
Oldendorf WH, Cornford ME and Brown WJ (1977) The large apparent work capability of the blood-brain barrier: A study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. *Annals of Neurology* 1(5): 409–417. DOI: 10.1002/ana.410010502.
Ordy JM, Wengenack TM, Bialobok P, et al. (1993) Selective vulnerability and early

progression of hippocampal CA1 pyramidal cell degeneration and GFAP-positive astrocyte reactivity in the rat four-vessel occlusion model of transient global ischemia. *Experimental Neurology* 119(1): 128–139. DOI: 10.1006/exnr.1993.1014.

Osumi T, Wen JK and Hashimoto T (1991) Two cis-acting regulatory sequences in the peroxisome proliferator-responsive enhancer region of rat acyl-CoA oxidase gene. *Biochemical and Biophysical Research Communications*. DOI: 10.1016/0006-291X(91)91645-S.

Owasil R, O'neill R, Keable A, et al. (2020) The pattern of AQP4 expression in the ageing human brain and in cerebral amyloid angiopathy. *International Journal of Molecular Sciences* 21(4). DOI: 10.3390/ijms21041225.

Pagano E, Romano B, Iannotti FA, et al. (2019) The non-euphoric phytocannabinoid cannabidivarin counteracts intestinal inflammation in mice and cytokine expression in biopsies from UC pediatric patients. *Pharmacological Research* 149. DOI: 10.1016/j.phrs.2019.104464.

Pan W and Kastin AJ (2007) Tumor necrosis factor and stroke: Role of the blood-brain barrier. *Progress in Neurobiology*. DOI: 10.1016/j.pneurobio.2007.07.008.

Pardridge WM, Boado RJ and Farrell CR (1990) Brain-type glucose transporter (GLUT-1) is selectively localized to the blood-brain barrier: Studies with quantitative Western blotting and in situ hybridization. *Journal of Biological Chemistry* 265(29): 18035–18040.

Park N (2019) Population estimates for the UK, England and Wales, Scotland and Northern Ireland, provisional: mid-2019. Available at: https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/bulletins/annualmidyearpopulationestimates/mid2019 (accessed 11 May 2020).

Parmentier-Batteur S, Jin K, Mao XO, et al. (2002) Increased severity of stroke in CB1 cannabinoid receptor knock-out mice. *Journal of Neuroscience* 22(22): 9771–9775. DOI: 10.1523/jneurosci.22-22-09771.2002.

Partyka PP, Godsey GA, Galie JR, et al. (2017) Mechanical stress regulates transport in a compliant 3D model of the blood-brain barrier. *Biomaterials* 115: 30–39. DOI: 10.1016/j.biomaterials.2016.11.012.

Paton WDM (1973) Cannabis and its Problems. *Journal of the Royal Society of Medicine*. DOI: 10.1177/003591577306600753.

Peinado MA, Quesada A, Pedrosa JA, et al. (1998) Quantitative and ultrastructural changes in gila and pericytes in the parietal cortex of the aging rat. *Microscopy Research and Technique* 43(1): 34–42. DOI: 10.1002/(SICI)1097-0029(19981001)43:1<34::AID-JEMT6>3.0.CO;2-G.

Peppiatt CM, Howarth C, Mobbs P, et al. (2006) Bidirectional control of CNS capillary diameter by pericytes. *Nature*. DOI: 10.1038/nature05193.

Perlmutter LS, Gall C, Baudry M, et al. (1990) Distribution of calcium-activated protease calpain in the rat brain. *Journal of Comparative Neurology* 296(2): 269–276. DOI: 10.1002/cne.902960207.

Pertwee RG (1988) The central neuropharmcology of psychotropic cannabinoids. *Pharmacology and Therapeutics* 36(2–3): 189–261. DOI: 10.1016/0163-

7258(88)90106-4.
Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacology and Therapeutics*. DOI: 10.1016/S0163-7258(97)82001-3.
Pertwee RG and Ross RA (2002) Cannabinoid receptors and their ligands. *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA)* 66(2–3): 101–121. DOI: 10.1054/plef.2001.0341.
Pieper C, Pieloch P and Galla HJ (2013) Pericytes support neutrophil transmigration via interleukin-8 across a porcine co-culture model of the blood-brain barrier. *Brain Research* 1524. Elsevier: 1–11. DOI: 10.1016/j.brainres.2013.05.047.
Pires PW and Earley S (2018) Neuroprotective effects of trpa1 channels in the cerebral endothelium following ischemic stroke. *eLife* 7(e35316.). DOI: 10.7554/eLife.35316.
Pisani V, Moschella V, Bari M, et al. (2010) Dynamic changes of anandamide in the cerebrospinal fluid of Parkinson's disease patients. *Movement Disorders* 25(7): 920–924. DOI: 10.1002/mds.23014.
Pisanti S, Malfitano AM, Ciaglia E, et al. (2017) Cannabidiol: State of the art and new challenges for therapeutic applications. *Pharmacology and Therapeutics* 175: 133–150. DOI: 10.1016/j.pharmthera.2017.02.041.
Pistis M and O'Sullivan SE (2017) The Role of Nuclear Hormone Receptors in Cannabinoid Function. In: *Advances in Pharmacology*, pp. 291–328. DOI: 10.1016/bs.apha.2017.03.008.
Popa-Wagner A, Badan I, Walker L, et al. (2007) Accelerated infarct development, cytogenesis and apoptosis following transient cerebral ischemia in aged rats. *Acta Neuropathologica* 113(3): 277–293. DOI: 10.1007/s00401-006-0164-7.
Potz BA, Sabe AA, Elmadhun NY, et al. (2017) Calpain inhibition decreases inflammatory protein expression in vessel walls in a model of chronic myocardial ischemia. *Surgery (United States)* 161(5): 1394–1404. DOI: 10.1016/j.surg.2016.11.009.
Raichlen DA, Foster AD, Gerdeman GL, et al. (2012) Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the 'runner's high'. *Journal of Experimental Biology*. DOI: 10.1242/jeb.063677.
Ransom BR and Ransom CB (2012) Astrocytes: Multitalented stars of the central nervous system. *Methods in Molecular Biology* 814: 3–7. DOI: 10.1007/978-1-61779-452-0_1.
Resstel LBM, Tavares RF, Lisboa SFS, et al. (2009) 5-HT 1A receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats. *British Journal of Pharmacology*. DOI: 10.1111/j.1476-5381.2008.00046.x.
Riva N, Mora G, Sorarù G, et al. (2019) Safety and efficacy of nabiximols on spasticity symptoms in patients with motor neuron disease (CANALS): a multicentre, double-blind, randomised, placebo-controlled, phase 2 trial. *The Lancet Neurology* 18(2): 155–164. DOI: 10.1016/S1474-4422(18)30406-X.
Rock EM and Parker LA (2013) Effect of low doses of cannabidiolic acid and ondansetron on LiCl-induced conditioned gaping (a model of nausea-induced behaviour) in rats. *British Journal of Pharmacology* 169(3): 685–692. DOI:

10.1111/bph.12162.
Rock EM, Limebeer CL, Mechoulam R, et al. (2008) The effect of cannabidiol and URB597 on conditioned gaping (a model of nausea) elicited by a lithium-paired context in the rat. *Psychopharmacology* 196(3): 389–395. DOI: 10.1007/s00213-007-0970-1.
Rodríguez-Cueto C, Santos-García I, García-Toscano L, et al. (2018) Neuroprotective effects of the cannabigerol quinone derivative VCE-003.2 in SOD1G93A transgenic mice, an experimental model of amyotrophic lateral sclerosis. *Biochemical Pharmacology* 157: 217–226. DOI: 10.1016/j.bcp.2018.07.049.
Rosenberg E, Bazelot M and Chamberland S (2018) Cannabidiol (CBD) exerts anti-epileptic actions by targeting the LPI-GPR55 signaling system potentiated by seizures. In: *American epilepsy society*, 2018.
Rosenberg GA (2012) Neurological diseases in relation to the blood-brain barrier. *Journal of Cerebral Blood Flow and Metabolism*. DOI: 10.1038/jcbfm.2011.197.
Russo EB and Marcu J (2017) Cannabis Pharmacology: The Usual Suspects and a Few Promising Leads. *Advances in Pharmacology* 80: 67–134. DOI: 10.1016/bs.apha.2017.03.004.
Ryberg E, Larsson N, Sjögren S, et al. (2009) The orphan receptor GPR55 is a novel cannabinoid receptor. *British Journal of Pharmacology* 152(7): 1092–1101. DOI: 10.1038/sj.bjp.0707460.
Saliba SW, Jauch H, Gargouri B, et al. (2018) Anti-neuroinflammatory effects of GPR55 antagonists in LPS-activated primary microglial cells. *Journal of Neuroinflammation* 15(1). DOI: 10.1186/s12974-018-1362-7.
Sattler R and Rothstein JD (2006) Regulation and dysregulation of glutamate transporters. *Handbook of Experimental Pharmacology*. DOI: 10.1007/3-540-29784-7-14.
Sawzdargo M, Nguyen T, Lee DK, et al. (1999) Identification and cloning of three novel human G protein-coupled receptor genes GPR52, ΨGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Molecular Brain Research*. DOI: 10.1016/S0169-328X(98)00277-0.
Schäbitz WR, Giuffrida A, Berger C, et al. (2002) Release of fatty acid amides in a patient with hemispheric stroke: A microdialysis study. *Stroke* 33(8): 2112–2114. DOI: 10.1161/01.STR.0000023491.63693.18.
Schmidt-Pogoda A, Bonberg N, Koecke MHM, et al. (2020) Why Most Acute Stroke Studies Are Positive in Animals but Not in Patients: A Systematic Comparison of Preclinical, Early Phase, and Phase 3 Clinical Trials of Neuroprotective Agents. *Annals of Neurology* 87(1): 40–51. DOI: 10.1002/ana.25643.
Schoknecht K, David Y and Heinemann U (2015) The blood-brain barrier-Gatekeeper to neuronal homeostasis: Clinical implications in the setting of stroke. *Seminars in Cell and Developmental Biology* 38: 35–42. DOI: 10.1016/j.semcdb.2014.10.004.
Schreibelt G, Kooij G, Reijerkerk A, et al. (2007) Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling. *The FASEB Journal* 21(13): 3666–3676. DOI: 10.1096/fj.07-8329com.
Schubert D, Kepchia D, Liang Z, et al. (2019) Efficacy of Cannabinoids in a Pre-Clinical Drug-Screening Platform for Alzheimer's Disease. *Molecular Neurobiology* 56(11):

7719–7730. DOI: 10.1007/s12035-019-1637-8.
Seifert G, Schilling K and Steinhäuser C (2006) Astrocyte dysfunction in neurological disorders: A molecular perspective. *Nature Reviews Neuroscience*. DOI: 10.1038/nrn1870.
Shaafi S, Sharifipour E, Rahmanifar R, et al. (2014) Interleukin-6, a reliable prognostic factor for ischemic stroke. *Iranian journal of neurology*.
Shi Y, Jiang X, Zhang L, et al. (2017) Endothelium-targeted overexpression of heat shock protein 27 ameliorates blood-brain barrier disruption after ischemic brain injury. *Proceedings of the National Academy of Sciences of the United States of America* 114(7): E1243–E1252. DOI: 10.1073/pnas.1621174114.
Shigemoto-Mogami Y, Hoshikawa K and Sato K (2018) Activated Microglia Disrupt the Blood-Brain Barrier and Induce Chemokines and Cytokines in a Rat in vitro Model. *Frontiers in Cellular Neuroscience* 12(December): 1–7. DOI: 10.3389/fncel.2018.00494.
Shim WS, Tak MH, Lee MH, et al. (2007) TRPV1 mediates histamine-induced itching via the activation of phospholipase A2 and 12-lipoxygenase. *Journal of Neuroscience*. DOI: 10.1523/JNEUROSCI.4643-06.2007.
Silver J and Miller JH (2004) Regeneration beyond the glial scar. *Nature Reviews Neuroscience*. DOI: 10.1038/nrn1326.
Sims NR and Yew WP (2017) Reactive astrogliosis in stroke: Contributions of astrocytes to recovery of neurological function. *Neurochemistry International* 107: 88–103. DOI: 10.1016/j.neuint.2016.12.016.
Sofroniew M V. and Vinters H V. (2010) Astrocytes: Biology and pathology. *Acta Neuropathologica*. DOI: 10.1007/s00401-009-0619-8.
Sparling PB, Giuffrida A, Piomelli D, et al. (2003) Exercise activates the endocannabinoid system. *Neuroreport* 14(17): 2209–2211. DOI: 10.1097/01.wnr.0000097048.56589.47.
Srinivasan B, Kolli AR, Esch MB, et al. (2015) TEER Measurement Techniques for In Vitro Barrier Model Systems. *Journal of Laboratory Automation* 20(2): 107–126. DOI: 10.1177/2211068214561025.
Stamatovic SM, Shakui P, Keep RF, et al. (2005) Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. *Journal of Cerebral Blood Flow and Metabolism* 25(5): 593–606. DOI: 10.1038/sj.jcbfm.9600055.
Stamatovic SM, Keep RF, Wang MM, et al. (2009) Caveolae-mediated internalization of occludin and claudin-5 during CCL2-induced tight junction remodeling in brain endothelial cells. *Journal of Biological Chemistry* 284(28): 19053–19056. DOI: 10.1074/jbc.M109.000521.
Stewart PA, Hayakawa K and Farrell CL (1994) Quantitation of blood-brain barrier ultrastructure. *Microscopy Research and Technique* 27(6): 516–527. DOI: 10.1002/jemt.1070270606.
Stone NL, Millar SA, Herrod PJJ, et al. (2018) An analysis of endocannabinoid concentrations and mood following singing and exercise in healthy volunteers. *Frontiers in Behavioral Neuroscience* 12. DOI: 10.3389/fnbeh.2018.00269.
Stone NL, England TJ and O'Sullivan SE (2019) A novel transwell blood brain barrier model using primary human cells. *Frontiers in Cellular Neuroscience* 13. DOI:

10.3389/fncel.2019.00230.
Stone NL, Murphy AJ, England TJ, et al. (2020) A systematic review of minor phytocannabinoids with promising neuroprotective potential. *British Journal of Pharmacology* 177(19): 4330–4352. DOI: 10.1111/bph.15185.
Story GM, Peier AM, Reeve AJ, et al. (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 112(6): 819–829. DOI: 10.1016/S0092-8674(03)00158-2.
Strong K, Mathers C and Bonita R (2007) Preventing stroke: saving lives around the world. *Lancet Neurology* 6(2): 182–187. DOI: 10.1016/S1474-4422(07)70031-5.
Sun Y, Alexander SPH, Garle MJ, et al. (2007) Cannabinoid activation of PPARα; a novel neuroprotective mechanism. *British Journal of Pharmacology*. DOI: 10.1038/sj.bjp.0707478.
Szalay G, Martinecz B, Lénárt N, et al. (2016) Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. *Nature Communications* 7. DOI: 10.1038/ncomms11499.
Taylor RA and Sansing LH (2013) Microglial responses after ischemic stroke and intracerebral hemorrhage. *Clinical and Developmental Immunology* 2013. DOI: 10.1155/2013/746068.
Tremblay MÈ, Stevens B, Sierra A, et al. (2011) The role of microglia in the healthy brain. *Journal of Neuroscience* 31(45): 16064–16069. DOI: 10.1523/JNEUROSCI.4158-11.2011.
Tyagi S, Gupta P, Saini A, et al. (2011) The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *Journal of Advanced Pharmaceutical Technology and Research* 2(4): 236–240. DOI: 10.4103/2231-4040.90879.
Urbi B, Broadley S, Bedlack R, et al. (2019) Study protocol for a randomised, double-blind, placebo-controlled study evaluating the Efficacy of cannabis-based Medicine Extract in slowing the disease pRogression of Amyotrophic Lateral sclerosis or motor neurone Disease: The EMERALD trial. *BMJ Open* 9(11). DOI: 10.1136/bmjopen-2019-029449.
Valdeolivas S, Navarrete C, Cantarero I, et al. (2015) Neuroprotective Properties of Cannabigerol in Huntington's Disease: Studies in R6/2 Mice and 3-Nitropropionate-lesioned Mice. *Neurotherapeutics* 12(1): 185–99. DOI: 10.1007/s13311-014-0304-z.
Van Der Flier WM and Scheltens P (2005) Epidemiology and risk factors of dementia. *Neurology in Practice*. DOI: 10.1136/jnnp.2005.082867.
Von Tell D, Armulik A and Betsholtz C (2006) Pericytes and vascular stability. *Experimental Cell Research*. DOI: 10.1016/j.yexcr.2005.10.019.
Wang Z, Li Y, Cai S, et al. (2018) Cannabinoid receptor 2 agonist attenuates blood-brain barrier damage in a rat model of intracerebral hemorrhage by activating the Rac1 pathway. *International Journal of Molecular Medicine* 42(5): 2914–2922. DOI: 10.3892/ijmm.2018.3834.
Yan A, Zhang T, Yang X, et al. (2016) Thromboxane A2 receptor antagonist SQ29548 reduces ischemic stroke-induced microglia/macrophages activation and enrichment, and ameliorates brain injury. *Scientific Reports*. DOI:

10.1038/srep35885.
Yang G, Meng Y, Li W, et al. (2011) Neuronal MCP-1 mediates microglia recruitment and neurodegeneration induced by the mild impairment of oxidative metabolism. *Brain Pathology* 21(3): 279–297. DOI: 10.1111/j.1750-3639.2010.00445.x.
Yousufuddin M and Young N (2019) Aging and ischemic stroke. *Aging*. DOI: 10.18632/aging.101931.
Yu SJ, Reiner D, Shen H, et al. (2015) Time-dependent protection of CB2 receptor agonist in stroke. *PLoS ONE* 10(7). DOI: 10.1371/journal.pone.0132487.
Zajicek J, Fox P, Sanders H, et al. (2003) Cannabinoids for treatment of spasticity and other symptoms related to multiple sclerosis (CAMS study): Multicentre randomised placebo-controlled trial. *Lancet*. DOI: 10.1016/S0140-6736(03)14738-1.
Zamberletti E, Gabaglio M, Woolley-Roberts M, et al. (2019) Cannabidivarin Treatment Ameliorates Autism-Like Behaviors and Restores Hippocampal Endocannabinoid System and Glia Alterations Induced by Prenatal Valproic Acid Exposure in Rats. *Frontiers in Cellular Neuroscience* 13. DOI: 10.3389/fncel.2019.00367.
Zani A, Braida D, Capurro V, et al. (2007) Δ 9-Tetrahydrocannabinol (THC) and AM 404 protect against cerebral ischaemia in gerbils through a mechanism involving cannabinoid and opioid receptors. *British Journal of Pharmacology* 152(8): 1301–1311. DOI: 10.1038/sj.bjp.0707514.
Zhang E, Chen Q, Wang J, et al. (2020) Protective role of microRNA-27a upregulation and HSP90 silencing against cerebral ischemia-reperfusion injury in rats by activating PI3K/AKT/mTOR signaling pathway. *International Immunopharmacology* 86. DOI: 10.1016/j.intimp.2020.106635.
Zhang W, Zhu L, An C, et al. (2020) The blood brain barrier in cerebral ischemic injury – Disruption and repair. *Brain Hemorrhages* 1(1). Elsevier B.V.: 34–53. DOI: 10.1016/j.hest.2019.12.004.
Zheng J (2013) Molecular mechanism of TRP channels. *Comprehensive Physiology* 3(1): 221–242. DOI: 10.1002/cphy.c120001.
Zhou Y, Yang L, Ma A, et al. (2012) Orally administered oleoylethanolamide protects mice from focal cerebral ischemic injury by activating peroxisome proliferator-activated receptor α. *Neuropharmacology*. DOI: 10.1016/j.neuropharm.2012.03.008.
Zimmermann KW (1923) Der feinere Bau der Blutcapillaren. *Zeitschrift für Anatomie und Entwicklungsgeschichte*. DOI: 10.1007/bf02593544.
Ziouzenkova O and Plutzky J (2004) Lipolytic PPAR activation: New insights into the intersection of triglycerides and inflammation? *Current Opinion in Clinical Nutrition and Metabolic Care*. DOI: 10.1097/01.mco.0000134358.46159.61.
Zlokovic B V. (1995) Cerebrovascular Permeability to Peptides: Manipulations of Transport Systems at the Blood-Brain Barrier. *Pharmaceutical Research: An Official Journal of the American Association of Pharmaceutical Scientists*. DOI: 10.1023/A:1016254514167.
Zuardi AW, Crippa JAS, Hallak JEC, et al. (2009) Cannabidiol for the treatment of psychosis in Parkinsons disease. *Journal of Psychopharmacology* 23(8): 979–983. DOI: 10.1177/0269881108096519.

Zygmunt PM, Petersson J, Andersson DA, et al. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature*. DOI: 10.1038/22761.