# Endocannabinoids modulate human blood-brain barrier permeability *in vitro*

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Running Title: Endocannabinoids modulate BBB permeability

**Summary**

**Background and purpose.** Endocannabinoids alter permeability at various epithelial barriers, and cannabinoid receptors and endocannabinoid levels are elevated by stroke, with potential neuroprotective effects. We therefore explored the role endocannabinoids in modulating blood brain barrier (BBB) permeability in normal conditions and in an ischaemia/reperfusion model.

**Experimental approach.** Human brain microvascular endothelial cell and astrocyte co-cultures modelled the BBB. Ischaemia was modelled by oxygen-glucose deprivation (OGD) and permeability was measured by transepithelial electrical resistance. Endocannabinoids or

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endocannabinoid-like compounds were assessed for their ability to modulate baseline permeability or OGD-induced hyperpermeability. Target sites of action were investigated using receptor antagonists, and subsequently identified via RT-PCR.

**Key results.** Anandamide (10μM, $P<0.05$) and oleoylethanolamide (OEA, 10μM, $P<0.01$) decreased BBB permeability (i.e. increased resistance). This was mediated by $CB_2$, transient receptor potential vanilloid 1 (TRPV1), the calcitonin gene regulated peptide (CGRP) receptor (anandamide only) and peroxisome proliferator activated receptor (PPARα; OEA only). Application of OEA, palmitoylethanolamide (both PPARα mediated) or virodhamine (all 10 μM) decreased the OGD-induced increase in permeability during reperfusion. 2-arachidonoyl glycerol, noladin ether and oleamide did not affect BBB permeability in normal or OGD conditions. *N*-arachidonoyl-dopamine increased permeability through a cytotoxic mechanism. PPARα and γ, $CB_1$, TRPV1, and CGRP receptor expression was shown in both cell types, but $CB_2$ mRNA was only present in astrocytes.

**Conclusion and implication.** These data show that endocannabinoids may play an important modulatory role in normal BBB physiology, and also afford protection to the BBB during ischaemic stroke, through a number of target sites.

**Abbreviations:**

AEA; Anandamide

2-AG; 2-arachidonoylglycerol

BBB; blood brain barrier

CB1; cannabinoid receptor 1

CB2; cannabinoid receptor 2

CGRP; Calcitonin gene related peptide

ECS; endocannabinoid system

FAAH; fatty acid amide hydrolase

LDH; Lactate dehydrogenase

NADA*; N*-arachidonoyl-dopamine

OEA ; oleoylethanolamide

OGD ; oxygen-glucose deprivation

PEA; palmitoylethanolamide

PPAR; peroxisome proliferator activated receptor

RT-PCR; real-time polymerase chain reaction

TEER; Transepithelial electrical resistance

TRPV1; transient receptor potential vanilloid 1

**Introduction**

The blood-brain barrier (BBB) is formed by brain endothelial cells that line the cerebral microvasculature, capillary basement membranes and astrocyte end feet, which surround 99% of the BBB endothelia and play an important role in maintaining BBB integrity. Tight junctions restrict the paracellular pathway for diffusion of hydrophilic solutes, allowing the body to control which substances can gain access to the brain (Abbott, 2002). Cerebral reperfusion following ischaemia initiates a cascade of events such as inflammation, protease activation, and oxidative and nitrosative stress, which increases the permeability of the BBB (Lo *et al.*, 2003). The compromised state of the BBB aggravates haemorrhagic transformation and vasogenic oedema, which has profound neurological consequences (Latour *et al.*, 2004). Indeed, uncontrolled cerebral oedema represents the leading cause of patient mortality within the first week following an ischaemic stroke (Hacke *et al.*, 1996).

The endocannabinoid system (ECS) is comprised of cannabinoid receptors ($CB_1$ and $CB_2$), endogenous lipid ligands (the endocannabinoids) and enzymes that synthesise and

degrade these compounds (Pertwee *et al.*, 2010). Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best studied endocannabinoids, but other, chemically similar, compounds have been suggested as endocannabinoids or endogenous cannabinoid-like compounds, including *N*-arachidonoyl-dopamine (NADA), noladin ether, oleamide, oleoylethanolamide (OEA), palmitoylethanolamide (PEA) and virodhamine. $CB_1$ and $CB_2$ receptors are not the only pharmacological targets for cannabinoids, which also display activity at the transient receptor potential vanilloid 1 (TRPV1), GPR55, and peroxisome proliferator activated receptors (PPAR)α and PPARγ (Pertwee *et al.*, 2010).

Some endocannabinoids have been shown to play a role in the regulation of BBB permeability in conditions other than ischaemia. Using *in vivo* and *in vitro* models, increased BBB permeability following chronic head injury and multiple sclerosis were decreased by the exogenous addition of 2-AG (Panikashvili *et al.*, 2006) or AEA (Mestre *et al.*, 2011), respectively. Oleamide has been found to inhibit gap junction coupling, thus increasing barrier permeability *in vitro* using pig brain microvascular endothelial cells (Nagasawa *et al.*, 2006). Interestingly, the effect of any other endocannabinoid or ECL on BBB permeability in normal conditions has not been investigated.

Components of the ECS are known to be altered by stroke. Human and animal *in vivo* data has shown increases in neurological levels of AEA (peripheral levels also elevated), 2-AG, OEA and PEA (Hillard, 2008; Naccarato *et al.*, 2010). The expression of cannabinoid receptors is upregulated in the rat brain following cerebral ischaemia, indicating that the ECS may play an important role in the endogenous response to stroke (see Hillard, 2008). Indeed, exogenous administration of 2-AG (Wang *et al.*, 2009), AEA (Wang *et al.*, 2009), OEA (Sun *et al.*, 2007; Zhou *et al.*, 2012) and PEA (Ahmad *et al.*, 2012b) offer neuroprotection against ischaemic stroke using *in vitro* and *in vivo models*, but the impact on BBB permeability in

stroke has only been assessed for OEA, where it was found to decrease *in vivo* BBB permeability via PPARα (Zhou *et al.*, 2012).

Since endocannabinoids offer neuroprotection from stroke and alter BBB permeability in various neurological disorders, we hypothesised that endocannabinoids might regulate *in vitro* BBB permeability in normal and ischaemic conditions. Our results show for the first time that AEA and OEA decrease permeability in normal conditions. When given before oxygen glucose deprivation (OGD), only OEA, PEA and virodhamine decreased BBB permeability. This study illustrates the important role that the ECS plays in regulating BBB permeability via several target sites of action.

## Methods

### *In vitro* BBB co-culture model

Human brain microvascular endothelial cells isolated from human brain tissue (HBMECs; Catalog #1000, ScienCell, USA) and human astrocytes isolated from human cerebral cortex (HAs; Catalog #1800, ScienCell, USA) were co-cultured in Endothelial Cell Medium (Catalog #1001; the concentration per ml is 10 µg apo-transferrin, 10 µg BSA, 2 ng FGF-2, 1 µg hydrocortisone, 2 ng IGF-I, 7.5 µg insulin and 20 nM progesterone. 2% FBS, 5.55 mM glucose and 10,000 units/ml of penicillin and streptomycin) and Astrocyte medium (Catalog #1801; the concentration per ml is 10 µg apo-transferrin, 10 µg BSA, 10 ng EGF, 10 ng FGF-2, 1 µg hydrocortisone, 5 µg insulin, 2 ng IGF-I, 0.5 ng IGF LR3 10-8 M retinoic acid & 2 ng VEGF. 5% FBS, Glucose 5.55 mM and 10,000 units/ml of penicillin and streptomycin) (both ScienCell, USA). HAs were seeded on the outside of collagen-coated 0.4 µm pore PTFE membrane Transwell inserts (12 well type; Corning Costar, USA) directed upside down and allowed to adhere to the membrane overnight. HBMECs were seeded on the inside of the

insert and cells were grown to confluence to create a contact co-culture model (Allen *et al.*, 2009; Mestre *et al.*, 2011).

**Measurement of BBB permeability**

Transepithelial electrical resistance (TEER) was measured as a marker of co-culture integrity and as a measure of paracellular permeability. The resistance across the membrane was measured using STX2 electrodes linked to an EVOM$^2$ resistance meter (World Precision Instruments, UK). Three readings were taken per insert and the average value used. A baseline TEER reading was taken (i.e. 0 h) and the percentage change from this value was calculated for subsequent readings. The average TEER was 30.23 ± 0.24 Ω/cm$^2$, similar to that previously reported using the same methodology (Allen and Bayraktutan, 2009).

To assess their impact on permeability, endocannabinoids were added to the luminal (endothelial) chamber and TEER was measured at various time points over 48 h, at which point the media was changed, endocannabinoids reapplied and TEER measured for another 48 h. Endocannabinoids that significantly altered permeability, had their mechanism of action probed using relevant receptor antagonists which were co-administered with the endocannabinoids. All molecular target nomenclature conforms to the British Journal of Pharmacology's Concise Guide to Pharmacology (Alexander et al., 2013).

**RT-PCR**

Presence of predicted sites of action was investigated at the mRNA level using reverse transcription followed by polymerase chain reaction (RT-PCR). Total RNA was extracted from HA and HBMEC cells using Allprep DNA/RNA kit with on column DNaseI treatment (Qiagen, Germany). Reverse transcription with and without reverse transcriptase was performed in 20 µl final volume using 2 µg of total RNA and random primers with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, UK) according to the

manufacturer's instructions. PCR reactions were carried out in a final volume of 25 μl with Zymotaq (ZymoResearch, USA) using 2 μl of reverse transcription product as template. Primer pairs used to amplify PPARα and PPARγ fragments (99bp and 87bp respectively) were as described in (Reynders *et al*., 2006); those for 128bp HPRT were from (Spinsanti *et al*., 2008); those for 303bp $CB_1R$ and 365bp $CB_2R$ were from (Cencioni *et al*., 2010); those for 511bp TRPV1 were from (Luo et al., 2008); and finally the 380bp CGRPR cDNA fragment was amplified using the primers reported in (Dong et al., 1999). After 5 min at 95°C, PCRs were performed for 40 cycles except those for CGRPR and $CB_2R$ that were carried out for 60 cycles. The cycles included 30sec at 95°C, 30sec at the annealing temperature optimal for each primer pair (56°C for $CB_1R$ and $CB_2R$; 60°C for PPARα, PPARγ and HPRT; 58°C for TRPV1; 61°C for CGRPR) and a final extension step of 30 sec at 72°C. Amplification products were separated by gel electrophoresis through ethidium bromide stained 2% agarose ($CB_1R$, $CB_2R$, TRPV1, CGRPR and HPRT) or 3% metaphore (PPARα and PPARγ) gels and visualised using a Biorad Chemidoc.

**Oxygen-glucose deprivation**

Ischaemic conditions were simulated using an oxygen-glucose deprivation (OGD) protocol. Cell culture media was replaced with glucose free RPMI medium (Invitrogen, UK) and the plates placed into a GasPak EZ Anaerobe Pouch (Beckton Dickinson, UK) with anaerobic conditions being achieved within 20 minutes, and the inserts were left in OGD conditions for a further 4 h in the incubator. No preconditioning was carried out on the cells. After OGD, TEER was read and the RPMI medium was replaced with the cells' normal medium and returned to the incubator. The permeability of the BBB was assessed throughout the reperfusion period. Medium samples were collected and stored at -80°C whenever the

medium was changed. Endocannabinoids were added before the OGD protocol to mimic an endogenous response to ischaemia.

**Lactate dehydrogenase assay**

Lactate dehydrogenase levels were measured using a commercially available kit according to the manufacturer's instructions (LDH-Cytotoxicity Assay Kit II, Biovision, USA). Medium samples were transferred into an optically clear 96-well plate and Reaction Mix (containing water soluble tetrazolium-1) was added to each well. After 30 min, absorbance was measured at 450 nm, subtracting the 650 nm reading to correct for optical imperfections in the plate.

**Drugs and Chemicals**

All endocannabinoids were purchased from Tocris (UK) and dissolved in ethanol to a stock concentration of 10 mM, except 2-AG which was purchased from Abcam (UK) and dissolved in acetonitrile. AM251, AM630, GW6471, GW9662 (all 100 nM), capsazepine, O-1918 (both 1 μM), (all dissolved in dimethyl sulfoxide) and calcitonin gene releasing peptide $(CGRP)_{8-37}$ (2 μM, dissolved in distilled water) were all purchased from Tocris (UK) and URB597 (1 μM, dissolved in dimethyl sulfoxide) was purchased from Sigma (UK). All dissolved to a stock solution of 10 mM .

**Statistics**

Data were compared with GraphPad Prism software (USA), using either Student's *t*-test or one-way ANOVA with Dunnett's or Bonferroni's *post hoc* test. Area under the curve (AUC) values were calculated using the trapezoidal method. In experiments conducted in control conditions, the baseline was set to be at the lowest value in the data sets, and the area above baseline was calculated. In the OGD experiments, the baseline was set to be highest value obtained in the data sets, and the area below baseline was calculated. $P<0.05$ was considered significant and error bars represent mean ± SEM.

## Results

### *Effects of anandamide on blood-brain barrier permeability*

AEA is a well characterised and frequently studied endocannabinoid, displaying effects on epithelial barrier permeability in BBB and non-BBB sites, therefore, this was the first compound investigated. AEA at 10 μM, but not 100 nM or 1 μM, decreased permeability (i.e. increased TEER/monolayer resistance) (see Figure 1A,B). In all subsequent antagonist studies, AEA (10 μM) also significantly increased TEER compared to vehicle in the same experimental set up as the antagonists. In these studies, the effect of AEA on BBB permeability was not inhibited by AM251 ($CB_1$), GW6471 (PPARα), GW9662 (PPARγ), or O1918 (novel endothelial receptor) (see Table 1 for AUC values). However, the effect of AEA (10 μM) was inhibited by the $CB_2$ antagonist AM630, the TRPV1 antagonist capsazepine and the CGRP receptor antagonist $CGRP_{8-37}$ (Figure 1 C-E). A synthetic $CB_2$ agonist HU308 and the steroid dexamethasone (as a positive control) were also able to significantly increase TEER in this BBB model (Figure 1F,G).

RT-PCR was carried out to profile the expression at the RNA level of these potential target sites of action in HAs and HBMECs. PPARs (α and γ), $CB_1$, TRPV1, and the CGRP receptor were found to be present in both cell types. By contrast, mRNA for $CB_2$ receptor was only present in the astrocytes (Figure 2).

Inhibition of the degradation of AEA by the fatty acid amide hydrolase (FAAH) inhibitor URB597 blocked the effects of AEA such that the change in TEER was no longer significantly different to that observed in the vehicle control inserts (Figure 3A,B).

Exposing the BBB to 4 h OGD increased permeability as shown by a reduction in TEER of approximately 35% (Figure 4). AEA did not alter the BBB permeability response to OGD when applied before (Figure 4A and B) or after the OGD protocol (Figure 4C and D).

***Effects of other endocannabinoids and endocannabinoid-like compounds on blood-brain barrier permeability given in normal conditions***

2-AG, noladin ether, oleamide, PEA and virodhamine did not alter BBB permeability when given in normal conditions (see Table 1). However, OEA significantly increased TEER (decreased permeability) in normal conditions at 10 μM ($P$<0.01, Figure 5A). A concentration-response curve showed that a significant response to OEA was only observed at 10 μM (Figure 5B). The effects of OEA (10 μM) were not inhibited by AM251, AM630, capsazepine or GW9662 (see Table 1), but were inhibited by GW6471, a PPARα agonist (P<0.05, Figure 5C and D).

After 48 h exposure to a single application of NADA (10 μM), BBB permeability was significantlyincreased (Figure 5E). Following a second application of NADA, BBB permeability remained significantly below that of vehicle for another 24 hours (see Figure 5E).Visual inspection using a light microscope showed apparent cellular damage, therefore levels of lactate dehydrogenase in the luminal (endothelial) medium from NADA-treated inserts were measured and found to be significantly greater than vehicle at 48 h ($P$<0.001, Figure 5F).

***Effects of endocannabinoids and endocannabinoid-like compounds on blood-brain barrier permeability given before oxygen-glucose deprivation***

OEA ($P$<0.01, Figure 6A and B), PEA (P<0.05, Figure 6C and D) and virodhamine ($P$<0.01, Figure 6E and F) given before OGD all significantly reduced the increase in permeability induced by the OGD protocol (see AUC values Figure 6B,D and F). The effect of these compounds were mainly observed in the reperfusion period rather than the initial increase in permeability (see Figure 6A,C and E). However, 2-AG, oleamide, NADA and noladin ether

had no effect on the permeability response to OGD (Figure 6G-J). In separate experiments, the protective effect of OEA and PEA given before OGD were inhibited by the PPARα antagonist GW6471 (Figure 7A, B).

## Discussion

The aim of this study was to investigate the potential roles that endocannabinoids play in the regulation of *in vitro* BBB permeability. Our results show that AEA (via TRPV1, $CB_2$ and the CGRP receptor) and OEA (via PPARα) decreased permeability in normal conditions, whilst NADA increased permeability. When given before OGD, OEA, PEA (via PPARα) and virodhamine decreased BBB permeability. Overall, this study illustrates the important role that the ECS plays in regulating BBB permeability, identifying a number of potential targets for future therapies.

### *Effects of anandamide on BBB permeability*

We found that AEA decreased BBB permeability (i.e. increases BBB resistance). This effect of AEA was observed within 2 hours of application, unlike the steroid dexamethasone, where a time-dependent increase in BBB resistance was observed. AEA has been shown to reduce BBB permeability using mouse *in vivo* and *in vitro* models, through attenuation of VCAM-1 levels via $CB_1$ activation (Mestre *et al.*, 2011). In contrast, we found that $CB_2$ activation by AEA was partly responsible for decreasing permeability and showed that a synthetic $CB_2$ agonist (HU308) was capable of inducing a similar acute increase in BBB resistance. PCR expression profiling revealed that the location of $CB_2$ is more likely to be on the astrocytes than endothelial cells. Activation of $CB_2$ has also been shown to decrease *in vivo* BBB permeability in mice following traumatic brain injury (Amenta *et al.*, 2012) or

inflammation (Ramirez *et al.*, 2012), and following stroke $CB_2$ agonists decrease infarct volume by reducing inflammatory infiltrate (Hillard, 2008). Therefore, activity at the BBB could account for some of the protective effects that $CB_2$ agonism has displayed in animal stroke models. It is worth noting that the lack of $CB_2$ in the human brain microvascular endothelial cells isolated from human brain tissue in the present study is in contrast to previous studies. This may be related to the source of human tissue. For example, in the work of Golech *et al.* (2004), the endothelial cells were isolated from brains of patients with idiopathic epilepsy. In the endothelium of human glioblastomas, $CB_2$ is expressed in about half of the cells (Schley et al., 2009). In multiple sclerosis, $CB_2$ has also been identified on the endothelium of cerebral arteries (Zhang et al., 2011). As $CB_2$ is known to be upregulated in pathologies and in response to inflammation and stress, this may explain the expression of $CB_2$ in these studies and not in the cells in the present study, which are derived from normal tissue. In support of this, Ramirez *et al.* (2012) showed little $CB_2$ immunoreactivity in healthy brain endothelium or on human brain microvascular endothelial cells, but that $CB_2$ was highly upregulated in patients with encephalitis or after an inflammatory insult, and was capable of reducing BBB permeability in these situations.

A recent study from our group showed that AEA activation of TRPV1 on the basolateral side of Caco-2 cells (human epithelial colorectal adenocarcinoma cells) reduced permeability, potentially via increases in tight junction proteins (Alhamoruni *et al.*, 2010), and the present study further demonstrates the permeability reducing properties of TRPV1 activation at epithelial barriers. Activation of TRPV1 by AEA is known to increase CGRP release (Zygmunt et al., 1999); therefore, AEA may mediate its effects via increased CGRP levels, as suggested by the effect of the CGRP receptor antagonist in the present study. Both TRPV1 and CGRP receptor mRNA were demonstrated in brain endothelial cells and in astrocytes. This is consistent with a recent study showing that CGRP activation decreased

cerebral oedema and BBB permeability following ischaemic stroke in the rat (Liu *et al.*, 2011).

Inhibiting the degradation of AEA using the FAAH inhibitor URB597 also inhibited the effects of AEA, which suggests that metabolic products of AEA degradation are also involved in AEA's effect on BBB permeability. This may seem contradictory to our data showing the effects of AEA are through activation of $CB_2$ and TRPV1, although this may not be the case. It has been shown for example that a cytochrome P450-derived epoxygenated metabolite of anandamide, 5,6-epoxyeicosatrienoic acid ethanolamide, is a selective $CB_2$ agonist (Snider et al., 2009) and epoxyeicosatrienoic acids derived from AEA can activate TRPV4 (Watanabe et al., 2003), so the possibility exists that in our model, it is the metabolites of AEA that modulate BBB permeability through $CB_2$ and TRPV1.

Although AEA reduced BBB permeability in normal conditions, it had no effect when given either before or after OGD. The increase in permeability caused by OGD was greater than the increase in TEER induced by AEA, suggesting the effect of AEA is not strong enough to alter permeability overall. Alternatively, the expression or function of the receptors involved, $CB_2$ and TRPV1, might be altered by the OGD protocol. Perhaps a negative effect of AEA on BBB permeability through activation of $CB_1$ is revealed is ischaemic conditions (Alhamoruni et al., 2012). Either way, protecting BBB permeability in ischaemia does not appear to underpin the neuroprotective effects of AEA observed in stroke.

***Effects of other endocannabinoids and endocannabinoid-like compounds on BBB permeability***

Like AEA, we found that OEA decreased permeability (increased TEER) when given in normal conditions or in the presence of OGD, and both of these responses were via PPARα activation. PPARα mRNA was confirmed in both the endothelial cells and astrocytes. This data in human

cells is consistent with *in vivo* mouse studies where OEA reduced infarct volume, oedema and BBB disruption following ischaemic stroke, through activation of PPARα (Sun *et al.*, 2007; Zhou *et al.*, 2012). Fenofibrate, a synthetic PPARα agonist, also protected a rodent *in vitro* BBB model from hyperpermeability following OGD (Mysiorek *et al.*, 2009). PPARα agonists inhibit VCAM-1 and ICAM-1 expression, downregulate MMPs and protease activity, and upregulate tight junction proteins (Deplanque *et al.*, 2003; Huang *et al.*, 2009; Marx *et al.*, 1999) all of which are beneficial to BBB integrity. OEA is produced by neurons and glial cells following ischaemia (Hillard, 2008). Hence, OEA activity at PPARα could form an important component of the body's innate defence following stroke, with data from the present study suggesting actions at the BBB may be crucial to this beneficial effect of OEA.

2-AG, noladin ether and oleamide did not affect BBB permeability. These endocannabinoids are known to activate receptors that we have shown to modify the permeability of the BBB in the present study (i.e. $CB_2$ and PPARα), so it is unclear why their effects are dissimilar to AEA and OEA. However, endocannabinoids are known to have complicated pharmacology which may be explained by a number of phenonomen including mechanisms of cell transport and trafficking, metabolism and pharmacologically active metabolites, agonist bias, allosteric modulation and activation of other target sites that might oppose any effects at $CB_2$ or PPARα (Alexander & Kendall, 2007; Kenakin, 2009; Fowler, 2013; Console-Bram *et al.*, 2012).

We found that PEA and virodhamine did not alter the permeability of the BBB in normal conditions, but they did decrease permeability following OGD. Similar to OEA, the permeability lowering effects of PEA were inhibited by the presence of a PPARα antagonist, further confirming a role for this receptor in modulating BBB permeability in ischaemia. PEA has been shown to reduce oedema and brain infarct size in mice using models of two separate diseases, both of which cause BBB damage; traumatic brain injury (Ahmad *et al.*, 2012a) and ischaemic stroke (Ahmad *et al.*, 2012b). Neurological PEA levels in human stroke patients

are increased following ischaemia (Schabitz *et al.*, 2002), which suggests a protective role for PEA in stroke, with permeability reducing effects on the BBB potentially forming part of this.

To date, no studies have investigated the effects of virodhamine on epithelial barrier permeability or stroke. However, virodhamine does inhibit neutrophil migration through $CB_1$ activation (McHugh *et al.*, 2008). Neutrophil accumulation and infiltration into the cerebral microvasculature plays a critical role in neuronal injury following cerebral ischaemia, especially during reperfusion (Sughrue *et al.*, 2004). Attenuation of neutrophil migration may be of benefit in treating hyper-acute stroke, but previous trials assessing compounds that inhibit neutrophilic function have been ineffective (Krams *et al.*, 2003).

NADA was found to increase BBB permeability with evidence of cell damage. In support of these findings, NADA has been shown to cause concentration-dependent cytotoxicity in human, murine or rat hepatic stellate cells (Wojtalla *et al.*, 2012), and human peripheral blood mononuclear cells (Saunders *et al.*, 2009). Interestingly, although NADA increased the permeability of the BBB in normal conditions, it did not further increase the permeability of inserts that were exposed to 4 hours OGD. This is most likely due to the fact that in this protocol, the cells were only exposed to NADA for 4 hours, compared to 96 hours in the non-OGD experiments, and that the cytotoxic effects of NADA are revealed with longer exposure times. NADA is known to be produced in bovine and rat brains (Walker *et al.*, 2002), but no studies have investigated whether stroke alters the levels of NADA, and what consequence this might have.

Of note, the effects of endocannabinoids were only observed in the high micromolar concentrations. This may be partly due to technical issues such as binding of endocannabinoids to cell culture plastic (see Fowler et al., 2004), interactions with FBS or BSA in culture medium, or the transport of endocannabinoids into and across the cell, which is particularly relevant for the PPARα activating endocannabinoids (Kaczocha et al., 2012). In addition, although studies have

investigated the levels of endocannabinoid in the brain, it is not known what the exact intracellular concentration of endocannabinoids is when their synthesis is stimulated. Despite this, the concentration of endocannabinoids required in the present study are in line with the known receptor affinity for AEA at $CB_2$ ($K_i$ up to 2 μM) and TRPV1 ($EC_{50}$ ranging from 0.63-4.9 μM depending on assay)(Pertwee *et al*., 2010), and for OEA and PEA at PPARα ($EC_{50}$ 3-4μM, O'Sullivan, 2007).

In conclusion, this study demonstrates that AEA, OEA, PEA and virodhamine (all 10 μM) decrease BBB permeability *in vitro* in human cells. Roles for $CB_2$, TRPV1, CGRP and PPARα activation are presented, which, in conjunction with existing literature, identifies them as potential targets to modulate BBB permeability.

**CONTRIBUTION STATEMENT**

WH was involved in the acquisition and analysis of data, and the preparation of the manuscript. CT, SIA and MN were involved in the acquisition and analysis of data. SOS contributed to the conception and design of the study and preparation of the manuscript. TE contributed to the conception and design of the study and critical revision of the manuscript. All approved the final version to the published.

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**Figures Legends**

**Figure 1.** The effects of increasing concentrations of anandamide (AEA) on blood brain barrier (BBB) permeability measured by TEER (A) with corresponding area under curve (AUC, B, *n* = 9 inserts from 3 separate experiments). The effects of capsazepine (cpz, C, *n* = 7 inserts from 3 separate experiments) or AM630 (D, *n* = 7-8 inserts from 3 separate experiments) or calcitonin-gene regulated peptide (CGRP, E, *n* = 5-6 inserts from 3 separate experiments) on the effect of AEA (10 μM). The effects of dexamethasone (n=6) and the $CB_2$ agonist HU308 on blood brain barrier (BBB) permeability over time (F) and expressed as AUC (G). Data are given as mean ± S.E.M. Statistics conducted using one-way ANOVA with Dunnett's (B) or Bonferroni's test (C,D,E). ***P<0.001, **P<0.01, *P<0.05 AEA compared to the vehicle treated inserts, †† P<0.01 AEA & antagonist compared to AEA alone

**Figure 2**. Expression profiling of potential target sites of action in HA and HBMEC cells. Shown are the ethidium bromide stained gels of the products obtained by RT-PCR using primers specific for PPARα, PPARγ, $CB_1R$, $CB_2R$, TRPV1, CGRPR and the control gene HPRT. cDNAs generated in the presence (+) or absence (-) of reverse transcriptase on total RNA from HA or HBMEC cells was used as template for the PCRs. The 100bp DNA ladder was used in all gels except for PPARα and PPARγ where a 10 bp ladder was used. Sizes are in base pairs.

**Figure 3.** The effects of anandamide (AEA) (*n* = 6) in the presence and absence of the FAAH inhibitor URB597 (n=6) on permeability in the BBB (A), with corresponding area under curve (AUC,B). Data are given as mean ± S.E.M. Statistics conducted on AUC using one-way ANOVA with Dunnett's test. **P<0.01, *P<0.05 AEA versus the vehicle, †† P<0.01 AEA & antagonist compared to AEA alone.

**Figure 4.** The effects of various concentrations of anandamide (AEA) either before (A - *n* = 7-12 inserts from 4 separate experiments) or after (C - *n* = 9-10 inserts from 4 separate experiments) 4 h oxygen-glucose deprivation (OGD) on permeability in the BBB, with corresponding area under curve (AUC, B and D). Data are given as mean ± S.E.M. Statistics conducted on AUC using one-way ANOVA with Dunnett's test.

**Figure 5.** The effect of oleoylethanolamide (OEA) over time on BBB permeability in the initial endocannabinoid screening (A; *n* = 6 inserts from 3 separate experiments), as a concentration-response curve (B - *n* = 4-6 inserts from 3 separate experiments), and in the presence of the PPARα

antagonist GW6471 (C and D - *n* = 5 inserts from 3 separate experiments). (E) The effects of *N*-arachidonoyl dopamine (NADA, 10 μM) on TEER values in the BBB model (*n* = 7-8 inserts from 4 separate experiments). (F) Absorbance values for lactate dehydrogenase assay conducted on cell culture medium obtained from the luminal (endothelial) chamber of the inserts at 48 h (*n* = 6 inserts from 3 separate experiments). Data are given as mean ± S.E.M. Statistics conducted using one-way ANOVA with Dunnett's or Bonferroni's test. ***$P$<0.001, *$P$<0.05 OEA compared to vehicles treated inserts, †† P<0.01 OEA & antagonist compared to OEA alone.

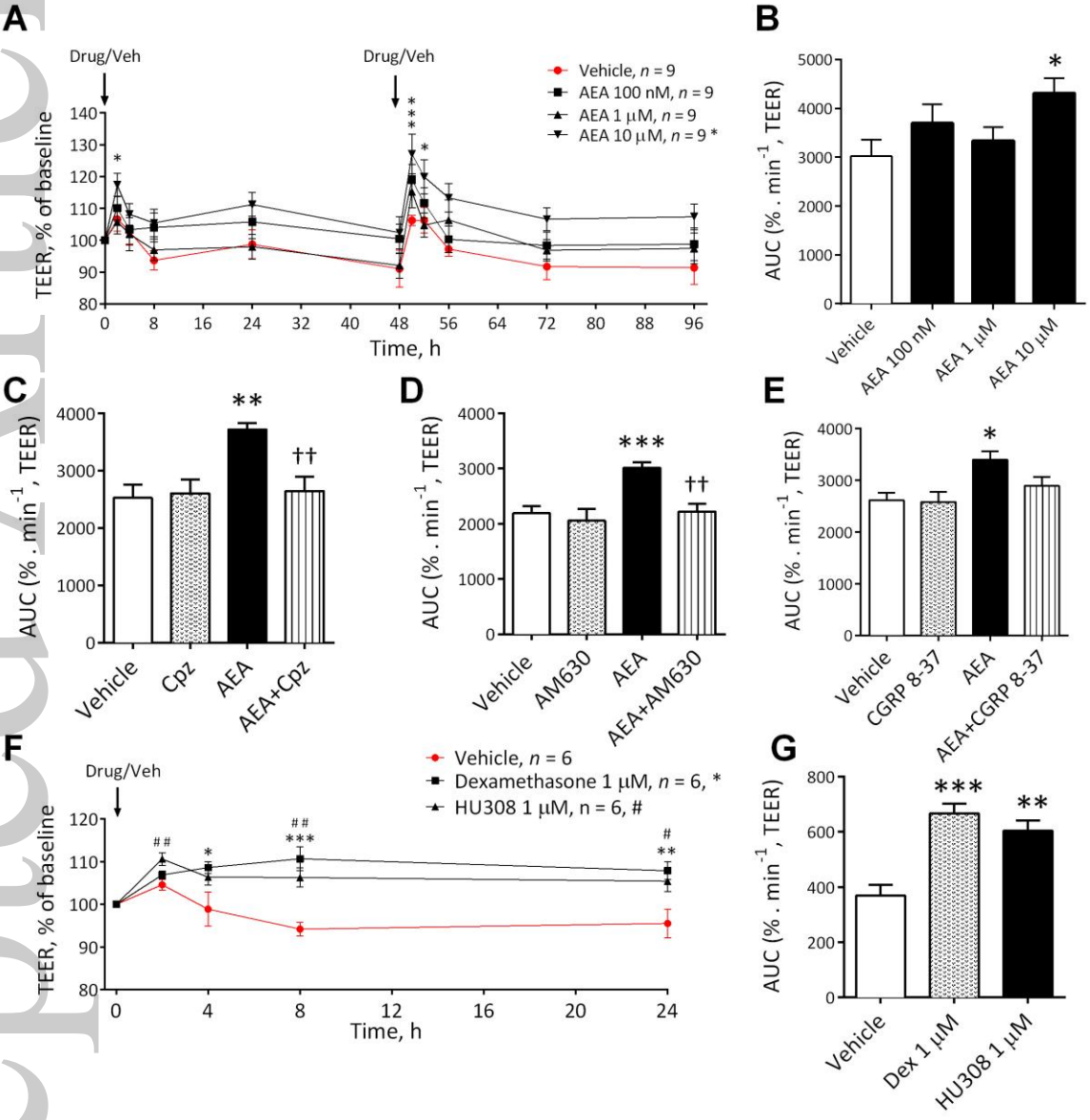
**Figure 6.** The effect of oleoylethanolamide (OEA, A and B - *n* = 6 inserts from 3 separate experiments), palmitoylethanolamide (PEA, C and D - *n* = 5-6 inserts from 3 separate experiments), virodhamine (E and F - *n* = 6 inserts from 3 separate experiments), 2-AG (G - *n* = 6 inserts from 3 separate experiments), Oleamide (H - *n* = 6 inserts from 3 separate experiments), NADA (I - *n* = 4-5 inserts from 2 separate experiments) and noladin ether (J - *n* = 5 inserts from 3 separate experiments) administered before 4 h oxygen-glucose deprivation (OGD) on TEER. Data are given as mean ± S.E.M. Statistics conducted on AUC using Student's *t*-test. **$P$<0.01, *$P$<0.05 compared to vehicles treated inserts.

**Figure 7.** The effect of oleoylethanolamide (OEA, A - *n* = 6 inserts from 3 separate experiments) or palmitoylethanolamide (PEA,B - *n* = 5-6 inserts from 3 separate experiments) alone or in combination with GW6471 before 4 h oxygen-glucose deprivation (OGD) on TEER. Data are given as mean ± S.E.M. Statistics conducted on AUC using one-way ANOVA with Bonferroni's test. *$P$<0.05 compared to vehicles treated inserts, † P<0.05 OEA & antagonist compared to OEA alone.

| | TEER (AUC) | |
|---|---|---|
| | **Vehicle** | **Endocannabinoid (10 µM)** |
| **Anandamide** | 3027 ± 328 | 4315 ± 303 * |
| +AM251 | 2355 ± 205 | 3380 ± 210 † |
| +GW6471 | 2841 ± 129 | 3595 ± 99 †† |
| +GW9662 | 2626 ± 148 | 3362 ± 145 † |
| +O-1918 | 2369 ± 60 | 3359 ± 240 †† |
| **OEA** | 1649 ± 124 | 2646 ± 133 *** |
| +AM251 | 1356 ± 171 | 2205 ± 208 †† |
| +AM630 | 1333 ± 164 | 2040 ± 50 †† |
| +Capsazepine | 1274 ± 262 | 2060 ± 57 † |
| +GW9662 | 1391 ± 182 | 2008 ± 129 † |
| **2-AG** | 2572 ± 104 | 2854 ± 351 |
| **NADA** | 2699 ± 104 | 2060 ± 204 * |
| **Noladin ether** | 2342 ± 208 | 2718 ± 192 |
| **Oleamide** | 2320 ± 205 | 2399 ± 176 |
| **PEA** | 2004 ± 104 | 2349 ± 350 |
| **Virodhamine** | 1850 ± 104 | 2183 ± 179 |

Table 1. Area under the curve (AUC) values for the effects of cannabinoids on TEER. All data are from experiments conducted in normal conditions (i.e. no OGD). Data are given as mean ± S.E.M. Statistics conducted using Student's *t*-test or one-way ANOVA with Bonferroni's test (antagonist experiments). ***$P$<0.001, **$P$<0.01, *$P$<0.05 compared to vehicle treated inserts. ††P<0.01, † P<0.05 endocannabinoid & antagonist compared to endocannabinoid alone. TEER (Transepithelial electrical resistance); AUC (area under curve); AM251: $CB_1$ antagonist; GW6471: PPARα antagonist; GW9662: PPARγ antagonist; O1918 (novel endothelial receptor antagonist); Capsazepine: TRPV1 antagonist.

There are no conflicts of interest.

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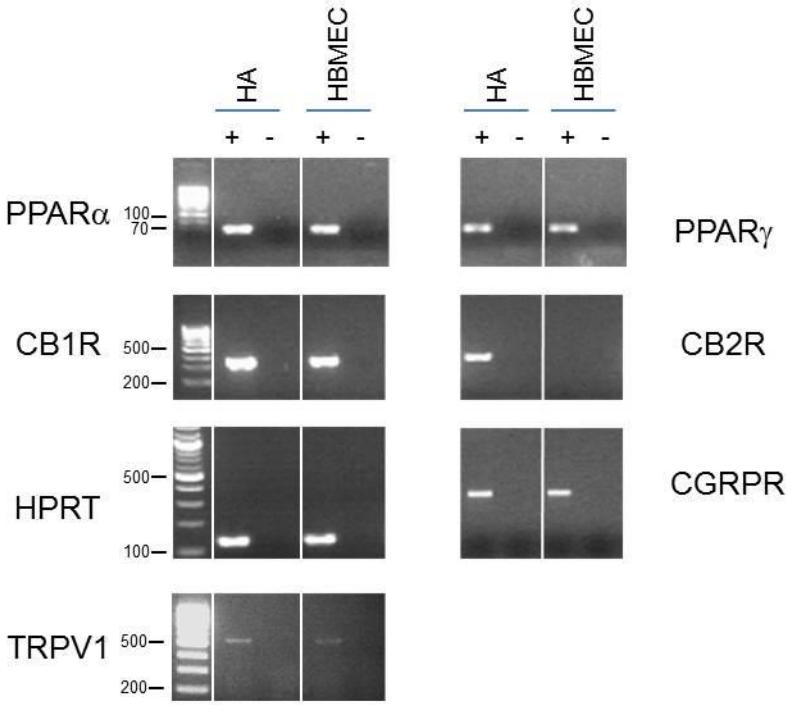


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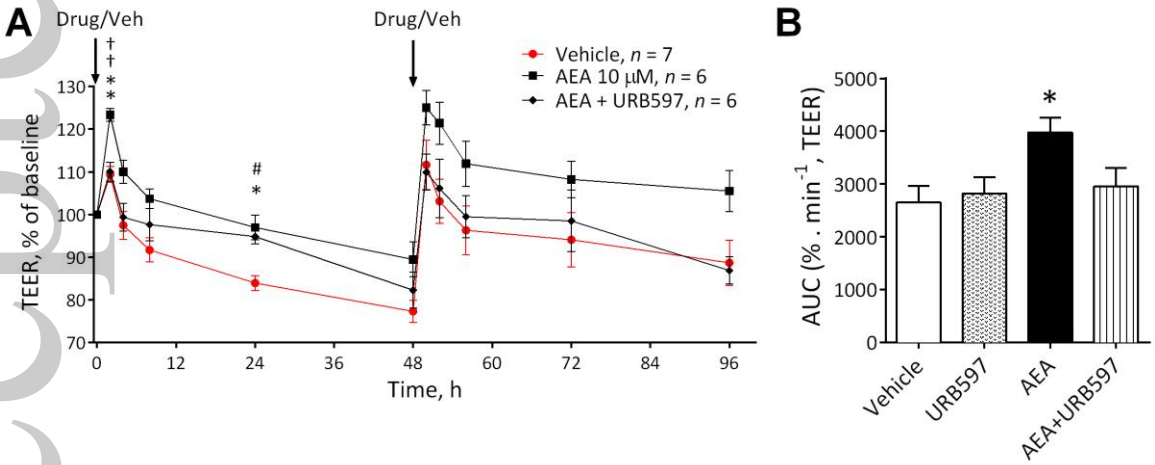


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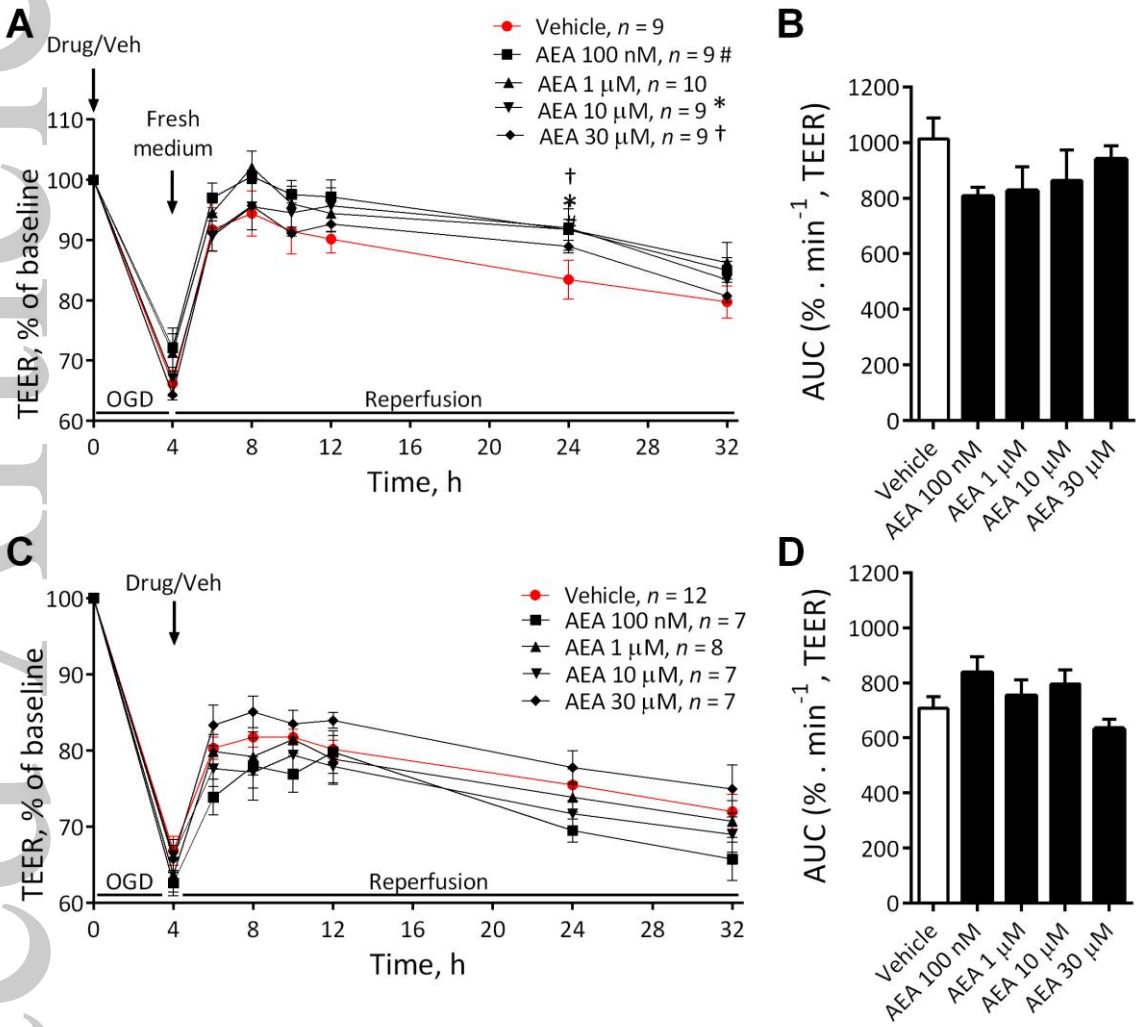


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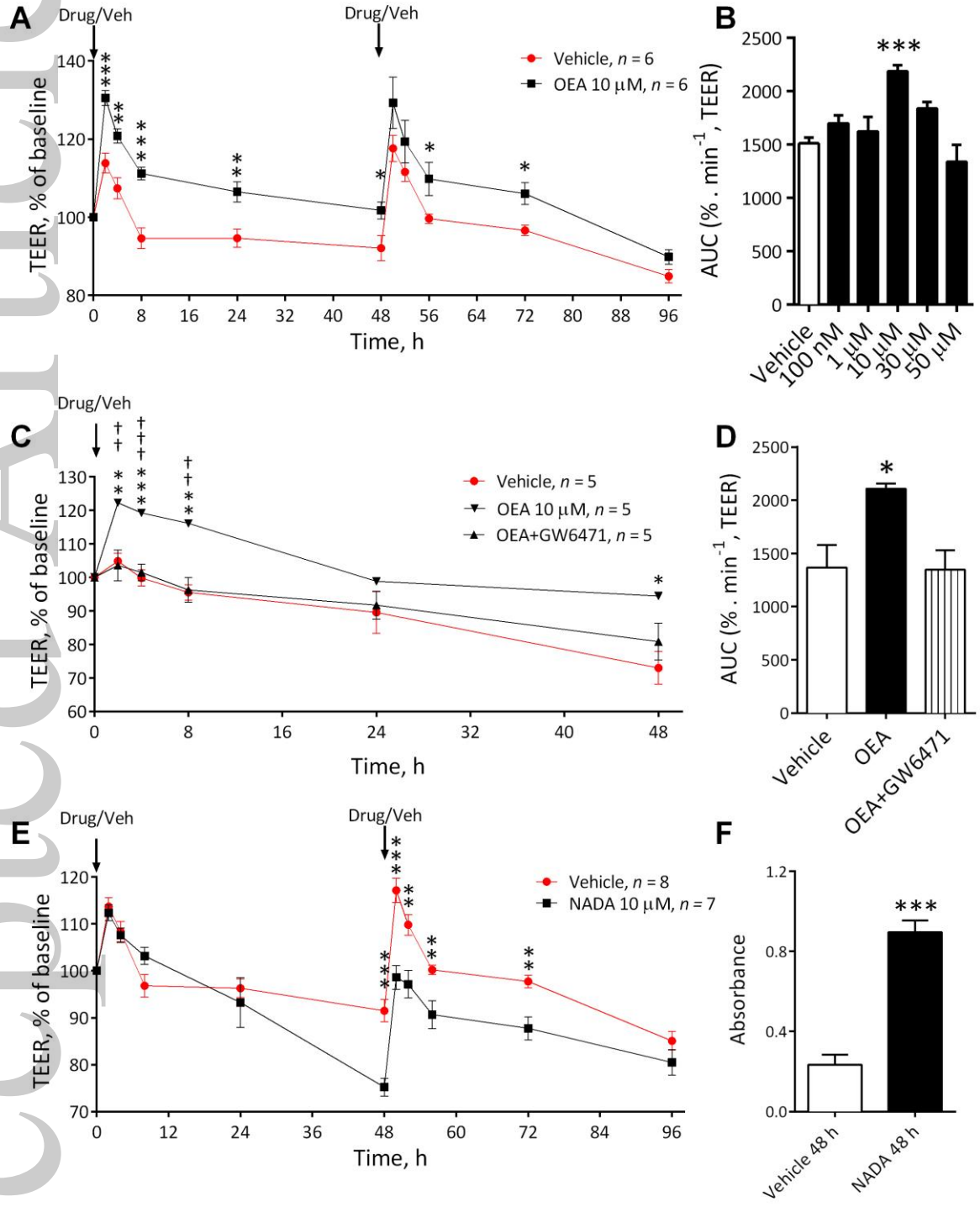


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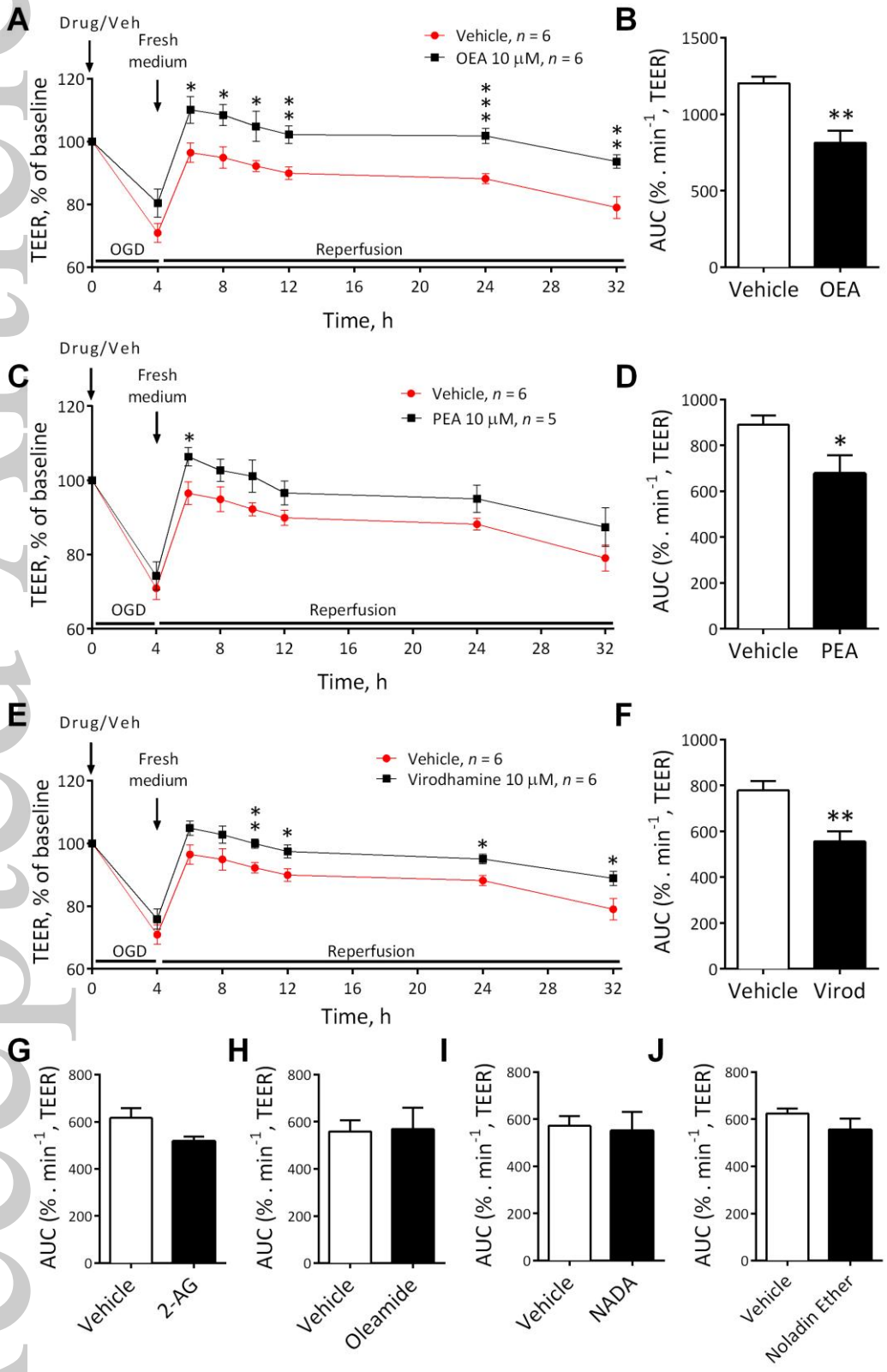


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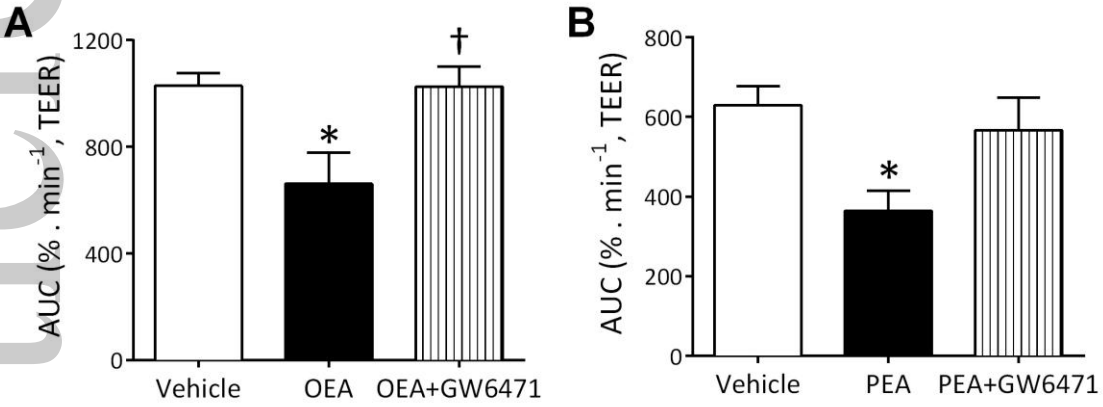


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