3.2.5 Western Blot

Cells were lysed in RIPA buffer (Tris-base 50 mM, NaCl 150 mM, EDTA 2 r
40 0.5 % v/v) supplemented with a protease inhibitor cocktail (SigmaF/
Protein samples (10 µg) were boiled in Laemmli buffer (LDS 5 % v/v, gl
v/v, Tris-HCl 1 M, bromophenol blue 2.5 mg, phenol red 2.5 mg and fic
v/v) supplemented with 2-β-mercaptoethanol (5 % v/v) and subjected t
(8 % gel). Following electrophoresis, samples were semi-dry transferr
buffer: Tris-base 50 mM, glycine 40 mM, methanol 20% v/v, SDS 0.037 %
onto PVDF membranes (Amersham, Buckinghamshire, UK) and blocke
TBS-T (Tris-base 10 mM, NaCl 100 mM, HCl 1 M, and Tween-20 0.1% v/v
5% w/v Marvel. Membranes were probed with a primary antibod
(dilution 1:1000) at 4 °C, washed with TBS-T and incubated with an
secondary antibody conjugated to horseradish peroxidase (HRP 1:250
Bands were visualised using chemiluminescence detection (H_2O_2, 250 mi
DMSO, 90 mM 4-iodophenylboronic acid in DMSO and 10 mM Tris-HCl) ar
captured using a Fusion Fx imaging system. Membranes were stripped ar
with HRP- conjugated β-actin antibody as a loading control (1:1000).

3.2.6 Permeability Assay

Permeability of the hBMEC and hCMEC/D3 monolayers were assessed u
Transwell plates (0.4 µm pore size, Corning, Lowell, MA). In brief, the upp
the transwell inserts were coated with matrigel (BD Biosciences; dilut
serum free media) at 37 °C for 30 min. HBMECs were seeded onto the up
($3x10^5$ cells per well), and medium added to the upper and lowe
Following overnight attachment, the upper chamber was re-seeded (3x
well) and allowed to attach overnight. Cells were then serum starved for
replacing the medium in the lower chambers with one containing F
dextran sulphate (10 mg/mL; Invitrogen). Simultaneously, the cells were
OA (10 nM), FTY-720 (5 µM), histamine (10 µM) or medium (Untx con
chambers. Samples (10 µL) were periodically removed from the upper ch
24 h, diluted 1:10 with water and fluorescence determined (ex λ: 485 nm
nm) using a fluorimeter (Thermofisher Scientific, Fluoroskan AscentFL).

3.2.7 Cell Transfection and CIP2A SET overexpression

hCMEC/D3s were seeded (2×10^5 cells per well) in 6-well plates 24 h before being transiently transfected with 1 μg of pcDNA-SET-FLAG-HA, pcDNA3.1_CIP2aflag_WT plasmid (pCIP2A), SET expression plasmid or a pcDNA.3.1 (pDNA.3.1) control plasmid using PolyFect (Qiagen) in serum and antibiotic free EndoGRO© medium (Millipore). Overexpression was confirmed 72 h post-transfection using qPCR.

3.2.8 PP2A phosphatase activity assay

A PP2A immunoprecipitation assay kit (Merck Millipore) was used to determine PP2Ac activity following extraction in RIPA buffer. Protein (100 μg) was incubated for 2 h at 4 °C with 4 μg of anti-PP2A (C subunit, clone 1D6) and protein-A-agarose. The immunoprecipitate was thoroughly washed prior to addition of malachite green and the absorbance read at λ 620 nm. PP2Ac activity was expressed in pmoles.

3.2.9 Data and Statistical Analysis

All data were normalised to the appropriate control and expressed as an absolute value, a percentage or ratio. Data were analysed by one-way ANOVA or two-way ANOVA with *post hoc* analysis (Bonferroni). The temporal effect of pharmacological and endogenous inhibitors of PP2A on VE-cadherin were summarised by calculating the area under the curve [625]. Protein and mRNA expression were normalised relative to β-actin and GAPDH respectively. Data are expressed as mean ± S.E.M., and a value of $P < 0.05$ was taken to indicate statistical significance.

3.3 Results

3.3.1 Effect of OA and FTY-720 on cell viability

Exposure of hBMEC to Histamine (10μM; positive control for the permeability assay) OA (10 nM), FTY-720 (5μM) or DMSO (0.001%) for 24 h did not alter cell viability compared to cells exposed to culture medium as determined using an MTT assay (Figure 3.1). Doxorubicin (5 μM; positive control) decreased cell viability by 81.9% compared to the Untreated (Untx) group.

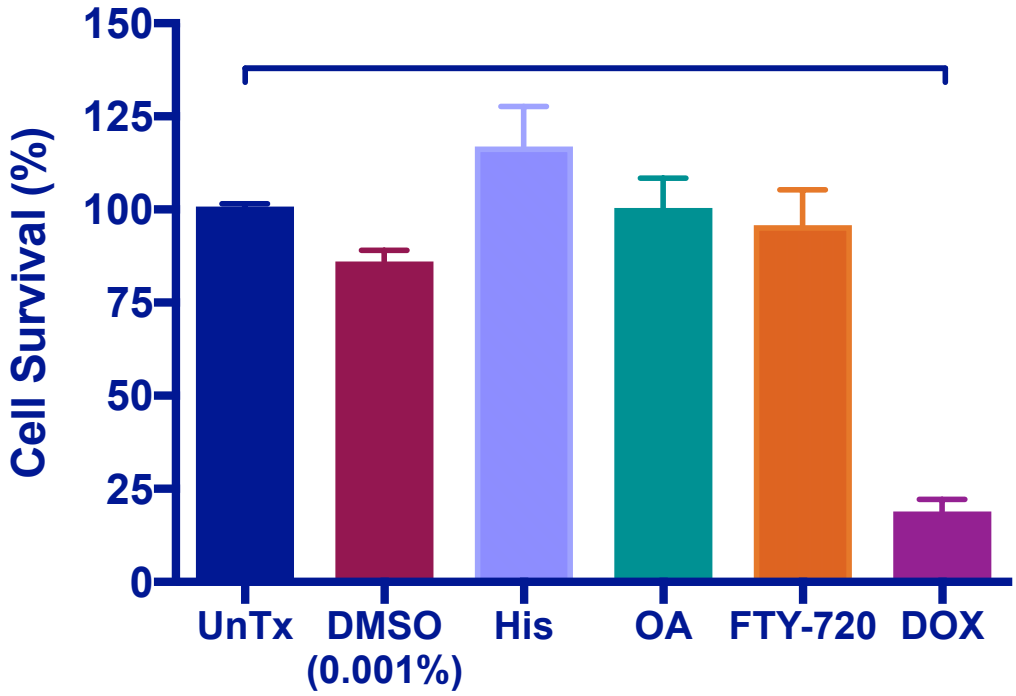

Figure 3.1: Cell Survival, determined by MTT assay.

hBMECs were exposed to DMSO (solvent control), histamine (10 μM), OA (10 nM), FTY-720 (5 μM) and DOX (5 μM, positive control) over 24 h. Data are represented as mean ± SEM; n=5. P < 0.05 indicated by the horizontal bars; data were analysed using one-way ANOVA with post hoc analysis (Bonferroni).

3.3.2 Effect of OA and FTY-720 on PP2Ac and posttranslational modification

In hBMEC, OA (10 nM) reduced PP2Ac activity by ~60% compared to the Untx and DMSO groups. Exposure of the cells to FTY-720 (5 μM) increased PP2Ac activity by ~45% compared to the control groups (Figure 3.2A; $P < 0.05$). PP2Ac activity was unaltered by DMSO (0.001%) compared to cells exposed to medium alone. In hBMEC, OA (10nM) and FTY-720 (5 μM) did not affect the abundance of PP2Ac compared to the Untx or DMSO groups (Figure 3.2B).

As OA (10nM) and FTY-720 did not alter PP2Ac abundance (Figure 2B), changes in PP2Ac activity could be due to altered methylation and/or phosphorylation. In hBMECs, 24 h exposure to OA (10nM) augmented the abundance of demethylated PP2Ac by 2.55 ± 0.3 fold relative to the Untx sample ($P < 0.05$) while FTY-720 (5 μM) had no effect (Figure 3.2C). OA (10 nM) increased phosphorylation of PP2Ac at Tyr^{307} by ~80% ($P < 0.05$; Figure 3.2D) compared to the Untx and DMSO groups. However, FTY-720 did not modulate the abundance of phosphorylated PP2Ac compared to Untx cells (Figure 3.2D).

As OA increased the demethylation of PP2Ac, this was investigated further to look at its effect on PP2Ac methylation modulations; PME-1 and LCMT-1. In hBMEC, OA and FTY-720 did not alter the abundance of PME-1 compared to either the Untx or DMSO groups (Figure 3.3A). However, OA reduced ($P < 0.05$; Figure 3.3B) the abundance of LCMT-1 by 79.49 ± 0.02% and 78.89 ± 0.02% compared to the Untx and DMSO groups respectively. Furthermore, LCMT-1 abundance was ~35% higher in the FTY exposed group compared to the Untx and DMSO groups ($P < 0.05$; Figure 3.3B). DMSO (0.001%) did not affect the abundance of any of the target proteins investigated compared to the Untx group (Figure 3.3A and B).

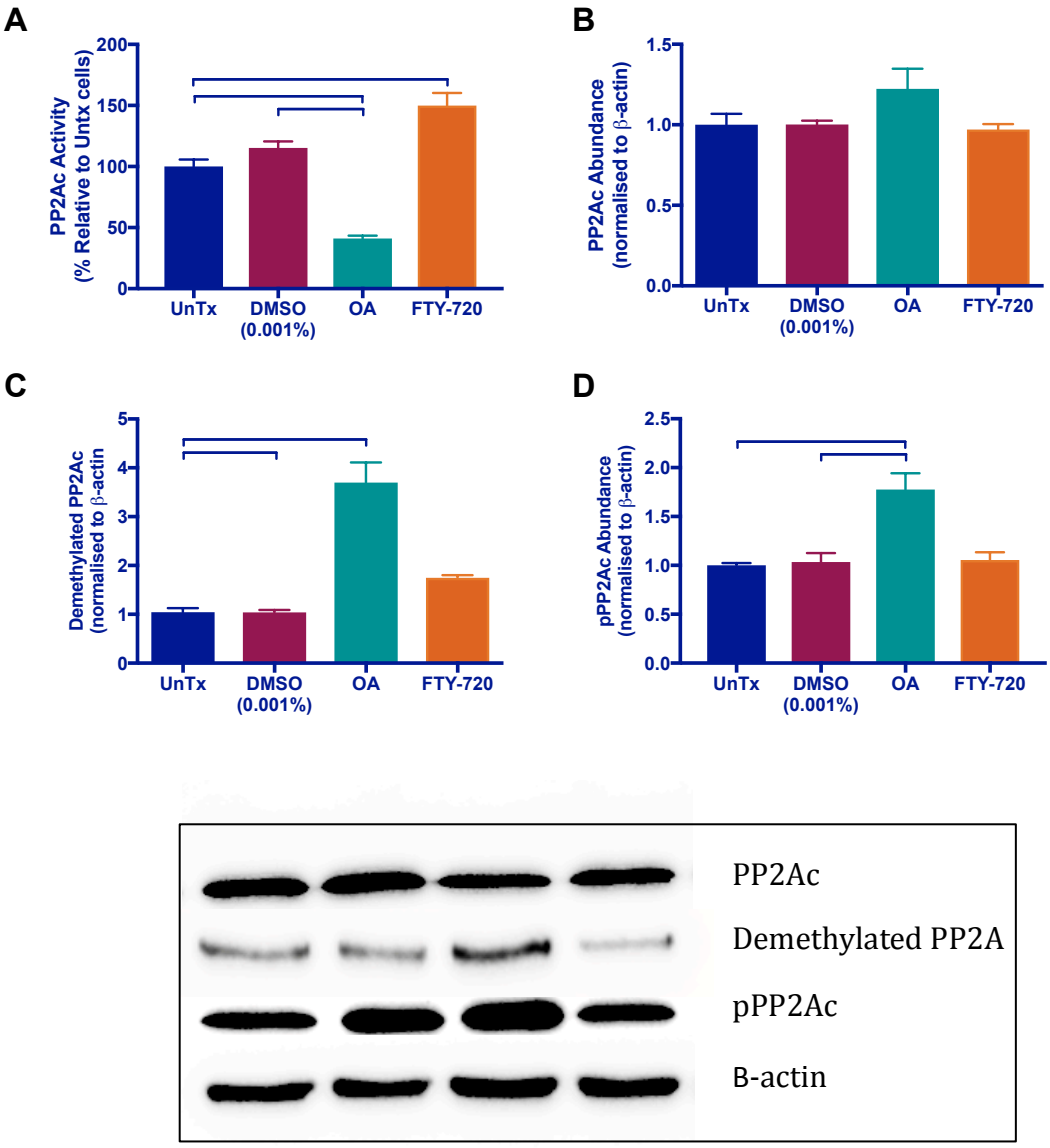

Figure 3.2: Effects of OA (10 nM) and FTY-720 (5 µM) on PP2A in hBMECs over 24 h.

PP2Ac activity was determined using an immunoprecipitation assay kit (Millipore), the results were normalised to the Untx control, which was set at 100% (A). PP2Ac total abundance was determined using western blot (B). The abundance of the inhibitory post-translational modifications, demethylation and phosphorylation of PP2Ac, was also determined by western blot (C, D). All western blots were normalised to the loading control β-actin and were analysed using one-way ANOVA with post hoc Bonferroni analysis. Data are represented as mean ± SEM; n=5. Horizontal bars indicate statistical significance at $P < 0.05$.

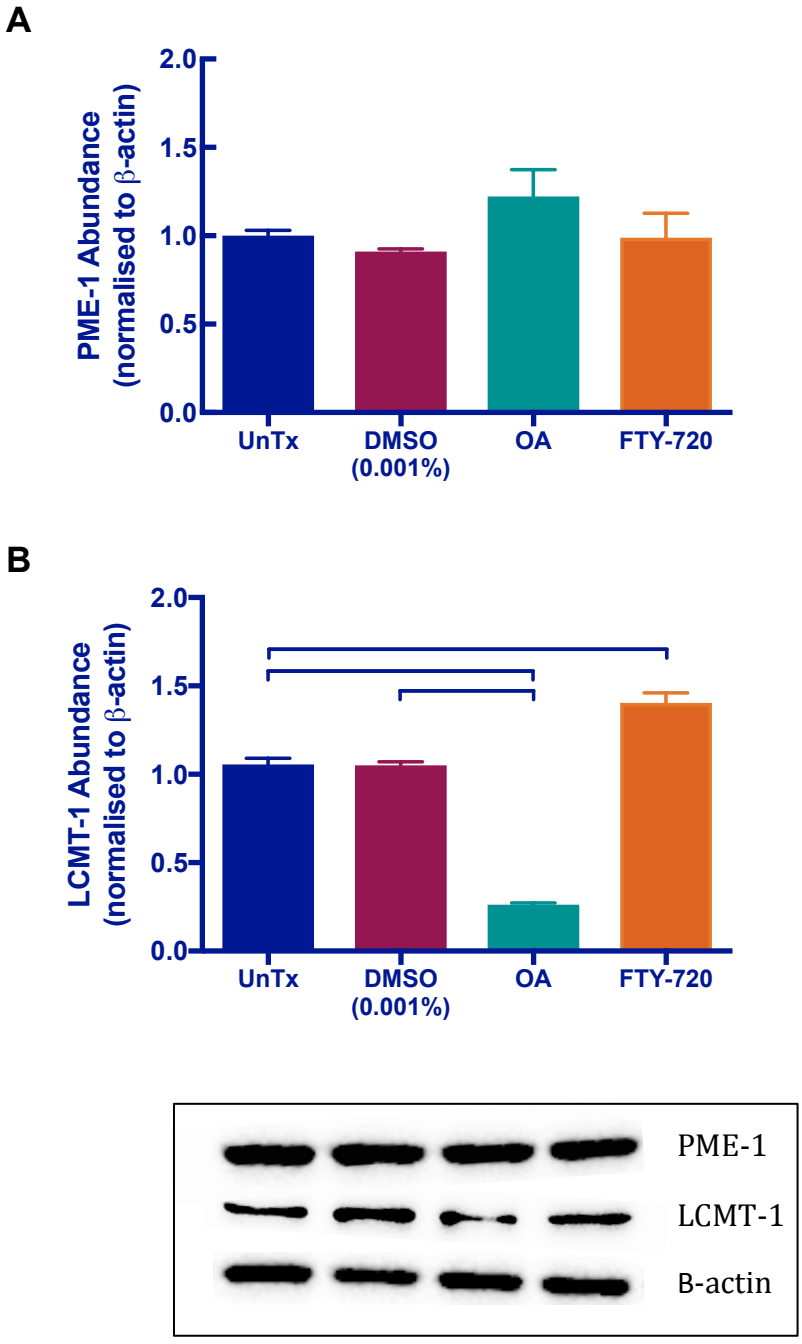

Figure 3.3: Effect of OA (10 nM) and FTY-720 (5 µM) on PME-1 (A) and LCMT-1 (B) abundance.

Abundance was determined by western blot, normalised to the loading control β-actin. Data are represented as mean ± SEM; n=5. P < 0.05 indicated by the horizontal bars; Data were analysed using one-way ANOVA with post hoc Bonferroni analysis.

3.3.3 Effect of OA and FTY-720 on VE-cadherin

In hBMEC, OA (10 nM) reduced the abundance of VE-cadherin to undetectable levels compared to the Untx and DMSO control ($P < 0.05$; Figure 3.4A), while FTY-720 (5 μM) increased it by ~2 fold ($P < 0.05$). OA augmented expression of VE-cadherin mRNA by ~2.7 fold ($P < 0.05$; Figure 3.4B) compared to the Untx and DMSO groups, while FTY-720 had no effect. DMSO did not alter the abundance or expression VE-cadherin compared to the Untx group (Figure 3.4A and B).

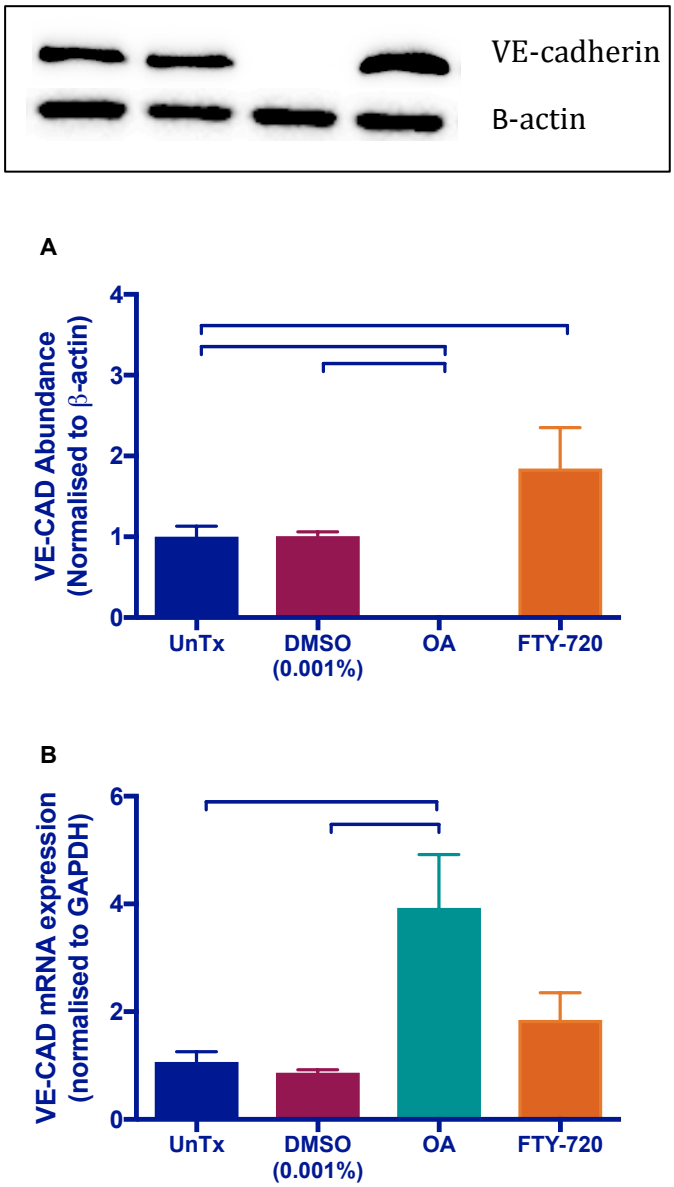

Figure 3.4: Effect of OA (10 nM) and FTY-720 (5 µM) on VE-cadherin.

Protein abundance was determined by western blot and normalised to the loading control β-actin (A). mRNA expression was determined by RT-PCR normalised to GAPDH (B). Data are represented as mean ± SEM; n=5. P < 0.05, significantly different as indicated by the horizontal bars; one-way ANOVA with post hoc Bonferroni analysis.

3.3.4 Effect of MG132 on VE-cadherin abundance

To investigate the mechanism by which OA reduced VE-cadherin abundance, the temporal effect of MG132 on its abundance was assessed over 24 h. The effect of OA (10nM) on VE-cadherin abundance in hBMEC was recorded as early as 6 h, resulting in a 44.0% decrease compared to the Untx and DMSO groups ($P<0.05$). At the 12 h time point there was a 86.4% loss in VE-cadherin as a result of OA (10nM) compared to the Untx and DMSO groups ($P<0.05$; Figure 3.5A). Finally at 24h OA resulted in an 83% loss in VE-cadherin abundance compared to the Untx and DMSO groups ($P<0.05$). In the presence of MG132 (2 μM) OA did not decrease VE-cadherin abundance at any time point studied or when summarised as the $AUC_{0\text{-}24h}$, compared to either the Untx or DMSO samples (Figure 3.5B). DMSO (<0.001%) did not alter VE-cadherin abundance compared to the Untx group in the absence or presence of MG132 (Figure 3.5A-B).

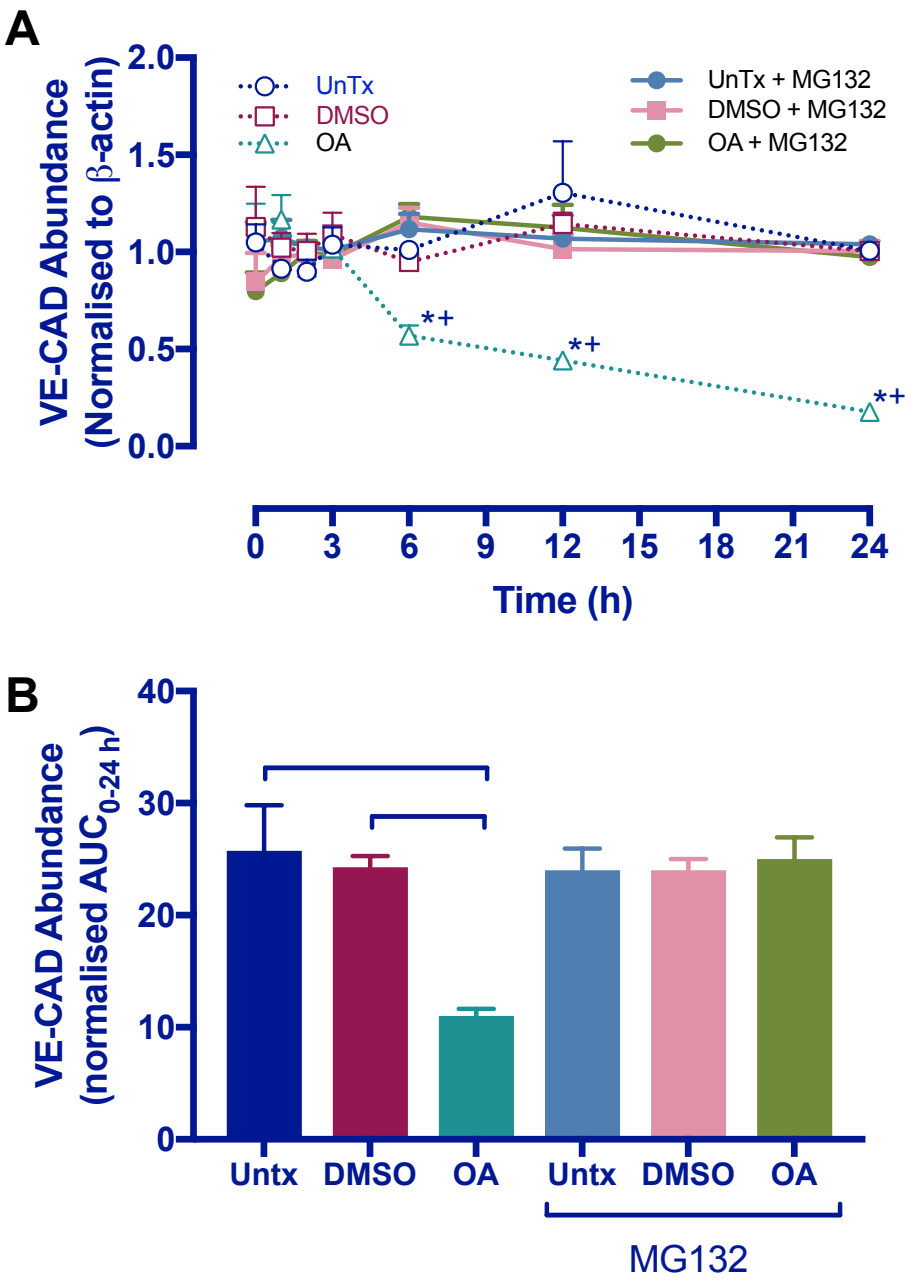

Figure 3.5: Investigation into how OA (10 nM) affects VE-cadherin abundance.

The role of proteasomal degradation (A-B) was determined using MG132 (2 µM) over 24 h. VE-cadherin abundance was quantified by western blot and normalised to β-actin. Data are further normalised to the Untx control and represented as a time course (A) and area under the curve (B) (mean ± SEM; n=5). P < 0.05, significantly different as indicated by the horizontal bars (* signifies OA compared to Untx, + OA compared to DMSO) Data was analysed by a two-way ANOVA with post hoc Bonferroni analysis.

3.3.5 Effect of CIP2A and SET overexpression on VE-cadherin abundance

To extend the physiological relevance of PP2A inhibition on VE-cadherin abundance, CIP2A and SET were overexpressed in hCMEC/D3 cells. Transfection of cells with either the CIP2A plasmid or SET plasmid augment CIP2A and SET mRNA expression by ~26 fold and ~6 fold compared to the mock and empty vector transfected groups ($P < 0.05$; Figure 3.6A, B). In hCMEC/D3 transfected with a CIP2A or SET plasmid attenuated PP2Ac activity by 54.25% and 58.25% compared to the pcDNA3.1 transfected groups ($P < 0.05$; Figure 3.6C). Importantly transfection with the empty plasmid did not alter CIP2A or SET mRNA expression or PP2Ac activity compared to the mock-transfected group.

Overexpression of CIP2A decreased VE-cadherin mRNA to undetectable levels ($P < 0.05$; Figure 7A). Protein abundance was also decreased compared to the mock (- 57.9%) and empty vector (- 57.6%) transfected samples ($P < 0.05$; Figure 3.7B). Similarly, over expression of SET attenuated VE-cadherin mRNA expression by 84.0% and 88.1 % compared to the mock and empty vector transfected samples ($P < 0.05$; Figure 7A). Finally, VE-cadherin protein abundance was also attenuated by SET overexpression compared to the mock (- 63.6%) and empty vector (- 63.07%) transfected groups ($P < 0.05$; Figure 3.7B). Empty vector transfection did not alter VE-cadherin mRNA expression or protein abundance compared to mock transfected cells.

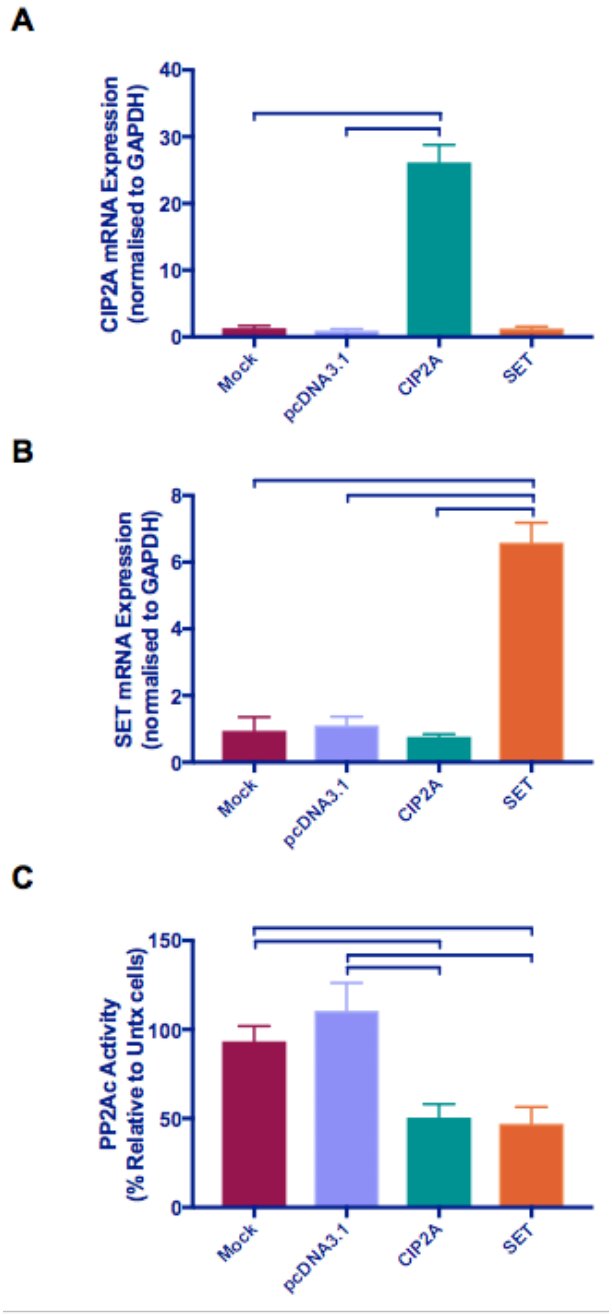

Figure 3.6: The effect of CIP2A and SET overexpression on CIP2A mRNA expression (A), SET mRNA expression (B) and PP2Ac activity (C).

hCMEC/D3 cells were transfected with pcDNA3.1_CIP2Aflag_WT and pcDNA3.1 SET plasmids for 72 h. Over expression was confirmed by RT-PCR and normalised to GAPDH. Data are presented as a normalised ratio/percentage of the Mock transfection control (mean ± SEM; n=5). P < 0.05, significantly different as indicated by the horizontal bars; one-way ANOVA with post hoc Bonferroni analysis.

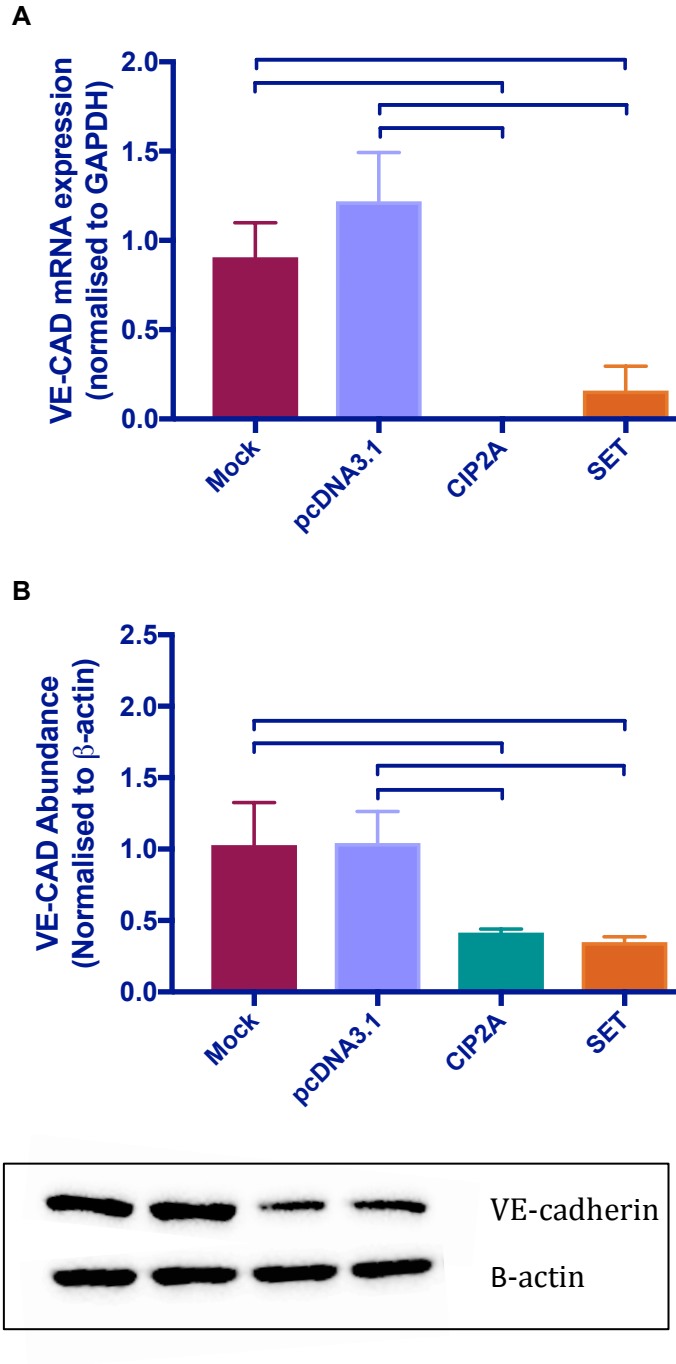

Figure 3.7: The effects of CIP2A and PP2A over expression on VE-cadherin mRNA expression (A) and protein abundance (B).

hCMEC/D3s were transfected with pcDNA3.1_CIP2Aflag_WT and pcDNA3.1 SET plasmids for 72 h. mRNA expression was determined by RT-PCR, normalised to GAPDH and calculated using the 2-ΔΔCt method. Protein expression was quantified by western blot and normalised to the loading control β-actin. Data are represented as mean ± SEM; n=5. P < 0.05, significantly different as indicated by the horizontal bars; one-way ANOVA with post hoc Bonferroni analysis.

3.3.6 Effect of PP2A modulation on microvascular endothelial cell permeability.

To assess a functional effect of OA and FTY-720 on brain microvascular endothelial cells, a transwell permeability assay was employed. OA (10 nM) increase hBMEC and hCMEC/D3 paracellular permeability in a time dependent manner compared to Untx and DMSO groups ($P < 0.05$; Figure 3.8 and Figure 3.9A). This was detectable from 19 h onwards, with permeability being increased by 127.1% and 113.9% in hBMEC and hCMEC/D3 respectively at 24 h. DMSO (0.001%) did not alter endothelial cell permeability at any time point, irrespective of the cell line used (Figure 3.8, Figure 3.9A). In hBMEC, FTY-720 (5 μM) did not alter permeability of the monolayer at any time point studied compared to the Untx and DMSO groups. Histamine (positive control) increased permeability of the hBMEC monolayer in a time dependent manner, which was detectable from 18 h onwards ($P < 0.05$; Figure 3.8).

To complement the data obtained following exposure to OA, I explored the effect of CIP2A and SET overexpression on permeability of a hCMEC/D3 monolayer. Transfection of the cells with the empty plasmid did not alter permeability of the monolayer at any time point studied compared to mock transfected cells (Figure 3.9B). Overexpression of CIP2A elicited a time-dependent increase in permeability, which reached significance from 22 h onwards compared to the mock-transfected group and from 23 h compared to the empty vector group ($P < 0.05$ Figure 3.9B). By 24 h, CIP2A overexpression had increased permeability by 27.6% and 29.9% compared to the mock and empty vector groups respectively ($P < 0.05$). SET overexpression caused a similar time-dependent increase in endothelial cell permeability, which was detected from 21 h onwards compared to mock transfected cells and from 22 h compared to the empty vector group ($P < 0.05$; Figure 3.9B). By 24 h monolayer permeability had increased by 30.1% and 32.5% compared to mock and empty vector transfected cells respectively ($P < 0.05$).

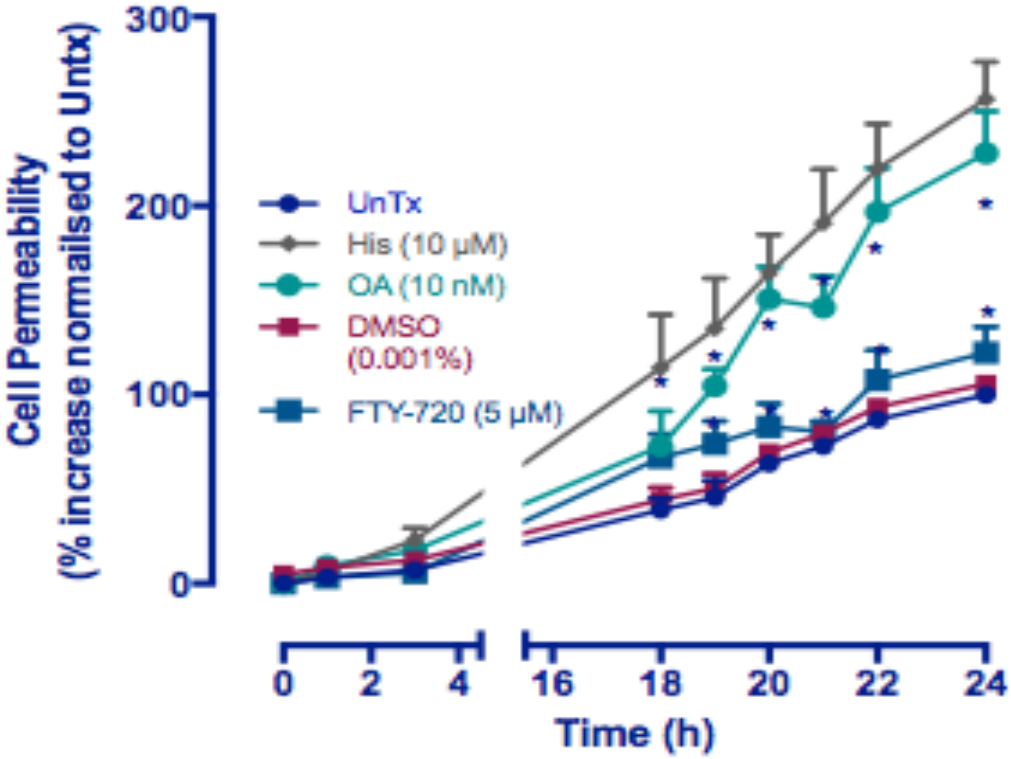

Figure 3.8: The effect of PP2A modulation on brain microvascular cell permeability.

Measured by the movement of a FITC-labelled dextran over 24 h through a monolayer of endothelial cells seeded on a transwell plate. hBMECs were exposed to OA (10 nM) and FTY-720 (5 µM), Histamine (10 µM) was used as a positive control. Data was normalised to the 24 h time point of the Untx control, which was set at 100% (mean ± SEM; n=5). * P < 0.05, significantly different from the relevant controls; two-way ANOVA with post hoc Bonferroni analysis

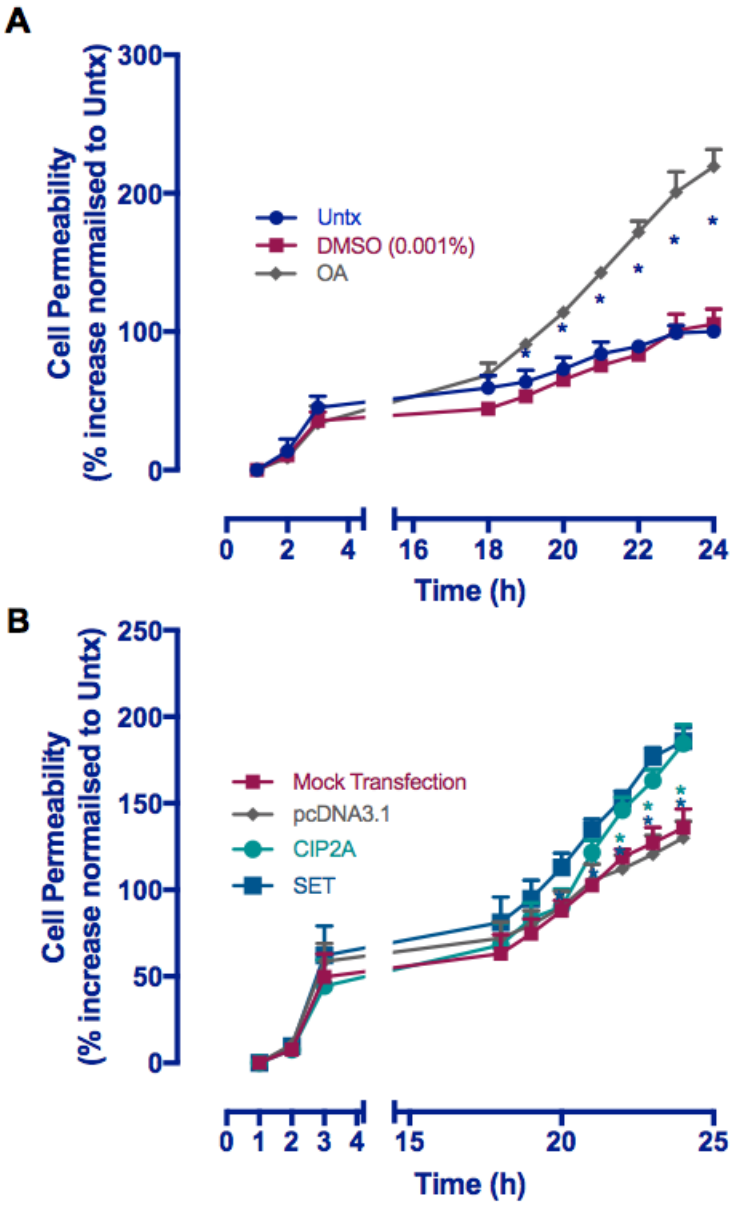

Figure 3.9: The effect of OA on endothelial cell permeability was confirmed using hCMEC/D3s (A).

Effect of CIP2A and SET over expression on hCMEC/D3 permeability was also determined (B). hCMEC/D3s were transfected with pcDNA3.1_CIP2Aflag_WT and pcDNA3.1 SET plasmids for 72 h. Permeability was represented by the movement of a FITC-labelled dextran over 24 h through a monolayer of endothelial cells seeded on a transwell plate. Data was normalised to the 24 h time point of the Untx control, which was set at 100% (mean ± SEM; n=5). * $P < 0.05$, significantly different from the relevant controls; two-way ANOVA with post hoc Bonferroni analysis.

3.4 Discussion

Understanding the regulation of VE-cadherin and its association with microvascular integrity is vital to combat diseases such as Alzheimer's disease and Multiple Sclerosis. The present study provides evidence that OA reduces VE-cadherin abundance in hBMEC cells while FTY-720 increases VE-cadherin abundance. These changes were not reflected at the level of transcription. While the effect of OA was associated with decreased PP2Ac activity, this was not due to a change in its abundance, but rather increased phosphorylation and demethylation of PP2Ac. The latter is consistent with OA-mediated attenuation of LCMT-1 abundance as PME-1 abundance was unaltered. Although FTY-720 increased PP2Ac activity this is not linked to altered post-translational modification despite a modest increase in LCMT-1 abundance. Importantly, co-incubation of OA with MG-132 prevented OA-mediated loss of VE-cadherin abundance. In hBMEC and hCMEC cell cultures, OA increased paracellular permeability in a time dependent manner, while FTY-720 had no effect. Importantly overexpression of CIP2A and SET in hCMEC/D3 cells, decreased VE-cadherin abundance and mRNA expression, and increased paracellular permeability.

Adherens junctions are a crucial dynamic structure intrinsically linked to maintenance of barrier function that are highly regulated through modulation of their phosphorylation status. While multiple kinases including protein kinase C and GSK3β are involved in this process, less is known about their de-phosphorylation mediated by PP2A. This is surprising as PP2A is the major phosphatase present in the brain [584, 585] and several neurological conditions including Alzheimer's and Parkinson's disease, which manifest a dysfunctional blood brain barrier and associated with decreased PP2A levels [515, 587, 626]. To address this, the present study utilised primary human brain microvascular endothelial cells as an *in vitro* model of the BBB in conjunction with an immortalised human brain microvascular endothelial cell line (hCMEC/D3) for confirmation and overexpression experiments.

While few studies have investigated PP2A-mediated regulation of VE-cadherin, several early lines of enquiry have implicated it in regulating E-cadherin, a structurally similar cadherin expressed in epithelial cells. Firstly, OA and microcystin-

LR which, are pharmacological inhibitors of PP2A dephosphorylate E-cadherin and cause it to translocate from the cell membrane to cytosol in keratinocytes and zebrafish [627, 628]. Secondly, and more convincingly as OA and microcystin-LR can inhibit other phosphatases [629], knockout of PP2Acα also caused redistribution of E-cadherin and β-catenin from the membrane to cytosol in mouse embryonic stem cells [630]. Importantly, Kasa *et al*. showed that VE-cadherin and β-catenin immunoprecipitate with the Bα regulatory subunit of PP2A in human pulmonary endothelial cells and that silencing of the Bα causes β-catenin to redistribute from the membrane to cytosol or nucleus [620]. However, it should be noted that OA and silencing of PP2A-A did not alter PP2Ac from associating with E-cadherin, or E-cadherin binding to α-catenin/β-catenin in HBME cells [631]. Our data demonstrates that OA (10 nM) decreases VE-cadherin abundance in hBMEC cells. This is in keeping with OA reducing E-cadherin abundance in MCF-7 breast cancer cells [632]. However, in another study utilising several human cancer cell lines including MCF-7, CS-11, a small molecule inhibitor of PP2A-β-catenin signalling increased E-cadherin abundance [633]. The disparity between these studies may simply reflect differences in pharmacological target as CS-11, inhibits the signalling cascade rather than PP2A.

Regarding the effect of FTY-720 on VE-cadherin abundance, our data complements that of others showing FTY-720 to increase VE-cadherin association with β-catenin and its co-localisation with α-catenin and β-catenin at cell-cell contact points [634] and with the tight junction protein ZO-1 in human lung endothelial cells [635]. Although, selenite which is another activator of PP2A attenuates E-cadherin expression in colorectal carcinoma cells [636].

In addition to the above, we also investigated the effect of CIP2A and SET overexpression on VE-cadherin abundance in hCMEC/D3 cells. CIP2A and SET are two endogenous inhibitors of PP2A which inhibit PP2A activity by binding to the scaffold (PP2Aa; R65) and regulatory subunits of PP2A [445], and catalytic subunit respectively [637]. Our data demonstrate that overexpression of CIP2A or SET reduced VE-cadherin abundance. This complements work by other groups showing

silencing of CIP2A increase E-cadherin abundance in clear cell renal cell carcinoma and laryngeal carcinoma cells [638, 639]. In contrast to our data, overexpression of SET increases E-cadherin abundance in a pancreatic cancer cells [640], while KO of SET decreases E-cadherin and β-catenin levels in colorectal adenocarcinoma [641]. The disparity in the literature between our data and that of others may reflect differences in the cell lines used (endothelial cells vs cancer cell lines). In the present study, OA increased VE-cadherin mRNA expression while CIP2A and SET overexpression had no effect. Interestingly, SET decreases E-cadherin mRNA expression in HN12 and Cal27 cells [642]. Although difficult to explain, our finding indicates that the decrease in VE-cadherin abundance is not a consequence of altered mRNA transcription. However, it may be due to posttranslational intervention such as a miRNA [643].

So, are the effects of OA, FTY-720 and CIP2A/SET on VE-cadherin associated with alterations in PP2Ac activity and abundance? Not surprisingly, OA and over expression of CIP2A or SET decreased PP2Ac activity while FTY-720 increased it without altering PP2Ac abundance as found by others [517, 644, 645]. Regulation of PP2A holoenzyme activity is a multifaceted phenomenon entailing not only transcriptional regulation and inhibition by CIP2A and SET, but also holoenzyme formation and post-translational modification of the PP2Ac catalytic subunit [449, 514, 646]. When the latter aspect was looked at in the present study we found OA to increase phosphorylation and demethylation of PP2Ac consistent with inhibition of activity as reported in studies [456, 526]. Although several kinases including protein kinase A and C are implicated in phosphorylation of PP2Ac we did not investigate this in the current study [525, 647]. Instead, we focussed on how OA and FTY-720 modulate the methylation state of PP2Ac. Interestingly, OA caused demethylation of PP2Ac, which was associated with a decrease in LCMT-1 abundance rather than an increase in PME-1 abundance. This expands previous work showing mutation of Leu^{309} to alanine in the C-terminal TPDYFL motif of PP2Ac, regulating enzyme assembly, affecting cell adhesion and translocation of cadherin and β-catenin in the Harderian gland in mice [648]. Similarly, OA inhibits carboxymethylation of PP2Ac and attenuates phosphatase activity in xenopus oocytes and insulin secreting cells

[649, 650]. Furthermore, FTY-720 mediated increase in PP2Ac activity in our study and that of others [517, 644], was not associated with altered phosphorylation or methylation of PP2Ac, despite a modest increase in LCMT-1 abundance.

How then does OA decrease VE-cadherin abundance in the present study? Using MG-132 to inhibit proteasomal degradation, we show that OA decreases VE-cadherin levels by increasing proteasomal degradation. To the best of our knowledge there is no information in the literature with which to compare this. Although several studies support translocation of E-cadherin/VE cadherin from the membrane to cytosol via clathrin-mediated endocytosis of phosphorylated VE-cadherin [317].

Although PP2A is critical for the maintenance of integrity of the blood brain barrier [221], there are divergent views on how it modulates paracellular permeability. The consensus is that inhibition of PP2A, whether through pharmacological inhibition or silencing of PP2Ac, attenuates paracellular permeability in RBMECs and mouse skeletal muscle microvascular endothelial cells [651, 652]. However, calyculin A and OA increase permeability in a dose and time dependent manner in rat pulmonary microvascular endothelial cells [653], while having no effect on paracellular permeability of BPAEC [654]. This disparity may simply reflect the fact that studies showing inhibition of PP2A to decrease paracellular permeability were in response to altering a stimulus response (OA/thrombin) compared to inhibition in the absence of an exogenous stimulus. Our data builds upon the earlier studies showing OA to increase paracellular permeability by verifying the effects of OA and clearly demonstrating that CIP2A or SET also increased paracellular permeability of hCMEC/D3 cells. Moreover, we and others associated the increase in paracellular permeability with decreased abundance of VE-cadherin or dysfunctional assembly of VE-cadherin [655].

It is not surprising that FTY-720 did not alter paracellular permeability in the present study as FTY-720 or its analogues have no effect or enhances barrier function in the retina [607], pulmonary endothelial cells [608], RBMEC and brain [656, 657]. This is despite altering occludin levels [656]. Although others indicate that they prevent a reduction in ZO1, occludin and claudin-5 levels [607]. However, the response is likely

to be concentration dependent as FTY720 enhances barrier function up to 1 μM, but decreases thereafter in HUVEC [610].

Regulation of paracellular permeability is multifactorial and includes not only alterations to adherens junction proteins but also tight junctional proteins. Although we attribute the effects of OA and CIP2A/SET on VE-cadherin to modulating paracellular permeability in hBMEC and hCMEC/D3 cells, effects on tight junctional proteins cannot be ruled out. For example, OA and PP2A can increase paracellular permeability by decreasing expression of ZO-1 [658], or by redistributing occludin and ZO1 from the intercellular junction [651, 659]; albeit the latter decreased cellular permeability. Additionally, other phosphatases such as PP2B rather than PP1 or PP2A may also be involved, as PP2B underpins thrombin-induced permeability in BPMECs via PKC [660].

In conclusion, the results of this study demonstrate a strong association between VE-cadherin abundance and PP2A modulation in human brain microvascular endothelial cell lines (summarized in Figure 3.10). OA mediated decrease in PP2A activity is facilitated through the post-translational modifications of the PP2A C-terminal tail. The decrease in methylated PP2A, due to the loss of LCMT-1 and increase in phosphorylation contributes to attenuated PP2A activity and increased VE-cadherin proteasomal degradation. Although there are disparities in VE-cadherin mRNA and protein expression, reinstating that functional protein abundance is imperative to counteract the increase in permeability associated with loss of VE-cadherin. CIP2A, SET and OA each confirm the parallel relationship between PP2A, VE-cadherin and endothelial integrity. Combatting this with FTY-720 will increase PP2A activity and loss of VE-cadherin, however further investigations are required to determine its efficiency to combat brain endothelial dysfunction.