Association of PP2A and VE-Cadherin in a Mono and Co-culture model with implications on Brain Microvascular Permeability

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Gillian Casey, B.Sc (Physiol.), M.Sc

Supervisor: Paul Spiers

Department of Pharmacology and Therapeutics, School of Medicine,
Trinity College Dublin

Coláiste na Tríonóide, Baile Átha Cliath
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Dedication

This thesis is dedicated to my family.

My sisters Yvonne, Ciara and Clodagh who not only helped with proof reading but also provided a listening ear when needed. My brothers Kieran and Ian who always provided a laugh even when times were tough. My fiancé Denis who has been there for me through it all. I appreciate all the love and support received over the last number of years especially from my parents Mary and Ciaran, without whom I know I couldn't have done this. Thank you all for being so patient with me. For my nieces Liah and Nia and nephew Alex. Thanks for playing games with me when I needed to get away from my work.

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Finally, I would like to that my aunt Eithne Casey and Pat Kelly for always being so supportive and caring.

Summary of Methods and Results

VE-Cadherin Regulation by Okadaic acid, CIP2A and SET in Human Brain Microvascular Endothelial Cells: Cell viability was determined using an MTT assay. hBMECs were exposed to histamine (10μM; positive control for induced permeability in the permeability assay), OA (10nM), FTY-720 (5μM) and DMSO (0.001%; solvent control for OA) for 24h. None of which altered cell viability compared to the untreated control (Untx). Doxorubicin (DOX; 5μM; positive control) results in an 81.9% loss in cell viability ($P<0.05$). Throughout this thesis 10 nM of OA and 5μM of FTY-720 was used and cells were treated for 24 h. hBMEC exposure to OA results in a ~60% loss in PP2Ac activity compared to the Untx group, as determined by a PP2Ac activity assay ($P<0.05$). FTY-720 increased PP2Ac activity by ~45% compared to Untx. OA, FTY-720 and DMSO (0.001%) did not alter the total protein abundance of PP2Ac as determined by Western-blot. Investigations into the post-translational modification of PP2Ac determined that exposure of hBMECs to OA increased the abundance of demethylated PP2Ac by >2.5 fold ($P<0.05$), while FTY-720 did not alter the abundance of demethylated PP2Ac. OA increased PP2Ac phosphorylation by ~80% in hBMECs compared to the controls ($P<0.05$), while FTY-720 had no effect. Abundance of both post-translational modifications was determined by Western blot. To determine how PP2Ac methylation is altered, protein abundance of LCMT-1 and PME-1 were also determined by Western blot. PME-1 abundance remained unchanged after exposure to OA and FTY-720 compared to the Untx group. LCMT-1 abundance was reduced by ~80% in hBMECs after exposure to OA ($P<0.05$). FTY-720 (5μM) elicited a ~35% increase in LCMT-1 abundance ($P<0.05$). The investigation was then extended to determine the effect of OA and FTY-720 on VE-cadherin protein abundance (Western blot) and mRNA expression (RT-PCR). OA reduced the abundance of VE-cadherin to undetectable levels compared to the Untx group, while FTY-720 (5 μM) increased it by ~2 fold ($P < 0.05$). VE-cadherin mRNA expression was increased by ~2.7 fold after exposure to OA compared to Untx ($P<0.05$); FTY-720 had no effect. The effect of OA on VE-cadherin abundance was first detected 6 h after OA exposure, resulting in a 44.0% decrease ($P<0.05$). At the 12 h time point there was an 86.4% loss in VE-cadherin as a result of OA. The combination of MG132 (2 μM) and OA did not decrease VE-cadherin abundance at any time point studied or when

summarised as the $AUC_{0\text{-}24h}$. hCMEC/D3s were transfected for 72 h with pcDNA3.1_CIP2aflag_WT or pcDNA-SET-FLAG-HA plasmids to overexpress CIP2A and SET respectively. A mock transfection and empty vector transfection were included in each experiment and did not affect any parameter investigated compared to the Untx group. CIP2A and SET overexpression was confirmed by RT-PCR. CIP2A and SET overexpression attenuated PP2Ac activity by 54% and 58% respectively ($P < 0.05$). Overexpression of CIP2A decreased VE-cadherin mRNA to undetectable levels ($P < 0.05$), while protein abundance was decreased by > 57% ($P < 0.05$). Similarly, over expression of SET attenuated VE-cadherin mRNA and protein expression by >80% and 63% respectively ($P < 0.05$). To assess if OA and FTY-720 had a functional effect on brain microvascular endothelial cells, a transwell permeability assay was employed. Histamine (positive control) for permeability. OA increased hBMEC and hCMEC/D3 paracellular permeability in a time dependent manner compared to Untx and DMSO groups ($P < 0.05$). 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. In hCMEC/D3s the overexpression of CIP2A and SET increased paracellular permeability over 24 h ($P<0.05$).

Modulation of PP2A and VE-cadherin in Human Brain Microvascular Endothelial Cells in a Macrophage Co-culture Model: Human Peripheral blood mononuclear cells were isolated from whole blood to create Mθ macrophages and M1 macrophages created after 14 h exposure to LPS (100 ng/mL) and IFN-γ (20 ng/mL). The M1 macrophage phenotype was confirmed by the increase in TNF-α and IL-1β as quantified by ELISA and RT-PCR. hBMEC and M1 co-culture increased PPP2CA mRNA expression by 36% in hBMECs compared to hBMEC in mono-culture ($P<0.05$). Mθ macrophages did not alter PPP2CA mRNA expression in hBMEC. Mθ and M1 macrophages co-cultured with hBMECs did not alter the abundance of PP2Ac compared to hBMECs in mono-culture. Mθ macrophages increased PP2Ac activity in hBMEC by 14%, while M1 macrophages decreased it by ~30% compared to hBMEC in mono-culture ($P < 0.05$). Post-translational modifications were also examined in the co-culture model. Co-culture of hBMEC with Mθ or M1 macrophages increased the abundance of phosphorylated PP2Ac by ~60% in hBMEC compared to hBMECs in mono-culture ($P < 0.05$). Although Mθ macrophages did not alter the abundance of

demethylated PP2Ac, M1 macrophages caused a 2.8 fold increase in demethylation of PP2Ac compared to hBMEC alone ($P < 0.05$). To explain the alteration in methylation state, PME-1 and LCMT-1 abundance were also quantified by Western blot. As in the first study, PME-1 remained unaltered in both macrophage co-culture models compared to hBMEC in mono-culture. LCMT-1 abundance in hBMECs co-cultured with Mθ macrophages was also unaffected, while LCMT-1 abundance decrease by 70% and 56% in hBMECs co-cultured with M1 macrophages compared to the mono- and Mθ co-cultured respectively ($P < 0.05$). Co-culture with Mθ macrophages increased VE-cadherin abundance (2.8 fold) and mRNA expression (60%) compared to hBMECs alone. In contrast, co-culture with M1 macrophages attenuated VE-cadherin abundance compared to hBMECs in mono-culture (~40%) and those co-cultured with Mθ macrophages (>60%; $P < 0.05$). VE-cadherin mRNA expression was increased (2.6 fold) in hBMEC co-cultured with M1 macrophages ($P < 0.05$). Similar to the first study, MG132 (2 μM) prevented the M1 macrophage induced loss in VE-cadherin abundance. hBMEC co-cultured with Mθ macrophages and MG132 did not alter the Mθ induced increase in VE-cadherin abundance. hCMEC/D3s co-cultured with M1 macrophages also resulted in a loss of VE-cadherin (~70%) compared to hCMEC/D3s in mono-culture ($P < 0.05$). PP2A overexpression in hCMEC/D3s was confirmed by Western blot (2 fold increase in protein abundance; $P < 0.05$). This was accompanied by a >4 fold increase in PP2Ac activity ($P < 0.05$) compared to the un-transfected and pCMV6 empty vector control groups. The overexpression of PP2A did not alter the abundance of VE-cadherin in hCMEC/D3s co-cultured with Mθ macrophages. The overexpression of PP2Ac prevented the loss of VE-cadherin induced by M1 macrophage co-culture. Transfection of hCMEC/D3 cells with the empty vector pCMV6 or pPP2Ac did not alter VE-cadherin abundance compared to mock transfected cells in mono-culture. M1 macrophage increased paracellular hBMEC permeability over a 24 h time-course compared to the mono-culture or Mθ macrophage co-culture. There was a 4.7 fold increase in permeability at 18 h, which continued to increase ($P < 0.05$). Mθ macrophages did not alter permeability in hBMECs over the 24 h epoch compared to hBMECs alone. The area under the curve is a summary of the time course, which demonstrated M1 macrophage increase paracellular permeability of hBMEC (>2.5 fold) compared to hBMEC alone or in co-culture with Mθ macrophages ($P < 0.05$). In hCMEC/D3s ci-cultured with M1

macrophage paracellular permeability was increased compared to mono- or Mθ co-culture. This was first detected at 18 h (>40%) and continued to increase for the duration of the experiment ($P < 0.05$). Mθ macrophages did not alter paracellular permeability compared to hCMEC/D3s in mono-culture. Overexpression of PP2A prevented the M1 induced increased in hCMEC/D3 permeability.

Effect of Okadaic Acid on the VE-Cadherin Interactome in Brain Microvascular Endothelial cells: In hBMEC, chlorpromazine hydrochloride (CPZ; 10 μg/mL), which prevents clathrin-mediated endocytosis, attenuated the OA (10nM) induced loss in VE-cadherin ($P < 0.05$). OA (10 nM) increased the abundance of phosphorylated VE-cadherin (Ser^{665}) by ~100% in hBMEC and hCMEC/D3 cells compared to the Untx group ($P < 0.05$). CIP2A overexpression also increased phosphorylated VE-cadherin (Ser^{665}) by ~100% in hCMEC/D3 ($P < 0.05$), confirming PP2A's role in dephosphorylating Ser^{665}. PP2A overexpression decreased the abundance of Ser^{665} phosphorylated VE-cadherin by ~60%. OA (10nM; 24 h) decreased the abundance of α-catenin by ~70% in hBMECs and ~30% in hCMEC/D3s ($P < 0.05$). CIP2A overexpression also caused a >50% decrease in α-catenin abundance ($P < 0.05$). The same treatment (OA, 10nM for 24h and CIP2A overexpression, 72 h) did not alter the abundance of β-catenin. OA (10 nM) reduced the abundance of P120 by > 57% in hBMECs and >38% in hCMEC/D3s ($P < 0.05$). Similarly, in hCMEC/D3s overexpression of CIP2A reduced P120 abundance by 50% ($P < 0.05$). A VE-cadherin pull-down assay was carried out to determine the effect of PP2A modulation on the VE-cadherin interactome. OA (10 nM for 24 h) reduced α-catenin bound to VE-cadherin by ~70% in hBMECs and ~30% in hCMEC/D3s ($P < 0.05$). In both cell lines, MG132 prevented the loss in VE-cadherin bound α-catenin. CIP2A overexpression also resulted in the loss of VE-cadherin bound α-catenin (>50%; $P < 0.05$). OA (10nM; 24 h) also reduced the abundance of β-catenin bound to VE-cadherin by ~ 50% in hBMECs and ~60% in hCMEC/D3s ($P < 0.05$). Like α-catenin, MG132 reversed the disassociation of β-catenin from VE-cadherin ($P < 0.05$). in hCMEC/D3s overexpression of CIP2A resulted in an ~70 % decrease in the association of β-catenin with VE-cadherin ($P < 0.05$). Regarding P120, neither OA (10 nM; 24 h) nor CIP2A overexpression altered the abundance of P120 binding to VE-cadherin in the

cell lines investigated. VE-cadherin pull-down confirmed a binding of PP2Ac to VE-cadherin. OA (10nM) decreases this binding by ~45% in hBMECs and ~166% in hCMEC/D3s ($P < 0.05$). Combining OA with MG132 reversed the disassociation of PP2Ac from VE-cadherin in both cell lines ($P < 0.05$). Overexpression of CIP2A reduced PP2Ac bound to VE-cadherin by > 50% ($P < 0.05$). OA (10 nM) and increased the abundance of ubiquitin attached to the VE-cadherin interactome by >90% hBMECs and >100% in hCMEC/D3s ($P < 0.05$). Overexpression of PP2Ac did not alter the abundance of ubiquitination of the VE-cadherin interactome. PP2A overexpression did not alter the total abundance of α and β-catenin but did increase P120 abundance by >130% ($P < 0.05$). Regarding the VE-cadherin interactome, overexpression of PP2A elicited a >2 fold increased in α-catenin, a >60% increase in β-catenin and >130% increase in P120 bound to VE-cadherin in hCMEC/D3s ($P < 0.05$). Finally over expression of PP2Ac increased the abundance of PP2Ac binding to VE-cadherin by ~2.5 fold ($P < 0.05$).

Papers in Preparation

Casey, G. and Spiers, JP. VE-Cadherin Regulation by Okadaic acid, CIP2A and SET in Human Brain Microvascular Endothelial Cells. *In preparation*

Casey, G. and Spiers, JP. Modulation of PP2A and VE-cadherin in Human Brain Microvascular Endothelial Cells in a Macrophage Co-culture Model. *In preparation*

Casey, G and Spiers, JP. Effect of Okadaic Acid on the VE-Cadherin Interactome in Brain Microvascular Endothelial cells. *In preparation*

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Oral Presentations

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*Finalist for the Roger Wadsworth Prize

Casey, G and Spiers, JP. The role of PP2A in brain microvascular permeability – Oral Presentation at the British Pharmacological Society Meeting, London 2017
http://www.pa2online.org/abstracts/vol16issue1abst606p.pdf

Poster Presentation

Casey, G and Spiers, JP. The role of PP2A and related proteins in Human Brain Microvascular Endothelial Cells in response to Pro and Anti-Inflammatory Cytokines – Poster at the British Pharmacological Society Meeting, London 2016
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Hakimjavadi. R, Burtenshaw. D, **Casey. G**, Di Luca. M, Cahill. PA, Enrichment of Dimethylation of Lysine 4 on Histone 3 and Resident Vascular Stem Cell Transition to Vascular Smooth Muscle Cells –Arteriosclerosis, Thrombosis and Vascular Biology Meeting 2016
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Hakimjavadi. R, Burtenshaw. D, **Casey. G**, Di Luca. M, Cahill. PA, Stem-cell derived myogenic progeny enrich for vascular smooth muscle cell epigenetic marks at the myosin heavy chain 11 promoter in vitro.- Poster at the Scotish Cardiovascular Forum Meeting, Dublin 2018
http://heart.bmj.com/content/104/Suppl_4/A9.2

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Abbreviations

AJ	Adheren junction
Ala-Asp-Thr	Alanine - Aspartic acid – Threonine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	adenosine monophosphate-activated protein kinase
ANG-1	Angiopoietin 1
ATP	Adenosine triphosphate
BACE1	Beta-secretase-1
BBB	Blood brain barrier
Ca2+	Calcium
CaMKII	Calcium/calmodulin-dependent protein kinase type II
cAMP	Cyclic adenosine monophosphate
CBD	Catenin-binding domain
CCL2	Chemokine C-C motif ligand 2
CIP2A	Cancerous inhibitor of PP2A
CNS	Central nervous system
CNS	Central nervous system
CSF-1	Colony stimulating factor-1
CVD	Cardiovascular Disease
Cys	Cysteine
DARPP-32	dopamine- and cAMP-regulated neuronal phosphoprotein
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DSP	Dual specific phosphatase
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signalling regulated kinase
EYA1-4	Drosophila eye-absent gene
FGF	Fibroblast growth factor
FOX01	Forkhead box protein O1
GLUT	Glucose Transporter
GMP	Guanosine monophosphate

GTP	Guanosine triphosphate
hBMEC	Human Brain Microvascular Endothelial Cells
His-Ala-Val	Histidine – Alanine – Valine
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
I/R	Ischemia/reperfusion
$I1^{PP2A}$	Inhibitor of PP2A
IFN-y	Interferon gamma
IL-1	Interleukin-1
JAM	Junctional adhesion molecules
JAM	Junction adheren molecule
JMD	Juxtamembrane domain
kDa	Kilo dalton
KO	Knock-out
LCMT-1	Leucine carboxylmethyltransferase -1
LPS	Lipopolysaccharide
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MDCKs	Madin Darby canine kidneys
MMP	Matrix metalloproteinases
mRNA	Messenger Ribonucleic acid
MS	Multiple sclerosis
N0	Nitric oxide
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-Methyl-D-aspartic acid or N-Methyl-D-aspartate
NVU	Neurovascular unit
OA	Okadaic acid
OGD	Oxygen and glucose deprivation
P gp	P-glycoprotein

PDGFR	Platelet-derived growth factor receptor
PECAM	Platelet endothelial cell adhesion molecule
PER	The period gene
PKC	Protein kinase C
PME-1	PP2Ac methyltransferase-1
PP2A	Protein phosphatase 2A
PPM	Metallo-dependent protein phosphatase
PPP	Phosphoprotein phosphatase
PSP	Serine/Threonine protein phosphatases
PTEN	Phosphatase and tensin homology
PTP	Protein tyrosine phosphatases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
S1PR1	Sphingosine-1-phosphate receptor 1
SEM	Standard error of the mean
Ser	Serine
SH2	Src Homology 2
siRNA	Small interfering ribonucleic acid
Thr	Threonine
TIM	Timeless gene
TJ	Tight junction
TNF-α	Tumour necrosis factor- alpha
Tyr	Tyrosine
VCAM-1	Vascular cell adhesion molecule - 1
VE-cadherin	Vascular endothelial cadherins
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VE-PTP, PTPRB	Vascular endothelial phosphatase tyrosine phosphatase
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
WHO	World Health Organization
ZO	Zona occluden

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Chapter 1

1 Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death globally, accounting for 30% of the total death count. The westernised lifestyle of Europe has resulted in CVD causing 4 million deaths, translating to 45% of total deaths annually. This is roughly 50% higher than the global average. (Figure 1) [1]. Risk factors that lead to the development of CVD include:

- Age [2].
- Physical inactivity [3].
- Diet: intake volume and composition. Overconsumption of sodium and alcohol is linked to an increase in hypertension. The increase in childhood obesity is also attributing to the continuing CVD fatalities [4].
- Genetics: Individuals whose parents have a history of CVD are 50% more likely to suffer from similar cardiovascular risks. Variants in DNA sequences are inherited contributing to the disease [5, 6].
- Other contributing factors: Tobacco, stress and diabetes [7, 8)]

The overall mortality rate of CVD has reduced globally but the burden of death and disability as a result of CVD remains a huge problem in low and middle class countries [8, 9]. The more fatal forms of CVD are myocardial infarction and stroke, resulting from blockage or restricted access of blood to the heart or brain respectively [10].

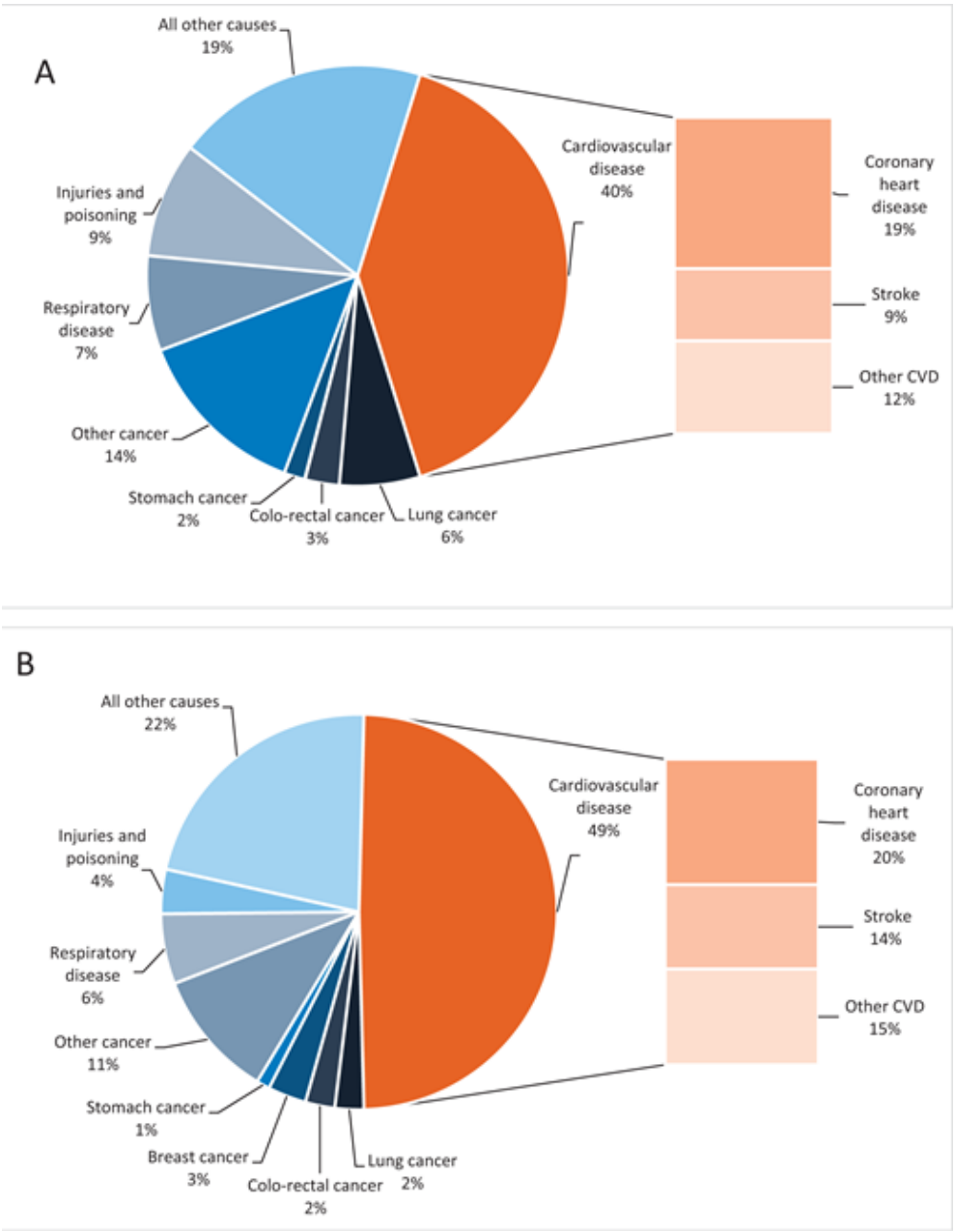

Figure 1.1: Cause of Death in Men (A) and Women (B) in Europe in 2015.

CVD accounts for 40% of deaths in men and a higher 49% in women. Data was summarised from the WHO mortality database. Taken from Townsend *et al* 2016. [1].

Our understanding of the mechanisms involved and pathogenesis of CVD have evolved through the development of better *in vivo* and *in vitro* models [11, 12]. Allowing for the design and development of therapies to combat CVD and guidelines to help reduce risk factors. The following sections will describe the vasculature system and further focus on the endothelium of the cerebrovascular network, which forms the blood brain barrier. It also protects the brain tissue by controlling the influx and efflux of biological materials in the circulating blood.

1.2 Vasculature

The vasculature is an extensive network that services the whole body. The understanding of its importance dates back to ancient Greece (3rd century BC), however the initial theories were somewhat flawed. Aristotle began describing the heart as the source of all blood vessels and the centre of the physiological mechanisms. Praxagoras and Herophilus differentiated between arteries and veins and finally Erasistratus described the heart to be the source of both vessel types; adding that arteries carry oxygen and that a punctured artery bleeds [13].

In the Roman Era, Galen made further observations. Such as, judging the dissipation of heat from the heart to the organs and periphery. Galen postulated that this proved blood originated in the heart and that oxygen in the arteries is at some point, consumed in the circulatory system. In the 1600's, from both Aristotle and Galen's theories of Physiology, Harvey first postulated the theory of vascular circulation. His investigations stemmed from the unknown function of the right ventricle and the theory that nature does not create what is not needed. Harveys research demonstrated that blood enters the limbs through the arteries and leaves through the veins [13, 14].

1.2.1 Vessel Structure

Both arteries and veins are composed of three layers; the adventitial, medial and intimal layer, making up the basic structure of the vasculature [15, 16].

The outermost layer of the vessels is the adventitial, composed of connective tissue, mast cells, nerve endings, fibroblasts and the vasa vasorum. The primary function of this outer layer is structural support, maintaining integrity of the vessel and to facilitate cell-cell signalling, cell trafficking and cross-talk between cells and the surrounding environment [6].

The medial layer of the vasculature consists mainly of vascular smooth muscle cells (VSMCs) [17]. VSMCs originate in the mesoderm of the developing embryo where the VSMC markers can first be detected for example α-actin, smooth muscle myosin heavy chain 11 and calponin 1. This continues to develop the medial layer consisting of a heterogenous population of VSMCs [18]. Some studies have reported the ability of the medial layer to undergo phenotypic switching, altering the primary function of the cells from a contractile to an angiogenetic/proliferative form [19].

Finally the luminal layer of an artery is the intimal, consisting of a single layer of endothelial cells in small vessels [20]. This is the first forming layer during vascular development, producing flat squamous and metabolically active cells that line the entirety of the vasculature [18, 21]. Endothelial thickening is a common side effect of ageing, frequently contributing to luminal narrowing and increasing blood pressure. A core function of the intimal endothelial layer is to sense pressure from passing blood flow, regulate the underlying vascular smooth muscle layer, which in turn alters vascular tone and vessel contractility. The endothelial layer not only reacts to mechanical stresses but also responds to metabolic conditions to produce molecules that alter vessel contractility such as nitric oxide (NO) and endothelin [15].

1.2.2 Endothelium

In the 1800's work by Von Rocklinghausen and Sterling resulted in the theory that the vasculature contained a narrow but selective physical barrier between the tissue and the vascular space [22]. This simple monolayer has the ability to recognise to both physical and chemical signals. The endothelium reacts by producing a wide range of factors with the ability to regulate cell adhesion, vascular tone, vessel inflammation and the underlining smooth muscle cell proliferation [23]. Furchgott and Zawadzki were the first to describe NO, which is a factor released from the endothelium to induce vasodilation. Endothelial NO synthase (eNOS) converts L-arginine into NO in the presence of co-factors such as tetrahydrobiopterin, required for the formation of NO [24]. NO acts through the activation of the underling smooth muscle cells to produce guanylate cyclase and cyclic GMP. This results in a decrease in intracellular calcium (Ca^{2+}), muscle relaxation and luminal dilation [25, 26]. The endothelial layer also reacts to mechanical signals. Shear stresses stimulate the activation of eNOS to produce NO resulting in vasodilation, decreasing blood pressure and also altering cardiac output [27]. There are also NO independent vasodilators produced by the endothelium, such as prostacyclin, which is a derivative of the cyclooxygenase system. Vasoconstriction is also regulated by factors released by the endothelium. Endothelin is a potent vasoconstrictor produced by endothelial cells responding to shear stress, hypoxia, thrombin and vasoactive factors such as angiotensin II. Endothelin binds to the endothelin receptors of which there are two isoforms. ETA is the most abundant (99%) of the receptor population causing vasoconstriction through stimulation of vascular GMP. ETB is less abundant (1%) and its activation results in an increase in NO synthesis and vasodilation [28]. The endothelium is a multifaceted organ with dynamic endocrine, paracrine and autocrine functions. It continually monitors and acts as a first response to local and systemic stimuli in order to maintain vascular homeostasis [29].

1.2.3 Hemostasis

Hemostasis is the physiological process that repairs damaged vessels, stops bleeding while maintaining systemic circulation. This process is fast acting and initiated within seconds of damage occurrence and remains localised [30]. In a healthy vessel, platelets are unable to bind to the endothelium. Binding and aggregation is prevented by the release of a number of vaso-relaxants and platelet inhibiting molecules. Healthy endothelial cells prevent the action of the coagulation factor thrombin. The action of thrombin is both blocked and neutralised by the release of heparin sulphate, which simulates anti-thrombin III [31]. Endothelial cells also express thrombomodulin, which binds to thrombin, activating the protein kinase C pathway in the endothelial cells and preventing the synthesis of pro-coagulation factors [32]. However, in a damaged vessel, platelets aggregate and a plug is formed at the site of injury. Release of factors from the endothelial cell promotes platelet binding to the damaged area. Thrombin is a serine protease involved at a number of points on the coagulation cascade. Thrombin stimulates the conversion of fibrinogen to fibrin and activates protease-activating receptors on platelets for platelet binding [33].

1.2.4 Endothelial Dysfunction

Endothelium dysfunction is linked to several diseases such as atherosclerosis, cancer vascular leakage, infection and stroke [23]. The ability of the endothelial layer to produce NO is required to maintain healthy vessels and tissue. Free radicals can prevent the action of NO and cause damage to the endothelium. A healthy body contains a supply of anti-oxidants that neutralise free radicals however a depletion or imbalance of anti-oxidants exposes the endothelium to injury [34]. Due to the extensive network of the vasculature, endothelial dysfunction can affect any part of the body [23, 24].

Hypertension is a predisposing risk factor for atherosclerosis. During atherosclerosis endothelial cells undergo phenotypic alterations into a pro-inflammatory and pro-thrombotic state. This is characterised by the increase in surface receptors such as VCAM-1, E-selectin and P-selectin for the adhesion of leukocytes and release of chemokines such as TNF-α, IL-1 and monocyte

chemoattractant protein-1 [35]. This stimulates monocyte and leukocyte adhesion to the vascular wall and penetration through to the tissue [36].
Atherosclerosis accounts for 75% of CVD deaths. An initial manifesting feature is thickening of the intimal medial, thickening as a result of risk factors such as ageing and a build-up of low-density lipoprotein [37, 38]. Progression of atherosclerosis involves recruitment of inflammatory cells such as monocytes, leukocytes and neutrophils to the vessel wall furthering the intimal thickening.
Monocytes are activated to express surface receptors, initiating the digestion of oxidised lipids, polarising the monocytes to a macrophage foam cell [39]. Endothelial dysfunction is also a contributing factor to the severity of inflammatory conditions during atherosclerosis manifestation. An Increase in C-reactive protein decreases the stability of eNOS mRNA and decreases eNOS-mediated NO production [40]. Endothelium dysfunction in the brain plays a pathogenic role in strokes. Strokes are the second most common form of death causing CVD (Figure 1), while also resulting in a high occurrence of disability [1].

Overall, we have an understanding of the vasculature and the complex regulatory role of the endothelium. This thesis focuses on the endothelium of the brain, which differs from the endothelium of the rest of the body [41-43] so the following sections will describe the neurovascular unit and components of the blood brain barrier.

1.3 Cerebrovasculature and the Blood Brain Barrier

Paul Ehrlich first described a barrier separating the brain from the periphery. Ehrlich infused dye into the vasculature and reported that the dye was detected throughout the body, but not in the brain or spinal cord [44]. It was not until the discovery of the electron microscope that the functioning BBB could be visualised. HRP was injected into the systemic blood and the cerebrovasculature. Investigating the luminal space of the microvessels in animals such as mice, it was observed that systemic HRP did not pass into the brain tissue. Brightman and Reese hypothesised that the restriction was due to epithelial-like tight junctions in the intercellular clefts [45, 46].

The brain has an extensive vascular network consisting of arterioles and collecting venules. The venules are similar to capillaries as they facilitate exchange of ions and materials. The brain is a complex organ, which requires specialised vasculature. Studies in rats have demonstrated that the vital areas of the brain for example the circumventricular organs contain a higher number of vessels (10 fold) compared to the areas containing white matter, which facilitates a higher rate of diffusion [47]. These areas are serviced by small arterioles and drained by collecting venules. The collecting venules facilitate the majority of material exchange due to their thin wall consisting mainly of endothelial cells [16]. Venules vary in size and diameter ranging from 15-400 μm in length and 3–10 μm in diameter [48]. Material delivered to the brain must first pass the brain specific endothelium [41]. An early study by Butt and Jones suggest that the brain capillaries are 100-500 times more restrictive than those in the muscle, skin or gut [49].

Further investigations determined that there are in fact 3 distinct barrier layers in the brain and spinal cord; the choroid plexus, the arachnoid epithelium and the microvascular endothelium. The choroid plexus is the vasculature found in the cerebral ventricles. These vessels contain fenestrations with no tight junctions and thus do not prevent the movement of small molecules. The arachnoid epithelium envelops the brain and contains tight junctions [50, 51] (Figure 1.2). Although

each layer contribute to the inhibition of ions and small molecules passing through to the brain tissue it is the endothelial of the microvasculature that is most selective and refined prevention of brain tissue damage [52]. The existence of the BBB is a common feature in vertebrates but regarded as a result of evolution. A study into ancient fish groups determined that glial cells replaced the endothelial-based barrier found in vertebrates. It is worth noting that the glial layer was unable to prevent the passage of HRP, a trait that led to the discovery of the BBB. This evolutionary cell type shift has resulted in the development of further barrier functions to create the robust and selective barrier we know [53].

While the importance of the microvascular endothelial layer is the primary foundation of the BBB, there are multiple cell types, which contribute to its function, collectively known as the neurovascular unit.

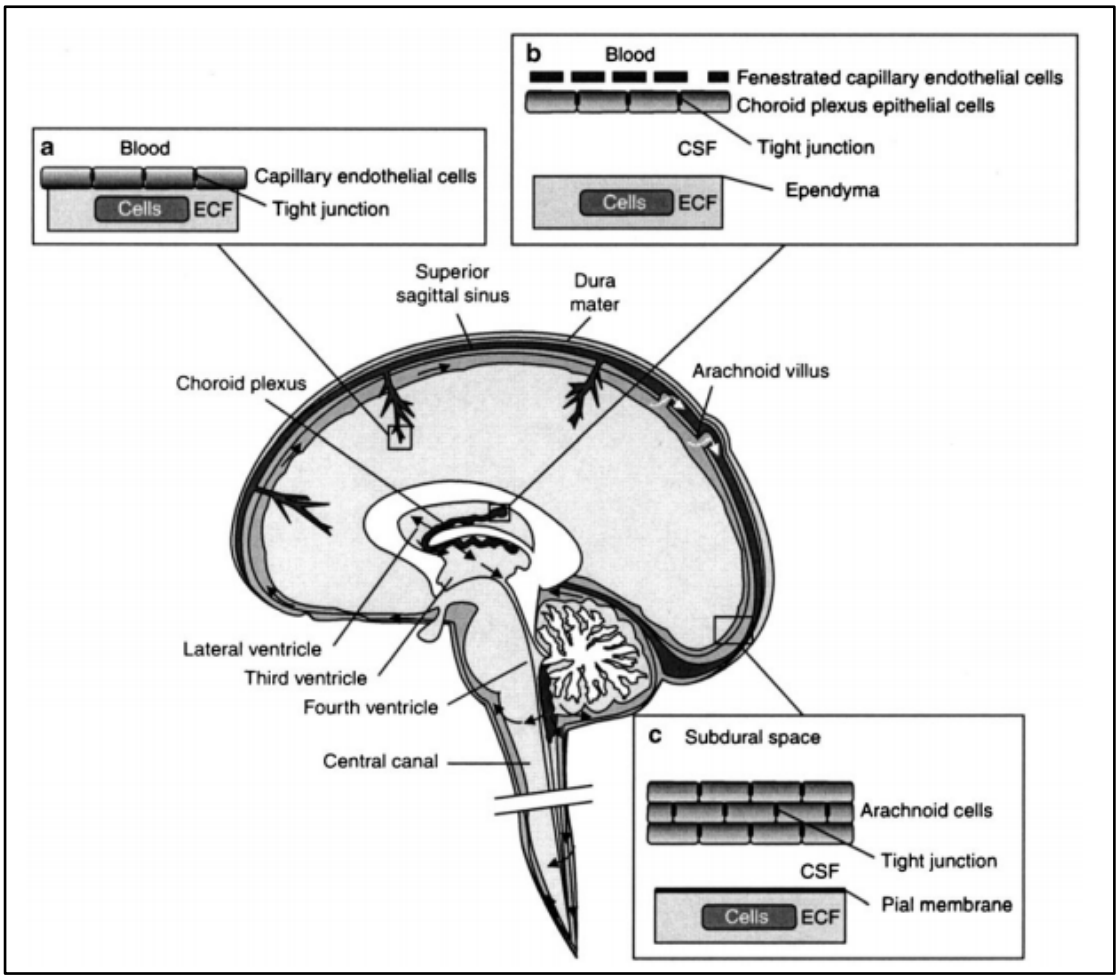

Figure 1.2: Barriers of the Cerebrovasculature.

The brain tissue is protected from the systemic blood by three protective barriers. The microvascular endothelium (a), where tight junctions between cells form a highly regulated and selective barrier. The choroid plexus (b) is found in the ventricles of the brain. This barrier regulated the movement from the blood to the cerebrospinal fluid. It connects fenestrated endothelial cells to underlying epithelial cells held together with tight junctions. The arachnoid epithelium (c) envelopes the brain with endothelial cells held together by tight junctions forming an effective seal. Barriers and c continue into the spinal cord. Taken from Abbot *et al* 2013 [50].

1.3.1 The Neurovascular Unit

The vasculature of the brain co-exists with a number of cells that work together, described as the neurovascular unit (NVU). The NVU consists of a number of cell types including microvascular endothelial cells, astrocytes, microglial, neurons and pericytes (Figure 1.3), which work together to communicate, maintain homeostasis and protect the brain from damage and are described in further detail in the following sections [54, 55].

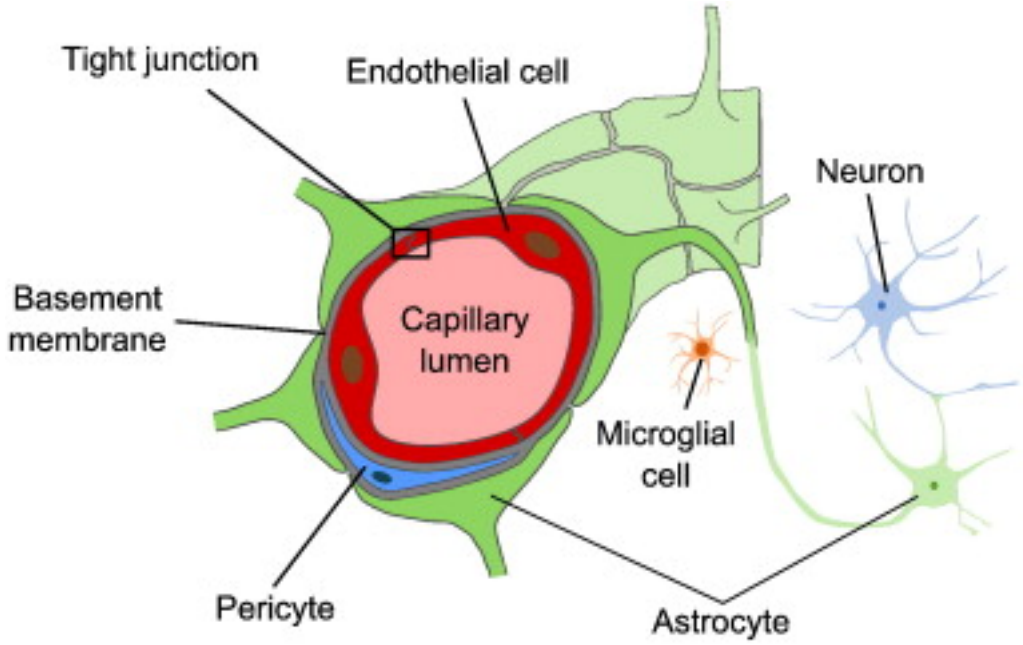

Figure 1.3: A schematic view of the Neurovascular unit (NVU).

The NVU are the cells involved in the blood brain barrier. The endothelial cells cover the luminal surface of the vasculature, which are held together by tight junctions. Pericytes and the basement membrane are in direct contact with the endothelial cells and are involved in contraction. Astrocytes facilitate communication between the endothelial cells and neurons. Microglial are the resident macrophage-like cells in the brain.
Taken from Heye et al 2014 [55]

1.3.1.1 Glial Cell

There are three forms of glial cells in the brain, microglial, astrocytes and oligodendrocytes [56].

1.3.1.2 Microglial

Microglial cells are named after the Greek word "glíal" meaning glue and their function was thought to be to glue neurons to the vasculature [56]. It has since been determined that microglial are the innate immune cells of the central nervous system and are activated during acute inflammation, similar to peripheral macrophages [57]. Microglial originate from yolk-sac progenitors, similar to macrophages whereas the other glial cells, such as astrocytes originate from the ectoderm tissue. Microglial account for between 33% and 66% of the total brain mass [58, 59]. They have been shown to have a role in synaptic pruning and modulation of synapses during development, healthy and diseased states [60-62].

Activated microglial produce reactive oxygen species (ROS) and promote the recruitment of peripheral immune cells. They monitor the CNS for potential threats, damage or pathological changes. Acting as the first line of defence.

1.3.1.3 Astrocytes

Astrocytes are the most abundant glial cell type in the adult brain [63]. They were first characterised as cells to support vascular and neuronal tissue and a sensor for diseased tissue. Astrocytes are involved in astrogliosis, a mechanism of CNS damage detection, which stimulates an increase in astrocyte abundance. Their functions have since been well documented and have a wide scope. Astrocytes form bridges between neurons and the vasculature, relaying nutrients and signals. The foot of the astrocytes also provides a structural and biochemical support to the endothelial cells [64]. Astrocytes regulate water and ion homeostasis while also facilitating an open communicative path between the brain vessels and neurons [52, 65, 66].

1.3.1.4 Oligodendrocytes

Mature oligodendrocytes are responsible for the support and insulation of axons in the central nervous system by the creation of the myelin sheath. These myelin-producing cells can service up to 50 neurons, wrapping them in an insulating layer to prevent ion leak and increasing the efficiency of the neurotransmission [67].

1.3.1.5 Pericytes

Pericytes are found throughout the body, however their highest density is found in the brain and retinas. They are in direct contact with endothelial cells (Figure 1.3)[68]. An increase in pericyte research over the last decade has determined that pericytes are involved in angiogenesis and vessel stabilisation, neurovascular coupling, blood flow regulation and maintenance of the BBB [69]. Their interaction through junctional complexes e.g. N-cadherin (Section 1.5.3) allow for the transmission of mechanical contractile forces and control of blood flow. Pericytes have macrophage like characteristics when required, carrying out phagocytosis to eliminate potential threats [70]. They have been linked to multiple pathological diseases such as Alzheimer's disease, multiple sclerosis, hypertension and tissue damage for example hypoxia-induced damage [71-73].

1.3.1.6 The Microvascular Endothelium

Starling recognised the inner lining of vessel walls as the selective physical barrier alluded to by Ehrlich [74]. Initially this layer was thought to be static, but subsequent studies unfolded its dynamic capabilities in autocrine, endocrine and paracrine signalling. It monitors the surrounding environment and is involved in regulating vascular homeostasis [23]. Brain microvascular endothelial cells are interconnected, anchor-dependent cells that line the cerebral-microvasculature. Consisting of a single endothelial cell thick and form the basis of the brain blood barrier. Characteristics of these cells differ from microvascular endothelial elsewhere in the body regarding the expression of specific ion transporters, number of fenestrations and pinocytotic capabilities. In comparison to peripheral endothelial cells, brain endothelial cells not only form an anatomical barrier, but also facilitates the transfer of information between glial and neurons [42, 43]. Endothelial cells adhere to one another through junctional structures described below. Transmembrane proteins are also linked to specific intracellular structural

proteins such as the actin cytoskeleton, further stabilising the junction and blood brain barrier. The endothelial layer is metabolically active, regulating vasomotor function and contributes to the nourishment of surrounding nerves [75]. As previously mentioned (section 1.1) damage to the endothelial layer has significant consequences, but also initiates a feedback loop causing further injury [76, 77]. Due to the importance of the endothelial layer, it has become a therapeutic target to protect against damage such as stroke [78].

1.3.1.7 Macrophages

Macrophages are derived from circulating monocytes involved in the detection of bacteria and other harmful factors resulting in phagocytosis and destruction. They are also involved in the initiation of inflammation and T cell stimulation by presenting antigens on their cell surface and in turn releasing cytokines [79]. Macrophage plasticity contributes to its ability to polarise from the Mθ (unstimulated) into:

- M1 pro-inflammatory phenotype, stimulated by factors such as LPS and IFN-γ. Whose function is to defend against foreign pathogens and stimulate leukocyte infiltration [80].
- M2 anti-inflammatory phenotype stimulated by factors such as interleukin 4 and 13. Their function is to stimulate anti-inflammatory signalling, stimulate wound healing and tissue remodelling [81].

There are four types of macrophage associated with the brain, perivascular macrophages, infiltrating systemic macrophages and the above-mentioned microglia and pericytes [68]. Peripheral macrophages and microglial carry similar characteristic functions, making knockout experiments complicated, as deciphering between the two populations is difficult. All microglia and pericytes have the ability to proliferate to a limited extent whereas macrophages cannot. There are also a few phenotypic expressional differences in microglial including MHC class II^{+}, $CD68^{+}$, $CD14^{-}$, $CD11c_{high}$ and $CD45_{low}$ [82-84]. Microglial also have morphological differences described as “spurred with spikes”, this morphology does not alter with age or activation [68, 84].

Systemic macrophages are recruited to the area of damage by chemoattractant molecules such as monocyte chemoattractant protein-1 (MCP-1) secreted by astrocytes [85]. The damaged area tends to have reduced tight junction proteins allowing for the infiltration of systemic macrophages through the BBB. The activation of macrophages have been linked to cerebrovascular disease [86]. A recent study also demonstrated how brain perivascular macrophages contribute to the detrimental effect of Aβ and a source ROS [87]. Peripheral macrophages have the ability to transport Aβ aggregates from the peritoneal cavity to the brain tissue resulting in further progression of Alzheimer's disease [88].

However, the role of macrophages in the BBB is multifaceted and their plasticity allows them to have both harmful and beneficial roles in the CNS. Removal of CNS macrophages result in impaired development and functions associated with controlling both vascular and neuronal morphogenesis [89]. While the recruitment of macrophages to the areas of Aβ builds up during Alzheimer's disease can also be neuroprotective as osteopontin released by macrophages aids in Aβ clearance [90].

1.3.1.8 Neurons

Neurons are the electrically excitable cells in the brain responsible for transmitting information through electrical and chemical signalling. In order to facilitate the transmission of electrical currents ions are required. The endothelium regulates sodium, potassium and calcium ions, which remain within a narrow range of concentrations [91]. Neurons are also required for the delivery of nutrients such as oxygen and glucose, which is vital for brain function as the brain consumes 2% of the body's glucose intake [92, 93]. Oxygen and glucose deprivation results in neuronal damage and the development of brain diseases such as stroke, Alzheimer's disease and motor neuron disease [94, 95].

1.4 Transmembrane Movement

The transport of material through the membrane has been studied extensively and this process has two main forms, paracellular and transcellular pathways.

Understanding this movement is imperative as it is the key protective mechanism. Each is described in detail below and represented in Figure 1.4.

1.4.1 Paracellular Pathway

Paracellular transport refers to movement of small water-soluble biological materials diffused between adjoining endothelial cells. This pathway is modulated by the presence and efficiency of junctional proteins (Figure 1.4a). Junctional proteins join neighbouring cells to form a seal (detailed below in section 1.5) [96]. Cell contact adhesive forces and cytoskeletal contractile forces also regulate paracellular transport [97]. Damage to the cell-cell contact points allows for paracellular movement, this is a passive process and is driven by a diffusion of biological material. Size is also a determining factor of paracellular movement as smaller molecules such as oxygen and carbon dioxide can pass freely [43]. Paracellular transport in the brain is more tightly regulated compared to the periphery and its dysfunction is the most common facilitator of increased BBB permeability and associated neurological diseases [43, 54, 55, 98].

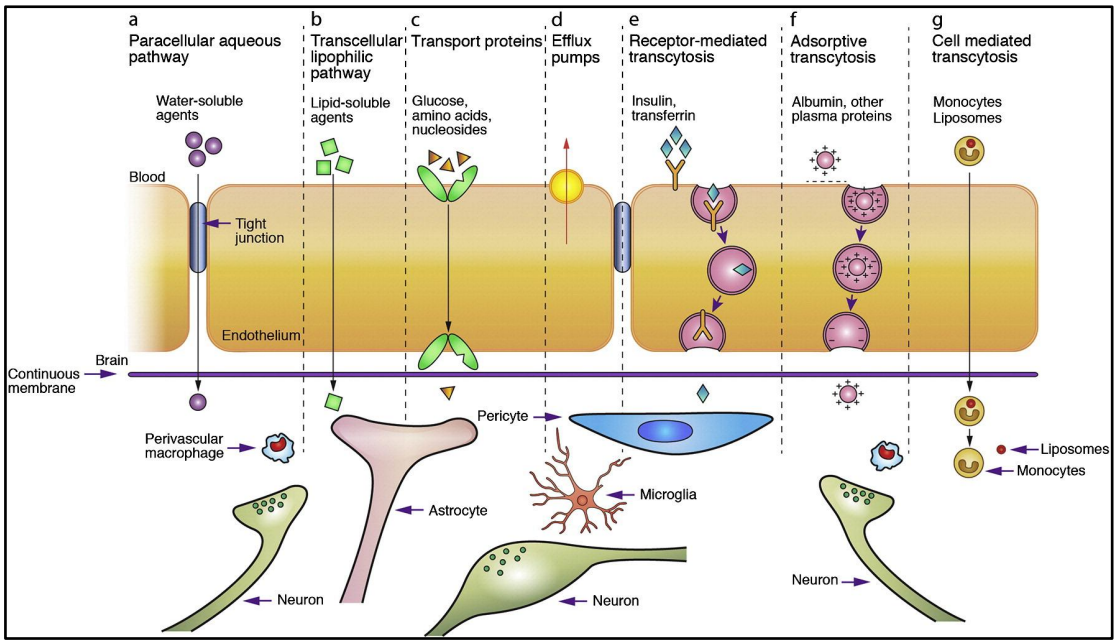

Figure 1.4: Schematic Overview of Blood Brain Barrier Transmembrane Movement.

Small molecules such as water, oxygen and carbon dioxide have the ability to move freely through TJ via the paracellular pathway (a). Small lipid-soluble molecules such as steroids move through the membrane via the transcellular lipophilic pathway (b). The transportation of large proteins such as glucose and amino acids is facilitated by specific transporter protein (c), this method moves substances against the concentration gradient and usually requires ATP. Efflux pumps (d) also require ATP and are responsible for the movement of drugs to and from the brain. Endothelial cells express receptors on the cell surface to facilitate receptor-mediated transcytosis (e) facilitating the movement of solutes such as insulin and transferrin by the binding to their specific receptors. Adsorptive-mediated transcytosis (f) is the main target for pharmacological drug delivery e.g. heparin sulphate proteoglycans and is stimulated by electrostatic interactions between cationic molecules and the anionic plasma membrane. Cell mediated transcytosis (g) is known for its transport of pathogens such as Cryptococcus neoformans and HIV, it requires immune cells such as monocytes as a carrier system to transport the enclosed material through the endothelial cell. Taken from Chen et al 2012 [99].

1.4.2 Transcellular Pathway

There are multiple transcellular pathways that facilitate the movement of molecules through the endothelial layer, the majority of which require additional transport carriers. The transcellular lipophilic pathway does not require an additional facilitator as it can only transport small lipid-soluble molecules such as steroids and alcohol and is driven by diffusion (Figure 1.4b). The transportation of larger proteins such as glucose and amino acids requires specific transporter protein (Figure 1.4c). Glucose binds to its receptors on the luminal membrane (GLUT) and transports it to the basal membrane [100]. This method often moves substances against the concentration gradient, which requires ATP. Similarly, receptor-mediated transcytosis (Figure 1.4e) selectively transport macromolecules and require the expression of specific receptors on the endothelial cell surface. The ligand binding stimulates the creation of membrane coated pit and internalisation, also known as endocytosis. This endosome is acidified and transported to the basal membrane where the endosome connects with the membrane and the ligand released. This pathway is commonly used for the transportation of insulin, transferin and growth factors [101, 102]. Absorptive-mediated transcytosis (Figure 1.4f), also known as pinocytosis is the main target for pharmacological drug delivery e.g. heparin sulphate proteoglycans and is stimulated by electrostatic interactions between cationic molecules and the anionic plasma membrane [103]. Finally, cell mediated transcytosis (Figure 1.4g) is known for its transport of pathogens such as *Cryptococcus neoformans* and HIV, it requires immune cells such as monocytes as a carrier system to transport the enclosed material through the endothelial cell layer. More recently studies are utilising this pathway for the delivery of therapeutic drugs [104, 105].

1.4.3 Enzymatic/ Efflux Barrier

Efflux pumps (Figure 1.4d) also require ATP and are responsible for the movement of drugs to and from the brain. These transporters consistently monitor the compounds being transported through to the brain tissue. Upon detection of harmful or toxic molecules the efflux pumps are activated for their removal. This protective transport pathway has developed through evolution to counteract the

passive influx of lipid-soluble compounds. P-gp, an ATP binding cassette transporter, and the multidrug resistant protein are efflux pumps constitutively expressed by the cerebral endothelium [106, 107]. Inhibition of the P-gp improves the delivery and ability of paclitaxel to penetrate the BBB as this prevents drug extrusion from the brain [99].

As previously described, in order to maintain a functioning BBB, a robust and selective endothelial layer is required. Junctional proteins, detailed in the following sections, are an integral part to the endothelium and hold the endothelial cells together.

1.5 Adheren/Tight Junctions

Tight junctions are found on the luminal side of adjacent endothelial cells. They monitor paracellular movement of ions and molecules and control cellular polarity. They have both transmembrane and intracellular components (Figure 1.5). The most abundant types are the claudins and occludin. For the purpose of this thesis I will be focusing on the association of adheren and tight junctions in the vasculature of the brain.

1.5.1 Occludin

Occludin is a 65kDa protein first described in chickens [108]. It is composed of nine domains, including four transcellular domains that separate both intracellular and extracellular components. The N- and C- terminus are both located intracellularly. Occludin is highly expressed in brain endothelium, which has an uninterrupted expression along adjacent endothelial cells. The C-terminus binds to scaffolding proteins known as zona-occludens (ZO-1 and -2), linking occludin to the cytoskeleton (Figure 1.5). C-terminal truncation results in the increase in paracellular permeability. Also, the phosphorylation if ZO-1 as a result of TNF-α and IL-6 stimulates its disassociation from occludin and increase in permeability. Suggesting the importance of ZO binding [109, 110].

Occludin mainly reinforces the protective barrier of the tight junctions and is involved in the stabilisation of adhesion. It is thought to be of less importance than claudins, as occludin deficient mice develop a normal barrier function, this is not

the case in claudin KO [108, 111]. Occludin is also not required for BBB development, as it is not expressed in foetal and newborn brain sections [108]. It is suspected that Occludin is not involved in age related dysfunction, as its expression does not alter in aging animals [112]. However, its expression level does vary throughout the vasculature and is reflective of the differences in permeability. Occludin is highly expressed in the brain compared to non-neuronal tissue, which would be more susceptible to 'leaky' junctions [113]. Occludin expression is also altered in different brain regions [114]. The expression of occludin is modulated by the binding of SP3 and YY1 to its promoter, resulting in transcriptional stimulation and inhibition respectively [115]. Once expressed, occludin is degraded by both MMP and Rac1 activity. Occludin expression is also increased by PKC [116-118]. Occludin assembly may also be altered, rendering it dysfunctional without effecting its expression [119, 120]. Occludin is degraded by its ubiquitination and proteasomal degradation [121].

The expression of occludin is associated with glucose abundance in the brain. Occludin stimulates GLUT-1 and GLUT-4 uptake through an increase in AMPK [122]. AMPK has also been shown to be a regulator of occludin as AMPK activation reverses loss of occludin due to LPS exposure in mice [123]. *In vivo* and *in vitro* studies demonstrated that glucose and oxygen deprivation results in the loss of occludin, ZO-1 and increased permeability [124-126]. Many studies have characterised how the loss of oxygen during ischemia/reperfusion (I/R) results in the loss and relocation of occludin [127, 128]. However, the production or administration of systemic erythropoietin or overexpression of BACE1 reverses this loss [129, 130].

Inhibition of occludin is a result of many different stimuli and a sign of multiple pathological neurological conditions. Histamine, a known permeability inducer results in the loss of occludin and ZO-1 [131]. Methamphetamine also results in the increase in BBB permeability through the endocytosis and degradation of occludin [132-134].

Alzheimer's disease, Parkinson's disease, stroke, hypothermia, infection, multiple sclerosis and diabetes have all been associated with a loss of occludin in the BBB [135, 136chai {Chai, 2014 #2237, 137-139]. *In vitro* and *in vivo* studies have also demonstrated that lead exposure is also associated with the loss of occludin [140]. Conversely, Romanitan *et al*. demonstrated an increase in occludin during Alzheimer's disease and vascular dementia. However, this increase in expression was found in the astrocytes and neurons instead of the endothelial layer [141].

In many studies occludin, along with the expression of other tight junction proteins such as claudin-5 are used as markers to determine breakdown of the BBB. Thrombomodulin was shown to attenuate the induction of vascular permeability by subarachnoid haemorrhage by the increasing in occludin through PKC activity [142]. A mouse study investigated the effect of explosive noises on the BBB. It was concluded that regular loud noises is associated with a loss of occludin and VE-cadherin, which could be reversed by modulating PKC activity, bryostatin-1 [143]. During traumatic brain injury the decrease in occludin can be prevented by progesterone (P4), which increases endothelial progenitor cells and in turn increases occludin expressing vessels [144]. Exercise also has the ability to reverse loss in occludin and the increase in MMP activity as a result of chronic cerebral impairment [145, 146].

Loss of occludin and vascular dysfunction occurs prior to neurological disorders [147]. Occludin can be detected in the blood plasma and this has been suggested as an early biomarker for damage to the BBB such as during ischemia and stroke [148, 149]. The use of occludin as a biomarker has also been suggested as a method to determine if newly developed vaccines effect the BBB [150]. Somewhat counter intuitively, mannitol-temozolomide a drug used to treat glioblastoma, has recently been investigated as a combined therapy to decrease occludin and ZO-1 for more efficient drug delivery [151].

Bile duct ligation and jaundice also results in the loss of occludin, through the increase in Rac1 activity and increase in occludin phosphorylation [152-154]. The inhibition of Src activity had been shown to prevent the phosphorylation of

occludin and in turn prevents BBB permeability [155, 156]. Although the exact phosphorylation sites were not determined, a study by Andras *et al* determined that glutamate resulted in the loss of occludin by increasing Tyr and decreasing Thr phosphorylation [157].

1.5.2 Claudins

Claudins were first discovered in 1998 [158] and have since been recognised as an integral part of paracellular permeability. It is a 24kDa protein with more than 25 members included in the claudin family. These members have a high sequence homology particularly in the first and fourth transmembrane regions. Each consists of two intra- and extra-cellular regions and four transmembrane domains [113]. The extracellular region is responsible for the binding of adjacent cells via homo and heterophilic interactions (Figure 1.5). Claudins have a similar distribution pattern as occludin [158, 159]. Due to the high number of claudin isoforms, their expression is tissue specific relating to the functional needs of the tissue. Early experiments carried out on Madin-Darby canine kidneys (MDCKs) determined that the induction of claudin-1 expression resulted in a four-fold drop in vascular permeability, while the induction of claudin-2 in MDCKs previously expressing claudin-1 and -4 resulted in an increase in permeability. Exposure of the brain endothelium to bradykinin regulates claudin-5 by Ca^{2+} induced Ca^{2+} release [160].

Claudin–3 and -5 are the most abundant forms detected in the brain [161]. The expression of claudin isoforms –1, -2 and -3 are highly expressed in the choroid plexus with less abundant -6, -9, -19 and -22 also present at lower abundance. Claudin-3 and in particular -5 are the focus of investigations into the brain endothelium. However -4 and -16 are also found in the microvessels. With ageing the abundance of the pore-forming isoforms, -2, -9 and -22 increase while the expression of -3 and -6 decrease [162]. Claudin 11 is less abundant and can be detected at the arachnoid blood, cerebral spinal fluid barrier [163]. Gender is a differential factor for claudin-5 as its expression is determined by oestrogen in women and expression is reduced post-menopause [164, 165]. Claudin-5 is

ubiquitously expressed and is essential for endothelial tight junction formation. Its expression is regulated by the transcription factors β-catenin and FOXO1 [166] and TNF-α induced loss of claudin-5 is due to NF-κB inhibition of the claudin-5 promoter [167]. A into HIV-1 study carried out by Mishra *et al* suggests that claudin-5 expression is governed by VE-cadherin expression [168]. Claudin-5 KO mice demonstrate a high birth mortality rate, with poor BBB development. Claudin-5 expression is dependent on astrocyte abundance and binding [169, 170].

Ischemia/reperfusion and glucose deprivation is known to have a debilitating effect on the BBB. This is associated with a loss in claudin-5 [124] [171, 172]. Although this may just be transient and claudin-5 recovers after the post-ischemia vascular remodelling has finished [173]. The use of the anti-oxidant picroside II or the angiopoietin-like protein 4, released during hypoxia, has been shown to prevent I/R induced loss of claudin-5 [174-176]. Also, pre-conditioning with isoflurane or the systemic release of erythropoietin by the kidneys increases claudin-5 abundance [177]. Shear stress also alters claudin-5 abundance as mild shear stress increases expression however; high velocity shear stress results in the loss of claudin-5 [178].

The loss of claudin-5, similar to occludin, is linked to multiple neurological diseases and is used as a marker for BBB damage; diseases such as inflammation, hypothermia, Parkinson's disease, Alzheimer's disease, multiple sclerosis, diabetes, lupus and stroke [125, 135, 169, 179-184]. During neuroinflammation claudin-5 is transferred to leukocytes at the BBB facilitated by endothelial vesicles. These vesicles can be detected in the blood and a potential biomarker for BBB dysfunction [185]. A study into Alzheimer's disease also demonstrated the transfer of claudin from endothelial cells to astrocytes resulting in an increase in the expression of claudin -2, -5 and -11 [186]. Vascular damage occurs prior to disease manifestation, suggesting the claudin-5 containing vesicles may be used as an early diagnostic marker [147]. Loss of claudin-5 also has clinical relevance to schizophrenia, autism and depression [187]. The schizophrenia gene mutation and claudin-5 gene are in a similar gene region, determining their association [188]. Other claudins are associated with diseases such as the loss of claudin-1 during

hypothermia as a result of increased cathepsin-L expression in astrocytes [189]. One rat study demonstrated how claudin 1 and 5 together are decreased during cerebral I/R and that this can be reversed by hyperbaric oxygenation [176].

As mentioned above claudin-5 is used as a marker of BBB damage and is also used to determine the effect of molecules on endothelium integrity. α-Melanocyte stimulating hormone is said to prevent cytokine induced BBB permeability by the prevention of claudin-5 loss [190]. Also, studies into potential MS therapies have highlighted that antagonising the P2x7 receptor, which is involved in neuroinflammation, also prevents the loss of Claudin-5 [191].

Environmental factors also have an effect on claudin-5 expression. Mice exposed to alcohol, traffic pollution and a high fat diet has a decreased expression of claudin-5 in the BBB, likely due to the increase in MMP activity [192, 193]. An *in vivo* study has demonstrated that cooling brain temperature results in increased permeability through the loss of claudin-5 [194]. Histamine exposure, which is known to be a permeability inducer, results in the loss of claudin-5 [131]. Exposing the endothelial layer to a low concentration of copper results in an increase in proliferation and viability. However, copper can also reach toxic levels, resulting in the increase in apoptosis. Copper concentration has a regulatory effect on claudins 1, 3, 5, and 12. Claudin 1, 3 and 5 have a tendency to decrease with the increase in copper concentration while claudin 12 has a tendency to increase [195]. Systemic Zinc has also been shown to contribute to the loss of claudin-5 as a result of ischemia [196].

Claudins are susceptible to post-translational modifications such as phosphorylation, which alter their protective function [197]. CCL2-induced permeability loss is associated with the increase is Ser/Thr phosphorylation of claudin-5 [198]. Inhibition of Ser/Thr kinases has prevented the ubiquitination and degradation of claudin-5 by E3 ubiquitin ligase [199]. AMPK is another kinase associated with claudin-5, loss of AMPK results in a loss is claudin-5 [200]. MMP-2 and MMP-9 activity is associated with the loss of claudin-5, likely through post-translational degradation [201]. Phosphodiesterase inhibitors have been shown to

prevent the loss of claudin-5 due to haemorrhage [202]. The KO of phospholipid transferase protein in mice also reduces expression of claudin-5 suggesting a regulatory role [203].

1.5.3 Cadherins

Cadherins are the most ubiquitously expressed adherens junctional protein along the endothelial layer of the vascular [204]. They are Ca^{2+} ion dependent and composed of intercellular, transmembrane and extracellular domains (Figure 1.5) [205]. They have a diverse number of functions including contact inhibition during cell remodelling and growth while also being a vital component in controlling paracellular transport [206]. Unlike the previously discussed occludin and claudins, cadherins are composed of a single intercellular and single extracellular domain separated by a single transmembrane region. Cadherins regulate cell-cell adhesion through the binding of adjacent extracellular regions; an integral component of cadherin homodimer binding is the attachment of intracellular accessory proteins such as catenins (Figure 1.6).

There are over 100 forms of cadherins that are expressed in a number of cell types, which can be classified into four main groups: classical, desmosomal, protocadherin and unconventional. The more abundant and common cadherins are E, P and N. Endothelial cells have a signature vascular endothelial cadherin (VE-cadherin), which is only expressed in the endothelial cells and the only cadherin expressed at BMEC cell-cell adhesion points [207]. In cell culture experiments VE-cadherin expression is increased at higher seeding densities due to a greater volume of cell-cell contacts [208]. Both *in vivo* and *in vitro* research have highlighted the pivotal role of VE-cadherin in endothelial and BBB integrity [209]. VE-cadherin expression is low during embryonic development of the BBB and increases postnatally, however VE-cadherin KO mice do not survive to birth due to deformities [210]. Elderly mice demonstrate a decline in VE-cadherin expression; during the recovery stages of ischemic stroke VE-cadherin expression is increased and is involved in vessel reconstruction [211]. VE-cadherin is regulated under the transcription factors serum response factor and myocardin related transcription factor and Fox03a [212]. VE-cadherin undergoes clathrin-

mediated endocytosis, which results in either proteasomal degradation or remains in the cytoplasm to be recycled to the membrane. VE-cadherin recycling at the BBB requires Rab11a [213]. N-cadherin is also expressed in endothelial cells. It is suspected that these two types have different functions as N-cadherin relocates to the apical and basal membranes in the presence of VE-cadherin [214]. N-cadherin is also expressed in astrocytes and pericytes and has a role in the binding of these cells to one another [215-217]. Cadherin 10 is found in the circumventricular organs and its loss has been linked to BBB dysfunction [218]. Protocadherins are cadherin related molecules, which are involved in cell adhesion, cell interaction, CNS development and highly expressed in hBMECs [219].

Catenins are a group of intracellular proteins associated with cadherins. They have four isoforms (α, β, δ and γ), the primary role of which is to attach cell junctions to the actin filaments. The cytoplasmic tail of VE-cadherin binds to β-catenin via the Src kinase in order to anchor it to the cytoskeleton; α-catenin is also involved in this process [220]. P120 is another member of the catenin family associated with VE-cadherin. It is an Src substrate homologous to β-catenin, which binds to the section of VE-cadherin coupled with the membrane. Interestingly, Src negatively regulates protein phosphatase 2A (PP2A) [221].

VE-cadherin expression is dependent on the presence of astrocytes [222, 223]. However, this may only be due to factors released by astrocytes as astrocyte-conditioned media also results in an increase in VE-cadherin [224, 225].

The loss of VE-cadherin is associated with multiple neurological diseases such as infection, multiple sclerosis, Alzheimer's disease, meningitis, ischemia, oedema, stroke and hypothermia [125, 226-231]. Total cell abundance does not represent VE-cadherin association with the cellular membrane and effect on permeability. Infection results in the increase in vascular permeability through the internalisation of VE-cadherin and accessory protein β-catenin without altering total cell abundance [232]. An increase in IFN-γ is associated with multiple sclerosis, while also increasing transendothelial migration and the relocation of VE-cadherin [233, 234], which can be prevented by IL-1β [235]. The loss of E-

cadherin in epithelial cells is also associated with neurological diseases such as traumatic brain injury (also an increase in VEGF) [236].

Extensive research has been carried out in an attempt to combat BBB damage relating to the loss of adheren junctions. Molecules such as angiopoietin-like 4, annexin A2, retinoic acid and memantine have shown a protective effect against brain oedema and ischemic stroke preventing VE-cadherin loss and decreasing MMP activity [174, 229, 237-239]. Growth factors have been associated with modulating VE-cadherin expression such as the hepatocyte growth factor [240]. The basic FGF has also been shown to protect against traumatic brain injury and OGD by the inhibition of S1PR1 and increasing VE-cadherin [241]. Contrary, VEGF is associated with a loss of VE-cadherin [236]. It appears that less research has focused on N-cadherin expression; however, adropin preserves BBB integrity after brain haemorrhage through the Notch/Hes1 pathway to increase N-cadherin expression [242, 243]. Cilostazol also prevents cerebral haemorrhage by increasing both N and VE-cadherin [244].

Factors such as diet have recently been shown to be associated with neurodegeneration. Hypoglycaemia, low foliate or B6/B12 intake has been associated with the loss of VE-cadherin as well as the intake of nicotine [245-247]. A sleep deprivation study discovered an association with VE-cadherin loss, which could be reversed by A2A receptor agonism [248]. Adropin, which is involved in lipid metabolism has been shown to reverse the loss of VE-cadherin and increased VEGF associated with ischemia [249]. Excessive “bombing” noises has also been linked to an increase in BBB permeability; in this study bryostatin1 was used to prevent the loss of VE-cadherin through PKC activity [143]. Stimulation of sphingosine kinase is another kinase linked to preventing VE-cadherin loss due to ischemia and stroke [250, 251].

VE-cadherin expression can undergo both pre and post-translation regulation. A recent study carried out into the leukoaralosis discovered two genetic small nucleotide polymorphisms (rs1801026 and rs16260) related to VE-cadherin dysfunction [252].

Caspase and MMP activity have also been associated with the degradation of VE-cadherin [253]. Increase in caspase-3 activity results in the translocation of VE-cadherin [254]. Mice suffering from West Nile's disease demonstrated an increase in MMP -1, -3 and -9, which is associated with VE-cadherin loss [255]. VE-cadherin has multiple phosphorylation sites responsible for its modulation, including Tyr^{645}, Tyr^{658}, Tyr^{685}, Tyr^{731}, Tyr^{733} and more recently Ser^{665} [256]. VEGF induces Tyr^{685} phosphorylation of VE-cadherin, resulting in its disruption, internalisation and degradation [257]. HIV infection result in the increase in both VE-cadherin and β-catenin tyr phosphorylation and disassociation of VE-PTP and SH2 [258]. Subarrachnoid hemorrage is associated with an increase in the ser/thr phosphorylation and in turn degradation of VE-cadherin [259]. VE-cadherin phosphorylation by Src2 is also induced by IL-2 exposure, resulting in the disassociation of β-catenin and P120 [260]. VE-PTP decreases VE-cadherin phosphorylation decreasing permeability and stroke size [261].

Targeting junctional proteins to increase drug delivery has previously been mentioned. Listeria monocytogen interlin breaches the BBB by binding to the extracellular domain of E-cadherin [262]. This method has been utilised for drug delivery. Two peptide sequences His-Ala-Val and Ala-Asp-Thr have been designed to target the extracellular domain of e-cadherin preventing cell-cell adhesion [263-266]. Previously we mentioned the association of the A2A receptor and VE-cadherin loss, which has also been investigated as a potential target to aid drug delivery [267]

1.5.4 JAMs and PECAM-1

Junctional adhesion molecules (JAM) are 40 kDa proteins are tight junction proteins. JAMs are involved in cell-cell adhesion via homo- and heterophilic interactions [96, 268]. They are composed of a short intracellular and a single membrane-spanning region. There is a large extracellular IgG binding domain responsible for the attachment of adjacent cells. JAM-1 is found in multiple cell types however the JAM-2 and -3 isoforms are endothelial cell specific [54, 269]. JAM expression is low during embryonic development and first expressed at 16 weeks gestation [270]. Once developed JAM-1 expression does not alter [271]. Similar to other TJ proteins, JAM regulates paracellular transport but is also involved in leukocyte adhesion during inflammation by interacting with a leukocyte function associated antigen-1. When expressed at the apical membrane JAM-1 has the ability to bind to leukocytes, neutrophils and monocytes to facilitate transendothelial migration. JAM-1 is relocated to the apical membrane through Rab4 and Rab5 activity [272]. JAMs association with increased vascular permeability is due to loss of expression. The exposure of endothelial cells to NO and the increase in NOS is known to induce BBB permeability and this is associated with the loss of JAM-1 [273]

The different JAM isoforms have been linked to BBB dysfunction. The loss of JAM-1 and -3 has been associated with hypothermia, oedema and ischemia/reperfusion in the brain and an increase in MMP activity [274-276]. Many studies into inflammation report that there is no alteration in JAM expression, Brooks *et al* demonstrate that 24 h of inflammatory induced pain did not alter JAM-1 expression. Extended inflammatory pain results in transient expression of JAM-1, at 48 h there was an increase in expression, which altered to a decrease in expression at 72 h [277]. Cathepsin S also induces increased vascular permeability and brain tumour invasion through the proteolytic processing of JAM-2 [278]. However, JAM-1 and -2 are thought to have opposing activities. HIV infection induces BBB permeability associated with an increase in JAM-2 and decrease in JAM-1 through MMP activity [279, 280].

Platelet endothelial cell adhesion molecule (PECAM) is a 130kDa transmembrane immunoglobulin. It is expressed in platelets, endothelial cells, monocytes, neutrophils and some T-cells [281]. PECAM-1 is expressed at birth and increases 1 week postpartum, PECAM-1 expression then decreases to a basal level after 2 weeks. Fluctuations in PECAM-1 expression increases with the maturation of the BBB [282-284]. Its main function in endothelial cells is to facilitate leukocyte, monocyte and neutrophil translocation and aid the adherence of dendritic cells [234, 285-288]. This function also facilitates disease progression and metastasis of acute lymphoblastic leukaemia into the CNS [289]. IFN-γ stimulates translocation through the relocalisation of PECAM-1 to the apical membrane [233]. It is highly regulated by phosphorylation of its intracellular docking sites, which regulates the interaction and recruitment of cytoplasmic components such SHP-2, β and γ-catenin [290, 291].

The relationship between PECAM-1 and neurological diseases is diverse. Infection, HIV, alzheimers disease, multiple sclerosis, ischemia and traumatic brain injury increases PECAM-1 expression [281, 292-296]. Multiple studies have demonstrated the increase in PECAM-1 expression to aid the transendothelial migration of monocytes during bacterial meningitis [297-299]. Conversely, treatment of the mouse hippocampus with LPS results in a decline in PECAM-1 expression after 12 h and expression was undetectable at 24 h [300]. The increase in PECAM-1 associated with neurological disease is also associated with PECAM-1 shedding, resulting in the ability to detect PECAM-1 in patient serum and enclosed in endothelial microparticles [301-303]. This serum PECAM-1 could potentially be used to also diagnose autism as children diagnosed have a decreased level of soluble PECAM-1 [304].

PECAM expression is also associated with addiction and associated decrease in BBB as seen with the increase in PECAM in those suffering from alcoholism and cocaine addiction [305-307]. As previously mentioned exercise aids in protecting BBB integrity, this is also the case with regards to PECAM-1. Exercise delays disease progression in multiple sclerosis patients, this associated with a decrease

in PECAM-1 expression. While smoking on the other hand results in an increase in PECAM-1 expression associated with BBB dysfunction [308-310].

PECAM-1 has been investigated as a target to prevent transendothelial migration. Antibodies specific to PECAM-1 bind to the extracellular region preventing the invasion of leukocytes, monocytes and neutrophils in disease such as Alzheimer's disease, multiple sclerosis and post-thrombotic brain oedema [297, 311-313]. Utilising PECAM-1 to deliver drugs through the BBB has also been investigated, by coating nano-particles with recombinant PECAM-1, facilitating drug internalisation [314].

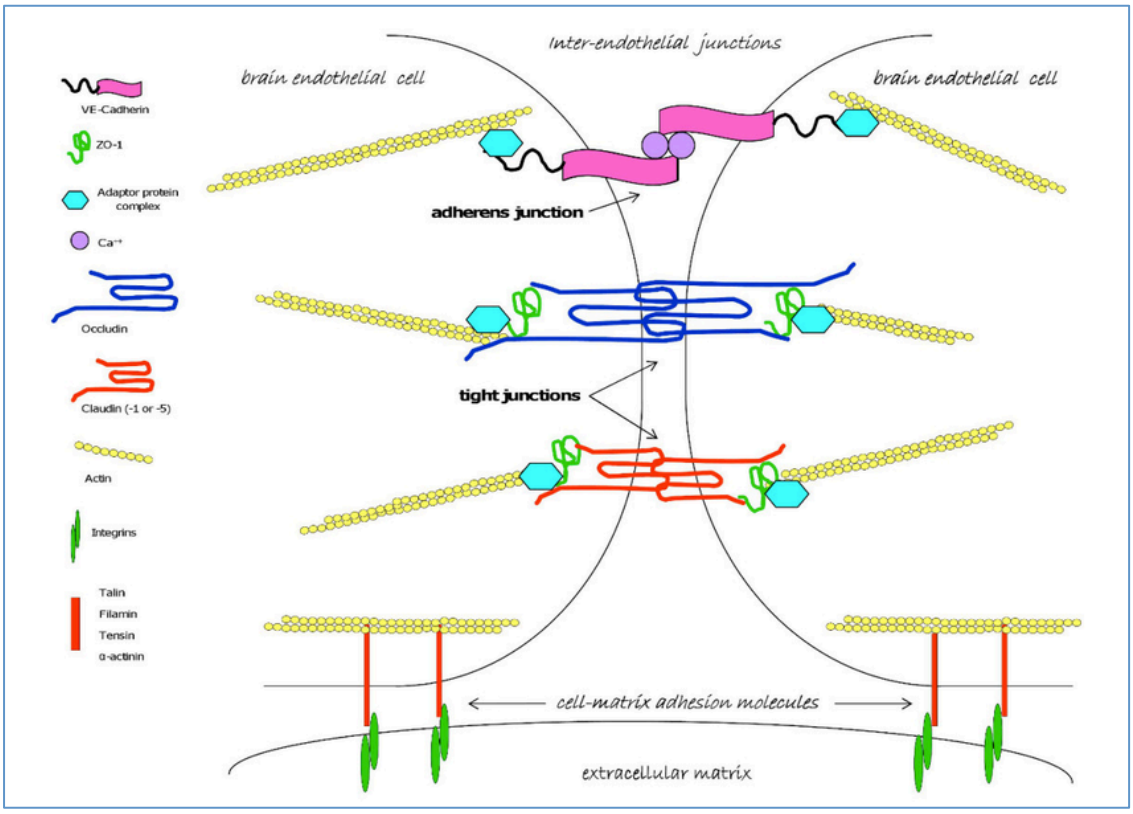

Figure 1.5: Endothelial junctions including tight and adheren junctions.

Brain endothelial paracellular permeability is regulated by inter-endothelial proteins including VE-cadherin (calcium dependent), Occludin and Claudins e.g. -1 and -5. Theses protein are linked to actin filaments to through accessory proteins such as ZO-1 and adaptor protein complexes. The endothelial cells are attached to the extracellular matrix through cell-matrix adhesion molecules. Talin, filamin, tensin, and α-actinin are linkers that connect endothelial cells actin filaments to integrins on the surface of the extracellular matrix.
Adapted from Polimeni *et al* 2014[315].

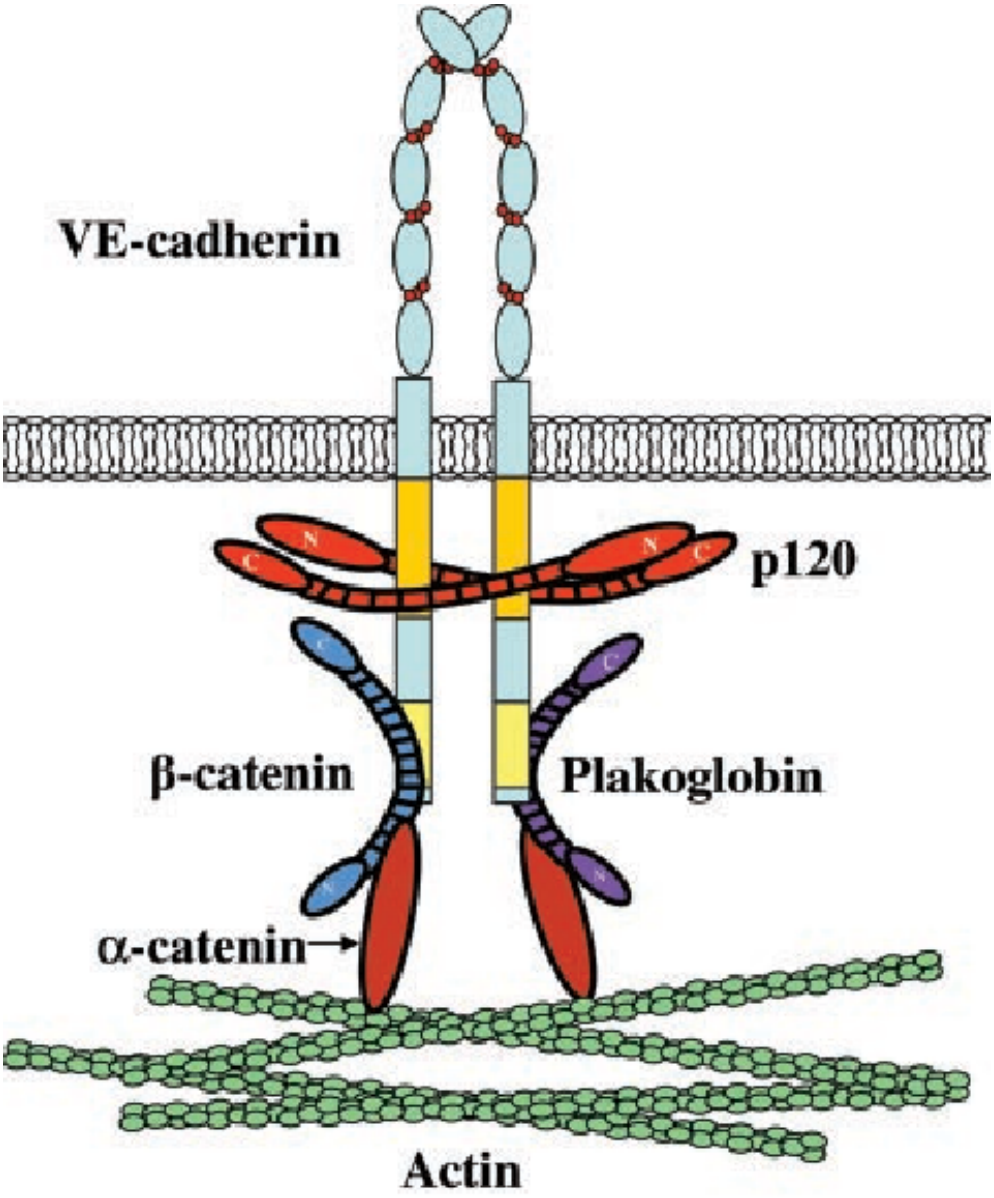

Figure 1.6: Structure of VE-cadherin.

VE-cadherin are composed of 3 domains; the extracellular, transmembrane and cytoplasmic domain. Accessory protein P120, plakoglobin and α- and β- catenin bind to the cytoplasmic domain of VE-cadherin to make up the VE-cadherin interactome. The accessory proteins contribute to the stability of VE-cadherin and connect VE-cadherin to the actin filaments.

Adapted from Vincent *et al* 2004. [316]

1.6 Lysosomal Degradation

The membrane of a cell is not static, in particular the area of the cell membrane in contact with adjacent cells. The cell membrane is frequently altering its surface proteins and rearranging in response to stimuli and cell signals. When cell contact is compromised junctional proteins are lost, this is most commonly due to endocytosis and degradation. VE-cadherin is the main research focus in this thesis it is therefore important to understand the mechanism and regulation of trafficking. Previous studies have shown how VE-cadherin accessory proteins are involved in endocytosis. Binding of P120 to VE-cadherin's cytoplasmic tail is essential for VE-cadherin membrane expression. Loss or disassociation of P120 results in the endocytosis of VE-cadherin [317-322]. P120 binds to a specific motif on VE-cadherin, which contains acidic residues. This motif also acts, as a site to facilitate endocytosis when P120 is not bound [323]. Additional lysine residues are targeted by the K5 ligase for ubiquitination during the process of endocytosis and degradation [324].

As previously mentioned (Section 1.5.3) the increase in Ser and Tyr phosphorylation of the VE-cadherin cytosolic tail induces internalisation of VE-cadherin [210, 221, 325-331]. Upon internalisation the VE-cadherin cytoplasmic tail is cleaved by calpain between the β-catenin and p120 binding sites. This cleavage favours the degradation of VE-cadherin over recycling [332]. Blocking of proteasomal degradation results in the build up of internalised VE-cadherin, β-catenin disassociates from the internalised VE-cadherin. Indicating that β-catenin disassociation may also be involved in the internalisation of VE-cadherin [324].

The Ras related in brain (Rab) GTPase play a key role in the regulation of cadherins (N, E and VE). Rab GTPases mediate the spatial and temporal recruitment of effector proteins to distinct cellular compartments through conformational changes in the protein. Active Rab binds to the endosome and promotes endocytic transport via the interaction of Rab effector proteins. Rab4-positive early endosomes are targeting them for recycling to the cell surface. Rab7 bound endosomes are transported to the lysosomes for degradation. Rab9 indirectly facilitates that transport of endosomes via the trans-golgi network. VE-

cadherin's transport to the cytosol is facilitated through Rab4 and Rab9. LPS inhibits Rab4 and activates Rab9 stimulating VE-cadherin degradation [333].

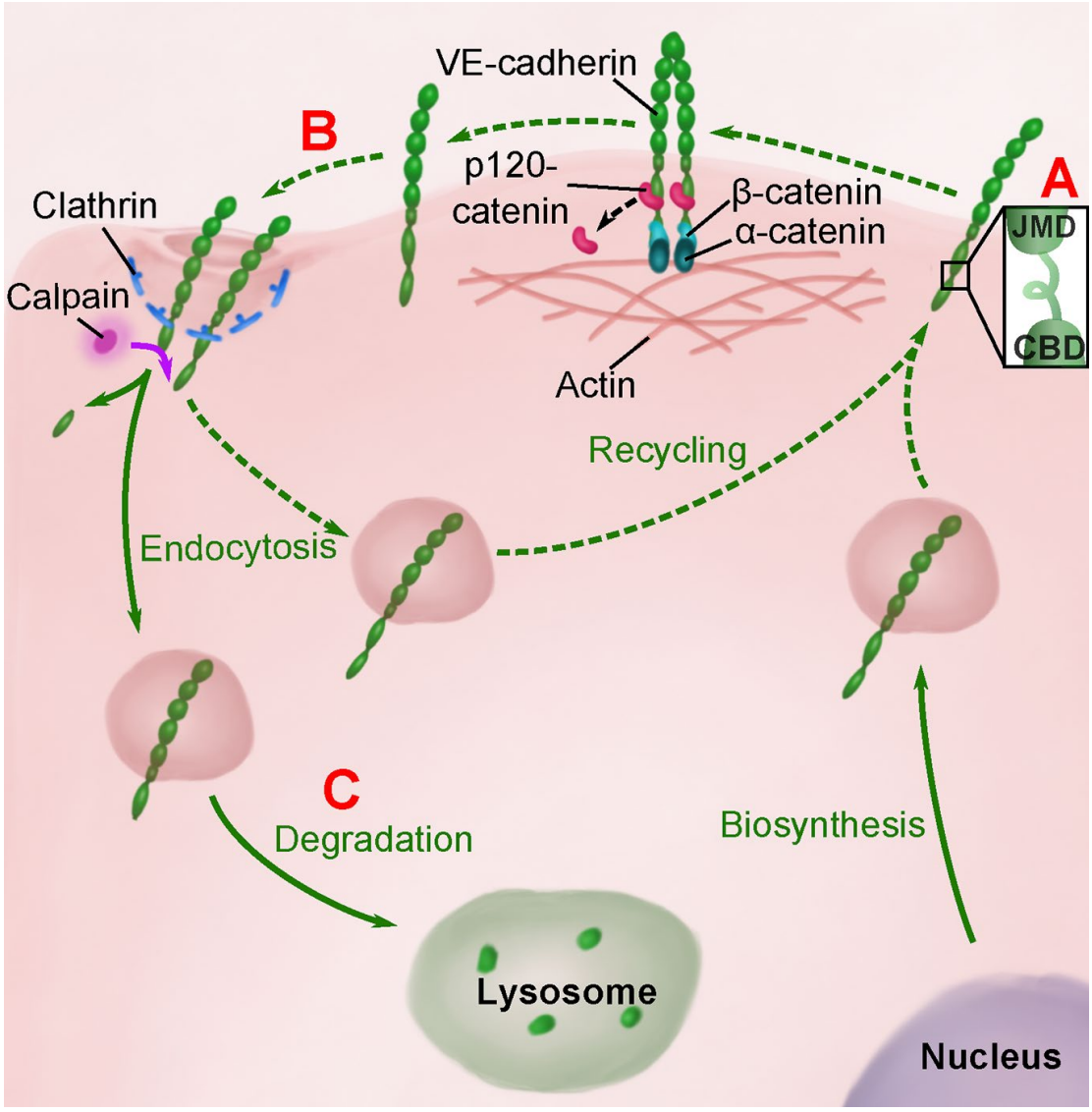

Figure 1.7: Schematic overview of VE-cadherin processing.

VE-cadherin is expressed at the cell surface. The highlighted section between the juxtamembrane domain (JMD) and the catenin-binding domain (CBD) is the target for calpain cleavage (A). Upon entry into the clathrin-coated pit calpain cleaves VE-cadherin (B). Cleaved VE-cadherin is shuttled to the lysosome for degradation (C). Figure adopted from Su *et al* 2017. [332]

1.7 Neuroinflammation

Inflammatory responses in the brain are initiated by; trauma, infection, ageing and toxins. The presence of inflammatory cells and cytokines compromises the integrity of the blood brain barrier. This was demonstrated in rat brains, where release of pro-inflammatory cytokines from chronically activated monocytes increased development of cerebral thrombosis and haemorrhage [334]. Common inflammatory cytokines released during neuroinflammation include interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). Their release is initially required for repair of damaged tissue, however prolonged or excessive exposure leads to neuronal damaged associated with stroke, multiple sclerosis, Alzheimer's disease and neuropsychological disorders [335-337]. Development and progression of such diseases have been linked to an alteration in overall phosphorylation status [338-341]. An accumulating body of evidence shows that infection or sepsis in the brain has both short and long term effect, and is linked to the development of depression, cognitive impairment and diseases such as multiple sclerosis and Parkinson's disease [54, 337, 342]

During endothelial injury there is an increased production of noxious substances and in turn platelet adhesion. Endothelial cells metabolise L-arginine to produce endothelial nitric oxide. NO can easily diffuse accros the BBB and causes vasodilation [343]. As mentioned in Section 1.5.4, JAMs are expressed on endothelial cells and are involved in the recruitment of leukocytes during inflammation. Redistribution of JAMs are induced by the chemokine C-C motif ligand 2 (CCL2) and is macropinocytosed when the inflammatory stimuli are not present. Allowing for the migration of the macrophages and monocytes through the blood brain barrier [272].

1.8 Protein Phosphatases

Almost one third of proteins in the cell are regulated by their phosphorylation status. Protein phosphatases control a multitude of functions, which stretch throughout the life span of the cell, including development, growth, cell division, proliferation, survival and death. Phosphorylation is a dynamic process facilitated by kinases and phosphatases, which add and remove phosphates respectively. Extensive research has been carried out on the role of protein kinases in cellular function compared to its phosphatase counterpart. In eukaryotes, protein phosphatases are classified according to their target protein. The amino acids that are most commonly phosphorylated are serine (Ser; 86.4%), threonine (Thr; 11.8%) and tyrosine (Tyr; 1.8%) [344]. This influences the phosphatase classification, which are protein Tyr phosphatases (PTPs) and the Ser/Thr phosphatases