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Tight Junctions of the Blood-Brain Barrier in Alzheimer’s Disease

A thesis submitted to the University of Dublin for the Degree of Doctor of Philosophy

By

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2015

Under the supervision of Professor Pete Humphries

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Declaration

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James Keaney
Summary

Located along the brain endothelium, the blood-brain barrier (BBB) is essential for regulating the exchange of ions and macromolecules between the blood circulation and neural tissue and restricting brain entry of potentially damaging blood-borne agents. To maintain central nervous system (CNS) homeostasis, brain endothelial cells tightly control entry and exit using specific transporter and receptor proteins for metabolite movement across cells (transcellular transport). In addition, brain endothelial cells are linked by tight junction (TJ) protein complexes that form a tight seal to limit movement between cells (paracellular transport). This has direct relevance to the pathogenesis of Alzheimer disease (AD) where failure to clear the pathogenic amyloid-β (Aβ) peptide across the BBB has been implicated as a key factor in disease progression. Impaired brain clearance of Aβ can also lead to the accumulation of Aβ around cerebral blood vessels, a condition known as cerebral amyloid angiopathy (CAA) that is found in over 80% of AD patients. In the context of Aβ transport at the BBB, the low-density lipoprotein receptor-related protein 1 (LRP1) mediates transcellular Aβ efflux from the brain, while the receptor for advanced glycation end products (RAGE) facilitates transcellular Aβ influx from the blood. While changes in transcellular endothelial receptors of Aβ are known to alter Aβ transport in AD patients and AD mouse models, alterations in TJ components of the paracellular pathway have also been documented in AD brains. Whether TJ dysregulation is specific to AD or a general feature of all neurodegenerative disorders is unknown. Furthermore, the consequences of changes in specific TJ components on Aβ transport and AD progression has not been explored.

In this study, levels of two transmembrane TJ proteins, claudin-5 and occludin, were shown to be reduced specifically in CAA-affected blood vessels of AD post-mortem brains with intact vascular claudin-5 and occludin immunostaining observed in brains of non-AD neurodegenerative patients and in non-CAA vessels of AD patients. Claudin-5 and occludin levels were also decreased in brain endothelial cell fractions isolated from aged APP-Swe mice, a transgenic murine model of AD. As in human AD brains, immunohistochemical analysis revealed that these TJ changes segregate with CAA. To further investigate the spatial relationship between Aβ accumulation and altered TJ protein levels, brain endothelial cells (bEnd.3) were treated in vitro with varying doses of recombinant Aβ1-40 monomer and dimer peptides. Time- and dose-dependent down-
regulation of claudin-5 and occludin and up-regulation of the intracellular TJ protein ZO-1 was observed in bEnd.3 cells following exposure to Aβ1-40 monomer and dimer. These findings indicated that reduced claudin-5 and occludin levels in AD/CAA patients could be directly related to the effects of Aβ accumulation on cerebral blood vessels and that Aβ itself may be able to regulate TJ protein levels. To assess how this claudin-5 and occludin down-regulation affects BBB permeability and Aβ movement, in vitro transwell permeability and RNA interference (RNAi) assays were used to measure Aβ transport across bEnd.3 cells in the absence of claudin-5 and occludin. Along with decreases in transendothelial electrical resistance (TEER) values, down-regulation of claudin-5 and occludin using small interfering RNAs (siRNAs) conferred a size-selective TJ that facilitated paracellular diffusion of soluble Aβ1-40 monomers (4.4 kDa in size) but not Aβ1-40 dimers (8.8 kDa). When applied to the in vivo BBB, RNAi-mediated co-suppression of claudin-5 and occludin in wild-type (WT) mice increased BBB permeability in a similar manner, to molecules 3-4 kDa but less than 10 kDa in size as measured by magnetic resonance imaging (MRI) and biotin-dextran permeability assays. Moreover, intravenous administration of soluble Aβ-containing hippocampal extracts, isolated post-mortem from an AD patient, in claudin-5- and occludin-deficient WT mice resulted in brain extravasation of soluble Aβ. In the context of AD where Aβ accumulates on the neural side of the BBB, the APP-Swe transgenic mouse model of AD was employed to measure the effects of claudin-5 and occludin co-suppression on Aβ movement across the BBB. Acute or long-term administration in young APP-Swe mice of claudin-5 and occludin siRNAs led to significantly increased plasma levels of Aβ1-40 compared to non-targeting (NT) siRNA-treated animals. Increased Aβ movement across the BBB along a concentration gradient coincided with decreased brain levels of Aβ1-40 and improvements in hippocampal-linked spatial memory. Importantly, siRNA treatment did not affect levels of the major transcellular Aβ receptors in the brains or livers of APP-Swe mice and no histopathological evidence of peripheral organ toxicity was observed.

The results obtained here from human AD brain samples, transgenic APP-Swe mice and in vitro and in vivo BBB permeability assays indicate that depletion of claudin-5 and occludin can size-selectively increase BBB permeability and enhance the passive paracellular clearance of Aβ1-40 across the BBB. In this respect, changes in TJ protein levels in brain endothelial cells of AD/CAA patients may therefore represent a cellular response to clear rising Aβ levels. In conclusion, these series of observations highlight a
novel method of Aβ movement from the brain to the blood and suggests that claudin-5 and occludin could represent novel therapeutic targets to modulate AD progression.
Acknowledgements

I have been lucky enough to receive the help, support and guidance of many people during the course of my PhD work. Firstly, I would like to thank my supervisor Professor Pete Humphries for giving me the opportunity to work on an exciting project in his laboratory. He has been a constant source of assistance and guidance, challenging me to explore the wider implications of this research while also allowing me to pursue the work in new directions. To Dr Matthew Campbell, whose guidance and direction has been invaluable during this project, spearheading the area of tight junction research in the laboratory and for having the time and patience to teach me complex techniques, answer my rambling questions and help me maintain focus to complete this work. I am deeply indebted to him for his support and friendship.

To Dr Marian Humphries for her help with genotyping, ordering and setting up my project, to Dr Anna-Sophia Kiang and Dr Lawrence Tam for all their assistance and advice before and during my research, to Dr Natalie Hudson for isolating mouse brain capillaries. To our collaborators, Professor Dominic Walsh and Dr Tiernan O’Reilly in Harvard for their insight into the fields of Alzheimer’s disease and amyloid biochemistry and supplying reagents, Professor Michael Farrell in Beaumont Hospital for supplying and helping to characterize human brain tissue and Professor Sean Callanan in UCD for assessing organ pathology in APP mice. I would also like to thank the Irish Research Council and Science Foundation Ireland for their financial support over the last four years.

To my fellow PhD students during my time in the lab, Dr Finn Hanrahan, Ema Ozaki, Dr Anh Nguyen, Dr Gareth O’Dwyer, Dr Matthew Carrigan – I am very grateful for all your help, discussions and friendship. To those in the animal unit: Caroline, Charlie, Dave, Rebecca, to Dr Christoph Blau and Rustam Rakhmatullin for their help with the MRI work, to Dave, Paul, Rachael and Brenda in the prep room. To the members of the Farrar and Campbell labs, and the other friends that I have made among students and staff in the Smurfit Institute over my six years here as an undergrad and postgrad - thank you.

Closer to home - Sean, Barry, Ronan, Niall, Fionn, Gareth, Alan, Darragh, Sarah – I have been lucky to count you as friends since childhood, you helped to relieve the stress. To Fiona, Barry and Olivia, to Robert and Sharon, to my grandmother Maureen, to Manon,
thank you all for being there. I love you very much. Finally, to my parents Miriam and Martin, for their love and support, for listening and for offering advice, for picking me up and for dropping me off. ‘Thank you’ does not begin to repay. This work is dedicated to you.
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Figure A8: Immunostaining of Aβ and microvessels in brain cortical regions of siRNA-treated WT mice following systemic injection of AD-TBS extracts

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Table A1: Sequences for siRNAs used during the study

Table A2: RT-PCR primer sequences for tight junction mRNAs

Table A3: RT-PCR primer sequences for capillary fractionation cell markers
Abbreviations

Aβ – Amyloid-β
AD – Alzheimer’s disease
APOE – Apolipoprotein E
APP – Amyloid precursor protein
BBB – Blood-brain barrier
bEnd.3 – Mouse brain endothelial cells
CAA – Cerebral amyloid angiopathy
CLDN5 – Claudin-5
CNS – Central nervous system
C/O – Claudin-5 and occludin
kDa – kiloDalton
LRP1 – Low density lipoprotein receptor-related protein 1
MRI – Magnetic resonance imaging
MW – Molecular weight
NT – Non-targeting
NVU – Neurovascular unit
Occ – Occludin
RAGE – Receptor for advanced glycation endproducts
RNAi – RNA interference
siRNA – small interfering RNA
TJs – Tight junctions
WT – Wild-type
Chapter 1: Introduction
1.1. The Blood Brain Barrier (BBB) and Neurovascular Unit (NVU)

1.1.1. Barriers of the central nervous system (CNS)

Neurons are the principal cellular unit of the central nervous system (CNS), communicating and transmitting information along neural networks through a combination of chemical and electrical signals. The specialized site where two neurons connect and transfer these signals is known as a synapse and strict regulation of the local microenvironment (for example ionic concentration and oxygen levels) around synapses and axon projections is crucial for proper neuronal function and signaling. As the organ responsible for cognitive functions and governing the physiological functions of the body, the risks posed by infection and inflammation in the brain are elevated with respect to their occurrence in peripheral organs. Since relatively low levels of neurogenesis (the generation of mature neurons from stem cell precursors or progenitor cells) exist in most regions of the adult brain, neuronal damage and cell death following brain entry of neurotoxic agents from the circulation would be potentially devastating (Abbott et al., 2010). To this end, three main cellular interfaces exist in the brain to form barrier sites between the blood and neural tissue: the blood-cerebrospinal fluid barrier (BCSFB), the arachnoid barrier and the blood-brain barrier (BBB). The BCSFB is formed by epithelial cells of the choroid plexuses in the lateral, third and fourth ventricles (Abbott et al., 2006; Figure 1.1). The choroid plexus epithelium secretes cerebrospinal fluid (CSF) which bathes the brain and spinal cord and functions as a shock absorber to protect brain tissue from injury as well as regulating solute levels and cerebral blood flow. A second barrier is conferred by the arachnoid epithelium, an avascular membrane located beneath the dura mater of the meninges and which envelops the entire CNS. This forms a seal between the CSF flowing along the subarachnoid space and the fluids of the rest of the body (Fig. 1.1). While villi of the arachnoid epithelium can project into venous sinuses located between layers of the meninges, this movement is unidirectional with CSF moving out of the brain into the blood only (Abbott et al., 2010). Located at the level of the brain endothelium lining cerebral capillaries, a third major interface is the BBB which separates the blood from the brain extracellular fluid (Fig. 1.1). With a surface area of approximately 12-18 m² in an average adult human brain, the cerebral microvessels and in turn the BBB form by far the largest surface area in the brain for molecular exchange with the blood (Abbott et al., 2010).
The blood-brain barrier (BBB) (and inner blood-retina barrier (iBRB) of the retina) therefore represents the main biological interface between the blood and the neural tissue of the brain and retina and as such play vital roles in maintaining CNS homeostasis. As well as regulating the exchange of ions and macromolecules between the blood and CNS microenvironment, these highly specialized endothelial cell structures protect the delicate neural tissue by restricting the entry of potentially damaging blood-borne agents such as neurotoxic chemicals, antibodies, pathogens, immune cells and anaphylatoxins. Among the early studies of the BBB, the landmark experiments of Paul Ehrlich and his student Edwin Goldmann provided the first evidence of a barrier between the CNS and the circulating bloodstream. When Ehrlich injected water-soluble dyes into the bloodstream he noted that all organs were stained except the brain and spinal cord. This was followed by Goldmann’s discovery that injection of trypan blue into the CSF stained cells of the CNS but did not penetrate the peripheral circulation. In the reverse experiment, intravenous injection of trypan blue resulted in colouration of the blood but not the brain or CSF (Figure 1.2A; Hawkins and Davis, 2005).

The CNS accounts for approximately 20% of the body’s oxygen consumption with the retina having the highest oxygen consumption per weight of any tissue (Zlokovic, 2008; Campbell et al. 2010). Since the BBB and iBRB are the primary structures that confer neuronal protection and regulate the brain microenvironment, the endothelial cells comprising the CNS vasculature bear properties distinct from endothelial cells in other tissues of the body. While other tissues and indeed the choroid plexus and arachnoid epithelial barriers of the brain possess tight junction (TJ) complexes to limit paracellular (intercellular) permeability, CNS endothelial cells are characterized by high electrical resistance TJs that form a tight seal limiting paracellular transport between adjacent endothelial cells of the neural vasculature. In addition, CNS endothelial cells lack fenestrae (small pores that allow rapid passage of molecules) and contain few transcytotic vesicles thereby restricting the transcellular flow of molecules from the blood to the brain (Saunders et al. 2012). Instead CNS endothelial cells express a variety of transporters to control both the selective transport of nutrients into the brain and the efflux of metabolites and toxins from the CNS (Daneman et al., 2010a). The factors responsible for the specification of CNS endothelial cells and the development and maintenance of the BBB have been the subject of intense research. Following the discovery of the BBB, a question arose of whether the properties of cerebral endothelial cells were intrinsic to this cell type
or whether signals from the surrounding brain microenvironment induced BBB formation. In a series of elegant transplantation experiments by Stewart and Wiley (1981), immature avascular brain tissue from embryonic quails was transplanted into the coelom (a fluid-filled cavity in the mesoderm) of chick embryos while conversely dorsal mesoderm from embryonic quails was grafted into the embryonic chick brain. While both tissue grafts became vascularized, only the brain tissue transplanted into the coelom developed endothelial cells with TJs and few transcytotic vesicles characteristic of the non-leaky BBB. Thus it became clear that interactions between the developing vascular tissue and surrounding neuronal microenvironment are crucial to the development of the BBB. This has given rise to the concept of a “neurovascular unit” (NVU) – the milieu of neurons, astrocytes, pericytes, microglia and other components of the brain parenchyma that communicate with endothelial cells to induce BBB formation and ensure proper cerebrovascular function (Fig. 1.2B).

1.1.2. Astrocytes

Astrocytes are glial cells that provide support and protection for neurons by controlling the ionic balance of the neural microenvironment (by removing excess ions, in particular potassium ions), recycling and clearing neurotransmitters, and regulating immune reactions (Obermeier et al., 2013). As well as their role in modulating synaptic transmission, astrocytes are known to interact with endothelial cells through their endfeet projections that encircle the abluminal side of cerebral capillaries and regulate the BBB (Fig. 1.2B). In the adult brain, such interactions are important in regulating brain water and electrolyte metabolism and synchronizing metabolite levels with cerebral blood flow and vasodilation (Zlokovic, 2008). For example the most abundant water channel protein, aquaporin 4 (AQP4), is predominantly expressed in astrocytic endfeet surrounding CNS vessels (Tait et al., 2008). The role of astrocytes in BBB development in the immature brain is more controversial. Janzer and Raff (1987) injected neonatal rat astrocytes into the anterior chamber of the eye and found that only vessels formed in the presence of astrocytes excluded Evans blue dye when compared to control injections of fibroblasts. Based on these results, the authors proposed that astrocytes were necessary for the formation of impermeable TJs but in a follow-up study by a separate group, Holash et al. (1993) reported no ultrastructural changes in endothelial cells following astrocyte grafts. In vitro co-cultures of endothelial cells with astrocytes or astrocyte-conditioned media have been shown to induce more complex TJs, elevated expression of transporters,
enhanced activity of metabolic enzymes and increased transendothelial resistance though these studies are limited by the use of adult endothelial cells (Saunders et al., 2012). Furthermore, the finding that astrocytes are not present in the developing brain during the time of initial vascularization lends support to a role for astrocytes in TJ maintenance and not formation (Daneman et al., 2010b). More recently however, production and release of retinoic acid (RA) by astrocyte precursors (radial glial cells) has been shown to act through the RA-receptor β on developing brain endothelial cells to induce BBB properties (Mizee et al., 2013). While transplantation and histological studies dominated early BBB research, our knowledge of how cells of the NVU interact at the molecular level to signal BBB development and maintenance is only now being advanced. For example a series of recent in vitro and in vivo molecular studies have revealed several effector molecules released by astrocytes that function to enhance and maintain barrier tightness, primarily members of the Hedgehog (Hh) family, the renin-angiotensin hormone system, and the cholesterol and phospholipid transporter molecule apolipoprotein E (APOE) as outlined further in sections 1.1.4 and 1.2.5 (Obermeier et al., 2013; Sohet and Daneman, 2013).

1.1.3. Pericytes
Attention in the BBB field is switching to the role of pericytes - perivascular cells of mesodermal origin whose elongated processes ensheathe vessel walls of capillaries, precapillary arterioles and postcapillary venules (Fig. 1.2B). The CNS vasculature has significantly higher pericyte coverage compared to vessels in peripheral tissues and it has long been known that pericytes have an important role in regulating capillary diameter, blood flow and extracellular matrix protein secretion and levels (Winker et al., 2011). For example a recent study by Hall and colleagues (2014) has highlighted the essential role of capillary pericytes in regulating cerebral blood flow – the neurotransmitter glutamate promotes the release of messengers including prostaglandin E₂ and nitric oxide that help dilate capillaries by actively relaxing pericytes. The importance of pericytes in BBB formation during embryonic development was demonstrated by Daneman et al. (2010b) using pericyte-deficient or platelet-derived growth factor receptor β (Pdgfrβ) knockout mice. It was found that pericyte coverage of the CNS capillaries regulates TJ formation, transendothelial vesicle trafficking and vascular permeability by inhibiting the expression of molecules that promote immune cell infiltration and increased vascular permeability. Intriguingly, pericyte effects on BBB formation preceded astrocyte differentiation by a week. Pericytes also regulate BBB integrity during adulthood since Pdgfrβ knockout
mice exhibit age-dependent BBB dysfunction as a result of reduced TJ protein expression while young adult mice with hypomorphic alleles of \(Pdgf\beta\) display defects in BBB integrity as a result of increased rates of endothelial transepithelization (Bell et al., 2010; Armulik et al., 2010). In another recent study, the contribution of pericytes to disease phenotypes such as neurodegeneration was shown with the observation that Alzheimer disease (AD) pathogenesis is accelerated following deficiency or degeneration of pericytes in mice (Sagare et al., 2013a). This again underscores the importance of pericytes in regulating neurovascular functions and hence proper neuronal behavior.

1.1.4. Development and maintenance of the BBB

As the cellular contacts necessary for BBB specification are elucidated, understanding the precise signaling pathways necessary for BBB formation and maintenance will be important. Research to date suggests that three main phases exist in the development of the BBB and that each phase relies on an intricate network of cross-talk involving virtually all cells of the NVU. This is achieved via a range of secreted morphogens and cell receptors that ultimately give rise to the unique barrier properties of the BBB.

Phase 1: Angiogenesis

In the embryonic vertebrate CNS, BBB development begins with the invasion of pre-existing blood vessels into the avascular neuroectoderm cell layer. These vascular progenitor cells are responsible for vascularization of the CNS through a process known as angiogenesis or the growth of new vessels from pre-existing ones. In the initial phase of neural angiogenesis, neural progenitor cells (also known as 'neuroblasts') guide the sprouting endothelial cells by secreting various factors that bind to endothelial receptors (Fig. 1.3A; Engelhardt and Liebner, 2014). The most well-studied angiogenic factor in CNS vascularization is vascular endothelial growth factor (VEGF) which binds to the VEGFR1 (also known as Flt-1) and VEGFR2 (Flk-1 encoded by \(Kdr\) gene) tyrosine kinase receptors on endothelial cells (Engelhardt and Liebner, 2014). Release of VEGF by neuroblasts in the subventricular neuroectoderm helps to establish a VEGF concentration gradient, thereby inducing endothelial cells at the tip of vascular sprouts to form filopodial extensions (Gerhardt et al., 2003). The importance of the VEGF pathway in embryonic angiogenesis is underscored by blood vessel malformations and early death of embryos in \(VeGF\) knockout and VEGFR2 (\(Kdr\)) knockout mice (Shalaby et al., 1995, Carmeliet et al., 1996). Separately, a series of papers linking the Wnt/\(\beta\)-catenin pathway...
to early BBB development has highlighted a close association between angiogenesis and barrierogenesis (Liebner et al., 2008, Stenman et al., 2008, Daneman et al., 2009). These studies found that the Wnt/β-catenin pathway is activated specifically in CNS endothelia but not non-CNS endothelia. In this pathway, Wnt ligands secreted by neuroblasts in the ventricular zone of the neural tube bind to Frizzled (FZD) receptors on endothelial cells and inhibit degradation of β-catenin. In response β-catenin translocates to the nucleus and induces transcription of several genes found upregulated in CNS endothelia only (Obermeier et al., 2013). Furthermore, interference with the Wnt/β-catenin pathway results in reduction of vessel number, loss of capillary beds and formation of hemorrhagic vascular malformations. Since Wnt/β-catenin signaling was also found to regulate expression of BBB-specific genes including the glucose transporter GLUT-1, this suggests that signals required for CNS angiogenesis can also induce barrier properties in CNS endothelia (Daneman et al., 2009). Tam et al. (2012) have since identified two downstream targets of Wnt/β-catenin signaling, the death receptors DR6 and TROY, that are enriched in CNS endothelial cells and regulate CNS angiogenesis, endothelium differentiation and BBB formation by interacting with the VEGF pathway. Two other regulators of brain-specific angiogenesis and barrierogenesis that have recently been identified are GPR124 and Nogo-A. Deletion of the endothelial-specific G protein-coupled receptor GPR124 leads to defective endothelial cell sprouting and migration as well as hemorrhaging in the developing forebrain and spinal cord (Cullen et al., 2011). GPR124 also regulates BBB integrity as its deletion disrupts GLUT-1 expression at the BBB (Cullen et al., 2011). In contrast, the axonal growth inhibitor Nogo-A found on the surface of neurons and oligodendrocytes is a negative regulator of CNS angiogenesis. Genetic ablation of Nogo-A was found to increase blood vessel migration, sprouting and density (Walchli et al., 2013). Further work will be needed to understand if and how signaling pathways between neural and endothelial progenitors interact and regulate each other in the embryonic brain.

Phase 2: Cross-talk with pericytes and astrocytes to differentiate and seal the barrier

In the second stage of BBB development, recruitment of pericytes and astrocytes by migrating CNS endothelial cells is crucial in promoting barrier properties in these immature blood vessels (Fig. 1.3B). The release of PDGFβ by endothelial cells of sprouting vessels promotes the recruitment and proliferation of PDGFβ receptor-positive
pericytes (Engelhardt and Liebner. 2014). This pericyte coverage of brain microvessels is vital to barrier differentiation as complete loss of pericytes in \( \text{Pdgf}^{\beta} \) or \( \text{Pdgfr}^{\beta} \) knockout mice results in CNS microhemorrhages, dysfunctional TJs and increased vascular permeability (Lindahl et al., 1997, Daneman et al., 2010b). Once pericytes are directed to nascent vessels, cross-talk between pericytes and neighboring endothelial cells is mediated by transforming growth factor-\( \beta \) (TGF\( \beta \)) and its receptor TGFR\( \beta \). When Li and colleagues (2011) generated a CNS endothelial-specific conditional mutant of \( \text{Smad}4 \), a downstream component of the TGF\( \beta \) signaling pathway, pericyte adhesion to vessels was disrupted and hemorrhages and BBB breakdown also observed. Following pericyte recruitment, astrocytes are another major NVU component that helps establish BBB characteristics. Release of the Sonic Hedgehog (SHH) ligand by astrocytes initiates activation of the Hedgehog pathway in endothelial cells via binding to its Patched-1 (PTC-1) receptor (Alvarez et al., 2011). In addition, activation of Src-suppressed C-kinase substrate (SSCKS) in astrocytes can stimulate production of angiopoietin-1 (ANG-1) which binds to TIE-2 tyrosine kinase receptors on cerebral endothelial cells (Lee et al., 2003). In both instances, pathway activation results in upregulation and redistribution of TJ proteins, decreased levels of transcytosis and decreased expression of leukocyte adhesion molecules (Obermeier et al., 2013). Thus pericyte and astrocyte recruitment to nascent vessels helps to induce properties vital to BBB integrity.

Phase 3: Barrier maintenance

While there has been a concerted effort in recent years to uncover the molecular and cellular mechanisms governing BBB formation during embryonic development, less is known about the NVU interactions that maintain BBB integrity and homeostasis in the adult. Of the first molecular cues that induce early CNS angiogenesis and barrierogenesis, it is known that expression levels of VEGF receptors (in mature CNS endothelium) and their ligands (in differentiated CNS parenchymal cells) are decreased in adult mice (Engelhardt and Liebner, 2014). However, the Wnt/\( \beta \)-catenin pathway may still have a role in differentiated CNS vessels since inactivation of the Wnt receptor FZD4 leads to disrupted BBB TJs and increased permeability in the retina and cerebellum of adult mice (Wang et al., 2012). More recently, Mfsd2a has been identified as a critical component of BBB formation and integrity. Mfsd2a is a brain endothelium-enriched receptor for the omega-3 fatty acid, docosahexaenoic acid (DHA), which is essential for normal brain growth and function (Nguyen et al., 2014). Interestingly, \( Mfsd2a \) knockout mice have...
reduced brain levels of DHA but also display a leaky BBB in embryonic and adult stages as a result of increased endothelial vesicular transcytosis (Nguyen et al., 2014, Ben-Zvi et al., 2014). Furthermore, pericytes were found to regulate endothelial levels of Mfsd2a and thus Mfsd2a is important for BBB function through an as yet unknown mechanism (Ben-Zvi et al., 2014). Apart from endothelial cells themselves, astrocytes, which appear at the BBB postnatally, are believed to be the main cellular support to the adult BBB. Along with the release of the Hedgehog pathway ligand SHH, astrocytes have been shown to regulate TJ formation via the renin-angiotensin hormone system (Fig. 1.3C). Angiotensinogen is expressed by astrocytes and is converted by renin to the inactive angiotensin I (ANG-I) and eventually to the effector molecule angiotensin II (ANG-II) (Wosik et al., 2007). ANG-II release from astrocytes leads to engagement and activation of AT1 receptors on endothelial cells and eventual post-translational modification and membrane localization of the TJ protein occludin (Wosik et al., 2007). The release of apolipoprotein E (APOE) from astrocytes has also been shown to regulate endothelial TJs by signaling through low-density lipoprotein receptor-related protein 1 (LRP1) on both pericytes and endothelial cells of cerebral microvessels (Fig. 1.3C; Bell et al., 2012). An often overlooked component of the NVU is the basement membrane (BM) – the extracellular matrix of structural proteins secreted by astrocytes, pericytes and endothelial cells. The BM helps to regulate crosstalk between NVU components and to this end, Yao and colleagues (2014) have recently shown that a brain-specific BM component, astrocytic-derived laminin, helps to regulate pericyte differentiation via binding to integrin α2 receptor on pericytes. Disruption of this BM protein in the brain converted pericytes from a resting to a contractile phenotype which in turn decreased AQP4 levels in astrocytic endfeet and decreased endothelial TJ expression, highlighting a complex interdependency among components of the NVU (Yao et al., 2014). Furthermore, Armulik and colleagues (2010) found that pericytes not only regulate mature BBB integrity but also function to guide astrocytic foot processes to cerebral vessel walls and mediate the polarization of astrocytic endfeet. These combined findings indicate that communication between cellular and molecular members of the NVU, first established during early CNS angiogenesis and barriergenesis, must remain in place to ensure robust BBB integrity in adulthood. Moreover, it suggests that if this fine balance is disrupted, it may directly or indirectly contribute to the pathogenesis of a variety of neurovascular and neurodegenerative diseases.
1.1.5. Macromolecule transport across the BBB

As well as sealing of the interendothelial clefts via TJ complexes, BBB formation during embryonic and postnatal brain development also involves establishing polarized expression of specific transporter proteins in the luminal and abluminal membranes of CNS endothelial cells. Because of the low rates of paracellular flux and transcellular trafficking, successful passive diffusion across the BBB is mostly limited to lipid soluble-molecules approximately < 180 Da in size and with fewer than 10 hydrogen bonds (Fig. 1.4B; Pardridge, 2009). Thus distinct BBB transcellular transport systems exist to tightly regulate the CNS entry and exit of hydrophilic molecules (Fig. 1.4). Carrier-mediated transport is the major route of transport for essential nutrients such as glucose and amino acids (Fig. 1.4C; Abbott et al., 2010). The apical-basal polarity of transporter localization and the direction of transport is determined by the particular substance’s concentration gradient. For example, the glucose transporter GLUT-1 is expressed exclusively in CNS endothelial cells and is asymmetrically distributed at the BBB (Devraj et al., 2011). GLUT-1 density is higher at the abluminal membrane to prevent glucose levels in brain ISF exceeding those of the blood. The importance of GLUT-1 at the BBB is also highlighted in human GLUT-1 deficiency syndromes where affected individuals suffer from infantile seizures and developmental delay (Zlokovic, 2008). The major amino acid transporters are also facilitative carriers and include the L1 system (for neutral essential amino acids like leucine, tyrosine and tryptophan), the y^+ system (for cationic amino acids like lysine) and the EAAT system (for excitatory acidic amino acids like glutamate) (Zlokovic, 2008). However for peptides and proteins, transcytosis involving vesicle formation is necessary for these large macromolecules to penetrate or depart the CNS.

For example, large molecular weight proteins such as transferrin, low-density lipoproteins and immunoglobulin G cross the BBB via receptor-mediated transcytosis (Fig. 1.4D; Abbott et al., 2006). In this process, both ligand and receptor are internalized into an endosome vesicle that is shuttled across the cell (avoiding the lysosome pathway) and exocytosed, thus separating the ligand and receptor again. Some plasma proteins such as albumin can also be cationized and this positive charge allows binding to sites along the negatively charged phospholipid membrane. This entry mechanism is known as adsorptive transcytosis (Fig. 1.4E; Abbott et al., 2010). In relation to Alzheimer’s disease, the major pathogenic component is a small 40-42 amino acid protein called amyloid-β (Aβ) that can be cleared from the brain interstitial fluid (ISF) across the BBB. As outlined further in section 1.5, research to date has focused on its transcellular movement across...
the BBB and this work has identified the receptors LRP1 (low-density lipoprotein receptor-related protein 1) and RAGE (receptor for advanced glycation end productions) as the major routes of receptor-mediated Aβ transcytosis across the CNS endothelium (Bell and Zlokovic, 2009). However, another route of entry and exit across the CNS is found between the endothelial cells – the paracellular pathway (Fig. 1.4A). This interendothelial space is sealed during BBB development by the formation of TJ complexes. However, there is now an increased awareness that dynamic changes in the distribution and levels of the protein components of TJs in health and disease can have a major impact on BBB permeability.

1.2. Tight Junctions
Positioned at the apical (luminal) side of the plasma membrane of adjacent epithelial and endothelial cells, tight junctions (TJs, also known as “occluding junction and zonula occludens”) are elaborate structures that function as sites for vesicle targeting, defining cell polarity, and regulating proliferation and transcription signals (Hawkins and Davis, 2005; Hanrahan et al., 2010). Most importantly, with regard to the BBB and iBRB of the CNS endothelium, TJs are responsible for limiting the paracellular flow of polar solutes and macromolecules between the blood plasma and brain extracellular fluid, thus maintaining the correct microenvironment for proper neuronal function. The interendothelial space also includes adherens junctions composed of cadherin proteins such as VE-cadherin that mediate adhesion of endothelial cells to each other, contact inhibition during angiogenesis, and regulate responses to cellular stress by interacting with the transcription factor β-catenin and the actin cytoskeleton (Hawkins and Davis, 2005). Disruption of adherens junctions can also lead to BBB breakdown (Abbruscato and Davis, 1999). However, unlike adherens junctions which are ubiquitous to endothelial cells, high electrical resistance continuous TJs are specific to CNS endothelial cells. While transendothelial electrical resistance (TEER) values for peripheral capillaries usually range from 2-20 Ω.cm², TEER values for brain endothelia can be as high as 1800 Ω.cm² (Abbott et al., 2006). TJs of the BBB are composed of at least three main types of transmembrane proteins – occludin, claudins and members of the junctional adhesion molecule (JAM) family of proteins – as well as several intracellular accessory proteins namely the zonula occludens (ZO) family members (Figure 1.5A; Anderson and van Itallie, 2008). Other important TJ components that have recently been revealed include
tricellulin and lipolysis-stimulated lipoprotein receptor (LSR) (Ikenouchi et al., 2005; Masuda et al., 2011; Daneman et al., 2010a).

1.2.1. Occludin

Occludin was the first transmembrane component of brain endothelial TJs to be discovered (Hirase et al., 1997). This 60- to 65-kDa protein consists of a conserved four transmembrane MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain, a long carboxyl-terminal (C terminus) cytoplasmic domain, a short amino-terminal (N terminus) cytoplasmic domain and two extracellular loops (ECLs) spanning the interendothelial cleft (Hawkins and Davis, 2005). Expression of C-terminal truncated versions of occludin disrupts its interaction with the intracellular scaffold protein ZO-1 and causes increased paracellular flux of low molecular weight molecules while deletion of the N terminus and extracellular loops reduces TEER and increases paracellular permeability (Balda et al., 1996; Bamforth et al., 1999). Though several studies have shown that occludin is both highly expressed at the BBB and not by non-barrier-forming endothelia, the precise function of occludin remains controversial (Hirase et al., 1997; Daneman et al., 2010a). Surprisingly, occludin knockout mice were found to develop physiologically and structurally normal TJ strands and maintain homeostatic transepithelial electrical resistance (Saitou et al., 2000). It is likely that normal or up-regulated expression of other junctional proteins may compensate for occludin loss but unfortunately brain endothelial transcriptome analysis has not been performed in these mice. Recent work by Raleigh and colleagues (2011) has proposed that occludin’s primary function is in regulating junctional dynamics as occludin phosphorylation remodels TJ protein interactions, resulting in changes in TEER values. However other phenotypes reported in occludin knockout mice including brain calcifications (thus a possible role in determining calcium flux across the BBB), testicular atrophy and postnatal growth retardation suggest roles for occludin beyond its junctional function (Zlokovic, 2008). Nevertheless, occludin downregulation has been shown to increase endothelial paracellular permeability in vitro (Tai et al., 2010). Furthermore, decreased occludin expression and increased BBB permeability has been observed in a number of animal models and CNS disease states with associated BBB dysfunction such as multiple sclerosis, stroke and Alzheimer’s disease (Wosik et al., 2007, Argaw et al., 2009, Bolton et al., 1998, Brown and Davis, 2002, Carrano et al., 2011).
1.2.2. Claudins

The claudin family of proteins have a similar membrane topology to occludin but lack sequence homology (Furuse et al., 1998). Claudins are 20- to 24-kDa proteins that are predicted to have four transmembrane domains, one intracellular loop and two ECLs (Fig. 1.5B). Claudins support TJ integrity through homophilic (between two molecules of the same claudin member) and heterophilic (between different claudin members) interactions with claudins of neighboring endothelial or epithelial cells (Piontek et al., 2008). Heterodimerization is thought to be mediated through their second ECL while homodimerization is conferred by a conserved amino acid motif (W-GLW-C-C) located in the first ECL (Fig. 1.5B; Piehl et al., 2010, Wen et al., 2004). The two cysteine residues of this motif likely form disulfide bonds with each other and other claudin molecules that are essential for paracellular sealing (Wen et al., 2004). Claudins also interact with other TJ components like ZO scaffold proteins within an individual cell through their C-terminus PDZ domain (Van Italie and Anderson, 2004). The extent of paracellular tightness in a given tissue is conferred by the particular combination of claudins. Of the 27 claudins identified to date in mammals, some members such as claudin-1 are widely expressed while other claudins display tissue-specific expression (Deli, 2009). Knockout studies have confirmed the role of individual claudins in regulating electrical conductance and tissue permeability. For example, claudin-14 regulates cation permeability in TJ strands of outer hair cells of the cochlea (ear) and deletion or mutation of claudin-14 in mice and humans results in deafness (Ben-Yosef et al., 2003). Claudin-11-null mice display defects in blood-testis barrier permeability, resulting in disrupted spermatogenesis and ultimately sterility in males (Gow et al., 1999). Knockout of claudin-1 disrupts the epidermal barrier causing excessive loss of water across the skin and death around postnatal day 1 (Furuse et al., 2002). Thus claudins are essential TJ components in limiting paracellular permeability to molecules as small as water. With regards the BBB, one member of the claudin family, claudin-5, is highly enriched in the endothelial cells of the brain and retinal vasculature with Nitta et al. (2003) reporting size-selective impairment (approximately less than 800 Da) of the BBB in claudin-5 deficient mice (Keaney et al., 2011). While these mice show no changes in blood vessel development or morphology, they die within one day of birth likely due to disruption of BBB paracellular permeability to small molecules. The expression of other claudin gene family members, claudin-1, -3, -11 and -12, has been detected in the cerebral microvascular endothelium but recent transcriptome analysis has
only found a definitive signal for claudin-12 at the BBB (Enerson and Drewes, 2006; Daneman et al., 2010a). Of the claudin subtypes in brain endothelial cells, claudin-5 is the most abundant with expression levels 593-fold greater than that of claudin-1, -3 or -12 (Ohtsuki et al., 2008).

1.2.3. JAMs and ZOs

JAMs are 40-kDa transmembrane members of the IgG superfamily and unlike tetraspanning claudins and occludin, JAMs have a single transmembrane domain. JAMs are localized to intercellular contacts where they are believed to mediate early attachment of adjacent cell membranes and establishment of endothelial cell polarity (Hawkins and Davis, 1995). In addition, JAMs can be tissue-specific with specific expression of JAM-1 and JAM-4 in the neuronal microvasculature (Mariano et al., 2011; Daneman et al., 2010a). The integral transmembrane components of TJs are linked to the cytoskeleton via the ZO intracellular scaffold proteins (Abbott et al., 2010). ZO intracellular proteins belong to the larger membrane-associated guanylate kinase (MAGUK)-like protein family and of the three main ZO proteins, ZO-1 forms heterodimers with ZO-2 and ZO-3 (Fanning et al., 1998). The ZO proteins are also thought to control the correct spatial distribution and assembly of claudins (via the PDZ binding domain of ZO proteins) and occludin (via their GUK domains) (Itoh et al., 1999, Umeda et al., 2006, Saitou et al., 1998). ZO-1 was the first protein to be positively identified with TJs (Stevenson et al., 1986). Dissociation of ZO-1 from the junctional complexes and reductions in ZO-1 expression have been associated with BBB breakdown and increased permeability in a variety of neurological disorders (Luissint et al., 2012).

1.2.4. Novel TJ components

Novel brain endothelial-specific components of TJs include tricellulin, LSR, Pard3 and cingulin-like 1 (Ikenouchi et al., 2005; Daneman et al., 2010a). Tricellulin (also known as marvelID2) was first identified as the central constituent of tricellular contact points in epithelial sheets where it acts to block the passage of macromolecules approximately 4-10 kDa in size (Ikenouchi et al., 2005; Krug et al., 2009). This was followed by the discovery of the integral TJ-associated protein LSR that functions to recruit tricellulin to tricellular TJ strands (Masuda et al., 2011). The recent finding that tricellulin and LSR were among the list of BBB-enriched TJ molecules in a mouse brain endothelium transcriptome analysis suggests that tripartite adhesions may be crucial to forming high
electrical resistance barriers and limiting paracellular flux of macromolecules where three brain endothelial cells meet (Daneman et al., 2010a). Thus identifying safe and reversible modifiers of tricellulin expression may serve as a potential avenue for delivery of large compounds across the BBB.

1.2.5. Regulation of TJ formation and maintenance

Correct assembly and maintenance of TJ complexes in CNS endothelial cells is crucial to the regulation of paracellular permeability across the BBB. Deciphering the pathways responsible for inducing BBB characteristics during embryonic development has also revealed the molecular and cellular components influencing TJ formation (see Table 1.1). Wnt/β-catenin signaling, which helps drive early brain angiogenesis and barrier induction, also plays a role in TJ formation in the embryonic and postnatal BBB. Liebner et al. (2007) showed that inactivation of endothelial β-catenin reduces claudin-3 levels in vivo while Wnt3a treatment of brain endothelial cells in vitro increases claudin-3 expression. As outlined in section 1.1, signals released from pericytes are vital to neurovascular function and BBB integrity, in part by regulating TJ formation. Cross-talk between endothelial cells and pericytes via the PDGFβ/PDGFRβ pathway induces TJ sealing by up-regulating occludin and claudin-5 while TGFβ/TGFRβ signaling has been shown to increase claudin-5 expression in vitro (Daneman et al., 2010b, Dohgu et al., 2005). Astrocytes are another NVU component that communicate with the brain endothelia to help establish and maintain TJ integrity. Sonic hedgehog (SHH), angiopoietin-1 (ANG-1) and angiotensin-II (ANG-II) are among the astrocyte-derived ligands identified to date that are involved in upregulation and redistribution of TJ proteins (Alvarez et al., 2011, Lee et al., 2003, Wosik et al., 2007). In addition, interactions between extracellular matrix (ECM) proteins of the vascular basement membrane and endothelial receptors activate signaling cascades that control TJ development. For example, binding of the ECM ligand laminin to the β1 integrin receptor induces claudin-5 expression in vitro (Osada et al., 2011). While the ligands and receptors involved in influencing TJ protein expression are starting to be elucidated, much of the downstream signaling including transcription factors and binding sites around TJ genes remains unknown. However, in addition to changes in TJ protein levels due to transcriptional up-/down-regulation, a number of signaling pathways regulate TJ protein integrity via post-translational modifications such as serine/threonine (Ser/Thr) and tyrosine (Tyr) phosphorylation. The protein kinase C (PKC) family of Ser/Thr
kinases differentially regulate endothelial TJs – activation of novel PKC isozymes (δ, ε, η, μ) generally contributes to TJ function while activation of conventional PKC isozymes (α, βI, βII and γ) causes barrier disruption (Andreeva et al., 2006, Harrington et al., 2003, Sonobe et al., 2009). Tyr-phosphorylation of occludin following exposure to glutamate increases permeability across rat brain endothelial cells in vitro while Tyr-phosphorylation of claudin-5 and occludin by the major angiogenic factor VEGF destabilizes TJs in brain endothelial cells (Andras et al., 2007, Antonetti et al., 1999, Argaw et al., 2009). Rearrangements of the actin cytoskeleton can also lead to indirect changes in TJ integrity. For example the small GTPase, RhoA, is known to increase paracellular permeability by reorganizing the actin filaments and thereby disrupting TJ proteins anchored to the cytoskeleton (Luissint et al., 2012). In response to inflammatory chemokines like MCP-1 and CCL-2, activation of RhoA and its effector kinase ROCK can also mediate Ser/Thr phosphorylation of occludin, claudin-5 and ZO-1 and increase barrier permeability (Stamatovic et al., 2006, Stamatovic et al., 2003). A recent study by Cristante et al. (2013) has also shown that the glucocorticoid anti-inflammatory messenger annexin A1 (ANXA1) maintains BBB integrity by binding to β-actin and anchoring it to the plasma membrane, thereby stabilizing TJs. Exogenous treatment of brain endothelial cells with ANXA1 also inhibited RhoA activity and enhanced TJ formation.

Understanding the physiological signals governing endothelial TJ complex formation in both the developing and adult brain is important when considering breakdown of the BBB during disease. Over the last 20 years, numerous studies in rodents and humans have shown that dysregulation of the BBB, such as changes in permeability following TJ disruption and impaired transcellular transport, correlates with and exacerbates synaptic and neuronal dysfunction, neuroinflammation and neurodegeneration (Zlokovic, 2008, Luissint et al., 2013, Obermeier et al., 2013). As such, a compromised BBB is considered a primary and/or secondary pathogenic component in a host of neurological disorders including multiple sclerosis, stroke, traumatic brain injury (TBI), amyotrophic lateral sclerosis (ALS) and others (mechanisms of BBB and TJ breakdown in CNS disease will be explored in sections 1.5 and 3.2). Included in this grouping is Alzheimer’s disease (AD), a multifaceted neurodegenerative disorder characterized by age-related memory decline and for which there is currently no cure or means of halting neuronal death. While cerebrovascular dysfunction has long been an associated clinical feature in AD, it is only
in recent years that molecular changes to the BBB and NVU have been elucidated in AD pathology (Bell and Zlokovic, 2009).

1.3. Alzheimer’s disease (AD)

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by a gradual decline in cognitive function and is the most common cause of dementia in the elderly. Indeed, there are over 35,000 people in Ireland and approximately 40 million people worldwide who suffer from AD (Alzheimer’s Society of Ireland, 2014, Brookmeyer et al., 2007, Prince et al., 2013). Furthermore, with life expectancy in the developed world increasing and AD being an aging-related condition, these rates are going to go up significantly in the coming years, such that the projected costs of AD in the US alone will be over one trillion dollars by 2050 (Alzheimer’s Association, 2014). AD can be distinguished from other dementias by its clinical history – onset over several months is followed by gradual but continuous decline in cognitive abilities over 7-10 years after AD diagnosis. Initial symptoms include impaired short-term memory, decreased attention and problem-solving abilities and in its latter stages mood swings, language dysfunction, exhaustion and long-term memory loss are apparent (Holtzman et al., 2011). At a cellular level, loss of neurons in AD is generally found in certain brain regions, in particular pyramidal cells of the entorhinal cortex and in the CA1 region of the hippocampus (Fig. 1.6A: Gomez-Isla et al., 1996). However, shrinkage and loss of neuronal processes like synapses, axons and dendrites is a major factor in brain volume loss in AD (Huang and Mucke, 2012). Indeed, degeneration of synapses and dendritic spines correlates better with AD dementia severity than other anatomical disturbances like neuronal loss (Palop et al., 2006). Synaptic dysfunction in turn disrupts vital neural networks, affecting patterns of circuit activity and increasing incidence of epileptic seizures in AD patients (Palop and Mucke, 2010). As outlined below, the current state of play suggests that these common cellular and structural markers of cognitive decline in AD stem from a multitude of diverse factors. However, a central pathological hallmark of AD is the extracellular accumulation of a small neurotoxic peptide called amyloid-β (Aβ) into plaques in the brain parenchyma and on the cerebral blood vessels (a condition known as cerebral amyloid angiopathy or CAA) (Fig. 1.6B-E: Benilova et al., 2012, Bell and Zlokovic, 2009). Another common feature of AD pathogenesis is the formation of intracellular neurofibrillary tangles (NFTs) composed of tau, a hyperphosphorylated microtubule-associated protein (Fig. 1.6D & E: Bloom, 2014). It is worth noting that
these characteristic neural pathologies of AD begin to develop many years before clinical symptoms, such as mild cognitive impairment (MCI) which often precedes AD, first appear (Jack et al., 2010, Bateman et al., 2012).

1.3.1. Genetics of AD

Genetic, epigenetic, immune-related and environmental factors have all been individually recognized as elevating risk of developing AD and this complex etiology and disease progression has made research into the causes, prevention and treatment of AD exceedingly difficult. The vast majority (~ 99%) of AD cases are ‘sporadic’ and late-onset with symptoms of dementia first recognised around 65 years of age and diagnosis often much later. Epigenetic changes in AD such as dysregulated DNA methylation and histone modifications have recently been discovered in post-mortem human tissues and transgenic animal models (Chouliaras et al., 2010). In addition, environmental risk factors have long been associated with AD and range from aging, the most well-known non-genetic risk factor, to head injury, hypertension, obesity and diabetes (Huang and Mucke, 2012, Barnes and Yaffe, 2011). However, a very small number of AD cases (< 1%) follow an early-onset (between 30 and 60 years of age) familial pattern and it was the discovery in the late 1980s of single gene mutations predisposing for early-onset AD that kickstarted modern research into the pathophysiology of AD at the molecular level.

1.3.1.1. Familial AD: amyloid precursor protein (APP) and presenilin (PS) mutations

First described in 1906 by the German psychiatrist Alois Alzheimer, senile ‘plaques’ were long known to be a major feature of AD neuropathology (Alzheimer, 1907, Holtzman et al., 2011). The discovery that these proteinaceous plaque cores were also present in the brains of Down’s Syndrome or trisomy 21 patients (the vast majority of whom develop dementia around 50 years of age) led to the hypothesis that the predisposing gene was located on chromosome 21 (Masters et al., 1985). Subsequently, the cloned complementary DNA for this ‘β-protein’ plaque component was found to match a short sequence within the larger amyloid precursor protein (APP) gene on chromosome 21 (Goldgaber et al., 1987, Tanzi et al., 1987). This protein isolated from the plaque cores was termed ‘amyloid-β’ (Aβ) and the role of Aβ in AD pathogenesis was further supported following the discovery that AD could be inherited in an autosomal dominant fashion as a result of mutations in the gene coding for amyloid precursor
protein (APP) (Goate et al., 1991; Karran et al., 2011). Aβ peptides are produced from the APP holoprotein in a series of sequential proteolytic cleavage events. There are several APP isoforms with the 695, 751 and 770 amino acid isoforms being most common (Holtzman et al., 2011). The APP transmembrane protein is first cleaved by β-site APP converting enzyme (BACE or β-secretase) which generates a C-terminal APP fragment (C99 or β-CTF) and a large secreted N-terminal soluble APP fragment (sAPP-β). β-CTF is further processed by the γ-secretase enzyme complex to form the APP intracellular domain (AICD) and various short Aβ peptides of which Aβ1-40 and Aβ1-42 are the most common neurotoxic species found in the AD brain (Fig. 1.6F; Karren et al., 2011; Obregon et al., 2012). As described below, while Aβ1-40 is the more soluble and more abundant Aβ isoform in the brain, Aβ1-42 is more fibrillogenic and the most common isoform found in senile plaques of the brain parenchyma (Jarrett et al., 1993, Ladecola, 2004). Missense mutations in APP that are positioned close to the sites of proteolysis by β-secretase and γ-secretase cause familial early-onset AD and in some cases the accumulation of Aβ around cerebral blood vessels or CAA (Fig. 1.7; Holtzman et al., 2011). This is due to increased production of Aβ1-42 relative to Aβ1-40 or in some cases, such as the Swedish APP mutation, the mutation increases levels of all Aβ isoforms (Citron et al., 1992). As well as the finding that Down’s Syndrome patients who carry an extra chromosome 21 (on which the APP gene resides) develop early-onset dementia, evidence for the involvement of Aβ in contributing to AD also comes from a recent pivotal study searching for protective APP mutations. Jonsson and colleagues (2012) searched for coding variants in APP from whole genome sequence data of approximately 2000 Icelanders and found that the A673T variant protects against the development of AD and also protects against cognitive decline in elderly people without AD. This A673T coding mutation is found adjacent to the β-secretase cleavage site and reduces the β-cleavage of APP, resulting in lower levels of Aβ1-40 and Aβ1-42 (Jonsson et al., 2012). Interestingly, the precise function of APP in the brain is not fully understood. Presently it is believed that APP plays a role in synapse formation, dendritic spine formation, axonal pruning, neuronal migration and in the processes of learning and memory (Westmark, 2013). Thus it is possible that familial mutations in APP may contribute to AD by mechanisms unrelated to Aβ generation.

However, mutations in two other genes again point to disease-causing changes in Aβ metabolism. Autosomal dominant mutations in genes encoding presenilin-1 (PS1) and

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presenilin-2 (PS2), protein components of the catalytic subunit of the γ-secretase complex, cause early-onset familial AD by altering APP processing and promoting the production of more neurotoxic Aβ species (Levy-Lahad et al., 1995, Huang and Mucke, 2012). Indeed mutations in PS1 are the most common cause of familial AD (Holtzman et al., 2011). Specifically, PS1 mutations cause a partial loss of γ-secretase function, resulting in less efficient APP cleavage and increased Aβ1-42 production (De Strooper, 2007). Mutations in APP, PS1 and PS2 also form the basis of human transgene mouse models of AD (outlined further in chapter 6) that mirror many aspects of AD including plaque formation and memory loss (Ashe and Zaks, 2010).

1.3.1.2. Apolipoprotein E (APOE)

As well as Mendelian-segregating single gene mutations in familial AD, there are a number of genetic factors that increase the risk of developing AD in both familial and sporadic cases. In humans there are three alleles of the apolipoprotein E (APOE) gene, ε2, ε3, ε4, which differ by only one amino acid at the protein level (Kanekiyo et al., 2014). The strongest genetic risk factor for sporadic late-onset AD is the ε4 allele with individuals carrying two copies of the ε4 allele having a 12-fold increased risk compared with the APOE ε3/ε3 genotype (Holtzmann et al., 2011). APOE ε4 inheritance also reduces the age of AD onset by around 5 years per ε4 allele and recent studies have highlighted how APOE genotypes differentially affect AD risk and onset by altering aspects of Aβ metabolism (Corder et al., 1993). The ε4 allele is associated with earlier and increased Aβ deposition and increased cerebrovascular amyloid deposition in both cognitively normal individuals as well as AD patients (Rebeck et al., 1993, Morris et al., 2010). Transgenic APP mice engineered to carry one of the human APOE isoforms also show isoform-dependent differences in brain Aβ levels and plaque formation (ε4 > ε3 > ε2) with presence of the ε4 allele associated with faulty Aβ clearance from the brain (Holtzmann et al., 2000; Castellano et al., 2011). APOE is a known regulator of lipoprotein metabolism in the plasma but its precise function in the brain is still not fully resolved. It is widely expressed in the CNS, in particular by astrocytes, and seems to have additional roles in cholesterol transport, neuronal plasticity and inflammation (Kim et al., 2009, Holtzman et al., 2011). With regards the influence of APOE on Aβ levels and AD pathogenesis, a number of hypotheses have been proposed. Histological analyses of post-mortem AD brains shows that APOE is co-deposited with Aβ in senile plaques and numerous in vitro and in vivo studies have suggested that APOE is an Aβ-binding
molecule that facilitates Aβ clearance in an isoform-dependent manner (Namba et al., 1991, Kanekiyo et al., 2014). APOE3 binds Aβ oligomers more strongly than APOE4 while APOE4/Aβ complexes are also less stable possibly due to the poorer lipidation status of APOE4-containing lipoproteins (Petrlova et al., 2011, Tai et al., 2013). Furthermore, Li and colleagues (2012) showed that recombinant APOE accelerates neuronal uptake of Aβ with APOE3 more efficiently mediating Aβ binding to the cell surface. These combined findings suggest that APOE isoforms directly affect Aβ aggregation and clearance.

However, despite this evidence, other groups have recently shown that APOE does not strongly bind to Aβ and instead may compete with (and slow down) Aβ binding to its receptors. When astrocyte-secreted or reconstituted APOE was mixed with Aβ at physiological concentrations (50:1 ratio of APOE:Aβ), 95% of Aβ remained free regardless of APOE isoform (Verghese et al., 2013). Instead cultured astrocytes from APOE-knockout mice cleared Aβ more efficiently than APOE-knockin mice and researchers actually found that the more APOE was added, the less the astrocytes could clear Aβ regardless of isoform (Verghese et al., 2013). It is known that APOE and Aβ share many of the same receptors for cellular uptake such as the low-density lipoprotein receptor protein 1 (LRP1) (Kanekiyo et al., 2014). Verghese and colleagues (2013) found that LRP1-deficient fibroblasts or treatment of astrocytes with LRP1 antibody cleared less Aβ but both processes were unaffected by APOE. These results suggest APOE affects Aβ metabolism by competing with Aβ for the same clearance pathway, namely LRP1-mediated removal by astrocytes. Whether APOE4 binds LRP1 on astrocytes with higher affinity than APOE2 or APOE3 is not yet known. As outlined later, APOE has other wide-ranging effects on Aβ clearance pathways, in particular Aβ phagocytosis by microglia and Aβ removal across the BBB.

1.3.1.3. Other genetic risk factors
Since around 50% of individuals with AD carry an APOE ε4 allele, other genetic components are likely involved in altering risk of developing AD (Holtzman et al., 2011). However, apart from the rare variants of large effect (ie. the Mendelian-segregating mutations in APP, PS1 and PS2 that cause AD) and common variants that significantly elevate AD risk (ie. APOE4 ε4), few other ‘high-risk’ genetic components have been identified (Fig. 1.8). In order to find genetic factors of smaller impact, researchers began
numerous genome-wide association studies (GWAS) involving thousands of AD case samples and nondemented elderly controls that evaluated millions of single nucleotide polymorphisms (SNPs). These studies over the last number of years have revealed approximately 20 new genetic risk loci with genome-wide significance for association with AD, albeit they increase AD risk by 2-5% (Harold et al., 2009, Lambert et al., 2009, Naj et al., 2011, Hollingworth et al., 2011, Guerreiro et al., 2013a). Interestingly, many of these common variants map to distinct biological pathways: immune system/inflammation (CD33, EPHA1, CLU, CR1, ABCA7), cholesterol/lipid metabolism (CLU, ABCA7, SORL1) and endocytosis (PICALM, CD33, BIN1, SORL1). While some of these variants likely affect Aβ metabolism, the clustering of signals for distinct processes like immune response and lipid biology indicates several distinct biological pathways are likely involved in AD etiology. With regards future AD therapeutics, it also suggests that these pathways may need to be targeted in combination to prevent disease progression in AD patients. Since twin studies have estimated that AD heritability stands at 60-80%, whole-genome and whole-exome sequencing is currently being adopted to find some of this “missing heritability” (Gatz et al., 2006). This has identified the first rare variant of intermediate risk – a rare missense mutation in TREM2 (triggering receptor expressed on myeloid cells 2) (Jonsson et al., 2013, Guerreiro et al., 2013b). TREM2 has a known anti-inflammatory role in the brain and is also involved in microglial-mediated Aβ clearance (discussed later) (Gandy and Heppner, 2013). How these genetic factors combine with environmental risks to alter AD risk is an area of research in need of attention. Some work has already shown that the combination of an APOE ε4 allele with head injury increases AD risk from an odds ratio of 2 (for head injury) to 20 (Holtzman et al., 2011). Furthermore, cognitively normal older adults with higher levels of exercise were found to have reduced levels of AD biomarkers and this correlation was more pronounced in APOE ε4 allele non-carriers (Liang et al., 2010).

1.3.2. Amyloid cascade hypothesis and soluble Aβ toxicity
Autosomal dominant mutations in APP, PS1 and PS2 are known to alter APP processing, resulting in increased production and/or aggregation of Aβ and early-onset AD develops in almost all of these mutant carriers. Genetic, biochemical, neurobiological and neuropathological findings have merged to create an amyloid cascade hypothesis – that AD pathogenesis is initiated by deposition of Aβ in the brain (Karran et al., 2011). Data from a vast longitudinal study recently concluded by the Dominantly Inherited Alzheimer
Network (DIAN) has given credence to this proposal – in people predisposed to developing familial AD, concentrations of Aβ1-42 in the CSF declined 25 years prior to expected symptom onset and positron-emission topography (PET) using Pittsburgh compound B (PiB) recorded Aβ deposition in brains 15 years in advance (Bateman et al., 2012). The estimated year for symptom onset was based on the parent’s age at symptom onset and these patients were clinically diagnosed with AD dementia on average 3 years after expected symptom onset. Importantly, increased brain atrophy was first detected 15 years in advance and impaired episodic memory and global cognitive impairment was found 5-10 years in advance (Bateman et al., 2012). While it remains to be determined how these clinical and biomarker changes correspond to sporadic AD, the overlapping clinical and neuropathological features suggests that deposition of Aβ in the brain is a crucial event in both forms of AD. Human brain Aβ can exist in a variety of assembly states depending on the extent of polymerization and aggregation. Beginning with monomeric (approximately 4.4 kiloDaltons in size) Aβ production in neurons from transmembrane APP, small soluble Aβ species can progress to higher-order oligomers, protofibrils and eventually insoluble mature fibrils that form the microscopically-visible plaques in AD brain sections (Fig. 1.9A; Benilova et al., 2012). Early proponents of the amyloid hypothesis envisioned senile plaques to be the critical mediators of Aβ neurotoxicity. However deposition of insoluble Aβ in senile plaques does not correlate well with neuronal loss and disease progression and changes in Aβ metabolism and plaque formation have been recorded years before the onset of clinical symptoms (Perrin et al., 2009). Instead soluble Aβ oligomers are increasingly being recognized as the drivers of AD neurodegeneration since Aβ oligomers can induce synaptic loss (a strong correlate of dementia severity in AD patients), impair hippocampal synaptic plasticity and predispose neurons to tau aggregation and NFT formation, with Aβ dimers found to be the smallest synaptotoxic species (Walsh et al., 2002, Shankar et al., 2008; Selkoe, 2011). Furthermore, certain mutations in APP located towards the middle of the Aβ peptide seem to enhance Aβ oligomerization in affected AD patients (Nilsberth et al., 2001, Tomiyama et al., 2008). It is also known that the major genetic risk factor APOE increases Aβ oligomer levels in an isoform-dependent manner and that APOE4 stabilizes Aβ oligomers more strongly than APOE3 (Hashimoto et al., 2012, Cerf et al., 2011). In this revised model, Aβ oligomers and plaques seem to exist in equilibrium. Oligomeric Aβ assemblies have been observed as a ‘halo’ surrounding the plaque edges suggesting...
that plaques can act as ‘reservoirs’ of Aβ oligomers that can then diffuse from plaque cores and cause synaptic dysfunction (Koffie et al., 2009; Selkoe, 2011).

How soluble Aβ oligomers impair synaptic transmission and induce dendritic spine/synaptic loss is not yet fully understood. A host of neuronal receptors have been implicated in vitro and in vivo in Aβ oligomer-mediated impairment of hippocampal synaptic plasticity and long-term potentiation (PirB/LirB2, FcγRIIB, PrPc) and in Aβ oligomer-mediated changes in excitatory synaptic transmission and collapse of glutamatergic dendritic spines (NMDA receptor, mGluR5, PrPc, EphB2) (Fig. 1.9B, Kim et al., 2013, Um et al., 2013, Shankar et al., 2007). However, whether the numerous Aβ receptors reflects the “sticky” nature of Aβ oligomers or specific pathological interactions is not yet known (Benilova and Strooper, 2013). At a network level, synaptic depression seems to co-exist with neuronal hyperactivity as soluble Aβ oligomers have been shown to actually enhance the number of both hypoactive and hyperactive neurons in certain neural circuits (Busche et al., 2008, Busche et al., 2012). Indeed, increased incidence of epileptic seizures in AD (in particular early-onset familial AD) suggests that neuronal overstimulation and excitotoxicity may contribute to neurodegeneration along with synaptic depression and loss (Palop and Mucke, 2010). With regards the brain vasculature, soluble Aβ species have also been implicated in vascular abnormalities in CAA and AD (more in section 1.4; Storkebaum et al., 2011).

The hypothesis that soluble Aβ oligomers are the primary mediators of synaptotoxicity and neurotoxicity, while convincing, is hampered by the dynamic nature of these species, poorly defined mechanisms of toxicity and different experimental protocols for generation and isolation of soluble Aβ oligomers. Much work is needed in the future to standardize studies of Aβ oligomerization and toxicity as well as understanding the composition of Aβ assemblies and their potential differences when generated in vitro or extracted in vivo. Current efforts to detect Aβ oligomers in AD patients have shown a 3- to 5-fold increase in Aβ oligomer levels in the CSF of confirmed human AD patients compared to healthy aged controls (Savage et al., 2014). This study used a two-site ELISA (enzyme-linked immunosorbent assay) with a 2500-fold increased specificity for Aβ oligomers compared to monomers. The prospect of CSF Aβ oligomer levels as a putative biomarker of Aβ oligomer-mediated neuronal dysfunction and loss is an exciting one.
1.3.3. Role of neuroinflammation in AD pathogenesis

Recent GWAS and whole-exome sequencing studies looking for genetic variants that increase AD risk identified a number of risk factors with immune system involvement: CD33, TREM2 and CLU among others (Naj et al., 2011, Hollingworth et al., 2011, Jonsson et al., 2013, Guerreiro et al., 2013b). It has long been known that microglia, the resident macrophages of the CNS that function to scavenge and phagocytose extracellular debris and foreign material in the brain, accumulate in senile plaques in transgenic AD mouse models and in brains of AD patients (Dickson, 1999). Genetic and biochemical analyses has indicated an essential role for microglia in AD pathogenesis and revealed a dynamic relationship between the activation status of microglia and their contribution to Aβ levels and neurodegeneration in AD. Microglia can exist in one of two active states: in the classically-activated M1 pathway, microglia are primarily pro-inflammatory and release pro-inflammatory cytokines like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) while in the alternative M2 pathway microglia are known to have an anti-inflammatory role, phagocytosing damaged neurons and foreign material and activating neurotrophic pathways (Aguzzi et al., 2013). To understand the contribution of microglia to AD pathology, Hickman and colleagues (2008) isolated microglia from transgenic APP-PS1 mice and found that the expression of the Aβ scavenging receptors CD36, SRA (scavenging receptor A) and RAGE (receptor for advanced glycation end products) was dramatically reduced in old, but not young, APP-PS1 mice. Instead, microglia in aged APP-PS1 animals produced higher levels of the pro-inflammatory cytokines IL-1β and TNF-α (Hickman et al., 2008). The authors concluded that while microglia recruitment in the early stages of AD likely promotes Aβ phagocytosis and clearance, chronic microglial activation in response to overwhelming Aβ levels converts them to an M1 phenotype and the resulting pro-inflammatory cytokine release accelerates neurodegeneration.

The concept of a microglial “double-edged sword” has been supported by subsequent studies looking at how AD genetic risk factors affect neuroinflammation. APOE for example has been shown to direct microglia towards the anti-inflammatory M2 phenotype by activating phagocytosis and migration (Baitsch et al., 2011, Cudaback et al., 2011). However the APOE4 isoform is less effective in promoting the M2 pathway in microglia compared to APOE3 (Zhu et al., 2013, Cudaback et al., 2011). The AD risk factor CD33 also plays a role in microglial activation. Griciuc et al. (2013) found that the CD33 variant rs3865444, which protects against AD, results in reduced CD33 expression in
microglia and also reduced insoluble Aβ1-42 levels in the brain. CD33 therefore appears to regulate microglial clearance of Aβ through an unknown mechanism and indeed CD33 protein levels and CD33-positive microglia are increased in the brains of AD patients (Griciuc et al., 2013). Thus microglia seem to be both helpful and harmful in AD pathogenesis – when M2 microglia keep up with Aβ accumulation, their scavenger receptors help to phagocytose Aβ. However once the microglia become ‘poisoned’ or overwhelmed by Aβ levels, conversion to an M1 phenotype appears to facilitate neuronal dysfunction and loss (Gandy and Heppner, 2013). Insights into how exactly Aβ deposition causes pro-inflammatory cytokine release from activated microglia has implicated the NALP3 inflammasome – a large multiprotein complex found in microglia that is required for caspase-1-mediated maturation and release of IL-1β (Heneka et al., 2013). Engineered Nlrp3−/− APP/PS1 mice were found to have reduced amyloid burden, improved cognitive abilities and increased amounts of anti-inflammatory M2 microglia (Heneka et al., 2013). This dynamic role of microglia in regulating Aβ levels and neuronal function highlights the need for careful anti-inflammatory drug design and timed delivery if neuroinflammation is to be targeted in AD treatment.

1.3.4. Tau and neurofibrillary tangles (NFTs)

Along with vast amounts of amyloid plaques, pathological analyses of post-mortem brain tissue from AD patients also shows the presence of neurofibrillary tangles (NFTs) (Fig. 1.6D & E: Huang and Mucke, 2012). These protein deposits consist of intracellular aggregates of a hyperphosphorylated microtubule protein called tau that contributes to the neurodegenerative process in AD. Indeed, increased concentrations of tau protein in the CSF and an increase in brain atrophy were detected 15 years before expected symptom onset in a longitudinal study of prospective familial AD patients (Bateman et al., 2012). Interestingly though, a follow-up study has shown that CSF biomarkers of neuronal death including tau and phosphorylated tau significantly drop after the estimated age of disease onset (Fagan et al., 2014). This suggests that as symptoms of AD are beginning to develop and progress, much of the neurodegenerative process has already taken place and brain atrophy may be slowing down (Fagan et al., 2014). Genetic and biochemical studies have indicated that abnormal tau aggregates act downstream of Aβ but that both tau and Aβ enhance each other’s toxicity. Firstly, mutations in MAPT, the gene encoding tau, cause frontotemporal lobar degeneration and dementia that is characterized by accumulation of NFTs but not Aβ plaques (Ballatore et al., 2007). In contrast, APP or PS
mutations cause AD with both NFTs and Aβ plaques prominent features of the neuropathology (Holtzman et al., 2011). Furthermore, oligomeric Aβ species exacerbate the development of tau aggregates and NFTs and the enrichment of tau in dendritic spines where it disrupts neurotransmission (Hoover et al., 2010). APOE4 can also enhance tau aggregation and its postsynaptic deposition (Andrews-Zwilling et al., 2010). However, the toxic effects of soluble extracellular Aβ on neuronal atrophy and synaptic dysfunction have been shown to depend on soluble cytoplasmic tau (Morris et al., 2011, Bloom, 2014). Intriguingly, there is evidence that soluble toxic aggregates of Aβ and tau may be able to self-propagate and spread from brain region to brain region in a prion-like manner (Eisele et al., 2010, Clavaguera et al., 2009, Bloom, 2014). While the effects of increased tau levels on the structure and function of the brain vascular system is a potential area of interest, the research described in this body of work will consider the influence of Aβ on neurovascular function and vice versa.

1.4. Neurovascular pathways in AD: amyloid-β transport across the BBB

In a healthy human brain and throughout life, soluble Aβ is produced and secreted by neurons and other cell types. Under physiological conditions, the rate of production and clearance of Aβ has been estimated to be 7.6% per hour and 8.3% per hour, respectively (Bateman et al., 2006). Aβ is cleared in the homeostatic brain along three major pathways with each pathway having been recognized as contributing to toxic Aβ buildup and neuronal dysfunction when disrupted (Kanekiyo et al., 2014). Firstly, intracellular and extracellular proteolytic degradation of monomeric Aβ is carried out by a number of Aβ proteases including neprilysin, insulin-degrading enzyme and angiotensin-converting enzyme (de Strooper, 2010). Genetic inactivation of any one of these Aβ-degrading enzymes in mice has been found to increase Aβ accumulation and plaque formation (Iwata et al., 2000, Farris et al., 2003). A second route of Aβ clearance is through lysosomal degradation by neurons, astrocytes and microglia. As outlined, reduced levels of Aβ-scavenging receptors in microglia and also in neurons (for example, knockout of LRP1) accelerates Aβ deposition and AD pathology (Li et al., 2012, Kanekiyo et al., 2013). The third major pathway of Aβ clearance involves the cerebrovascular system in the brain. This can be divided into: (1) drainage of interstitial fluid (ISF) containing Aβ by the so-called ‘glymphatic’ system and (2) clearance of Aβ across the BBB. In the ‘glymphatic’ system, subarachnoid CSF enters the brain parenchyma along paravascular spaces surrounding arteries (Virchow-Robin spaces) and mixes with the ISF containing
extracellular solutes and metabolites that can then be cleared along paravenous drainage pathways (Fig. 1.10; Iliff et al., 2012). This process is driven by arterial pulsation and requires astrocytes as deletion of the water channel protein AQP4 in astrocytes reduces ISF (and Aβ) clearance (Iliff et al., 2012). Interestingly, this exchange of solutes between the CSF and ISF is regulated by sleep – extracellular space volume in the brain increases during sleep and is associated with increased Aβ clearance levels compared to a waking state (Xie et al., 2013). Thus, impairments in the diurnal fluctuation of Aβ and its clearance through paravenous flow may contribute to the mis-accumulation of soluble Aβ in AD. To this end, it is known that amyloid deposition in the preclinical stage of AD in humans appears to be associated with worse sleep quality and that chronic sleep deprivation increases amyloid plaque formation in AD mouse models (Ju et al., 2013, Kang et al., 2009).

The pool of Aβ in the brain exists in equilibrium with Aβ in the CSF and systemic Aβ in plasma. With a surface area of approximately 18 m², the cerebral microvessels function as a major site for brain-blood exchange (Abbott et al., 2010). Thus the BBB is a major route for exit of Aβ from the brain – Ito and colleagues (2013) have estimated that the clearance rate constant of Aβ1-40 in the mouse cerebral cortex is 3.21 x 10⁻²/min with efflux of Aβ1-40 at the BBB occurring at a rate of 1.48 x 10⁻²/min. Since sporadic late-onset AD cases typically do not display increased production of Aβ (in contrast to early-onset familial AD), models of neurovascular disease pathways in AD pathogenesis have proposed that impaired brain clearance of Aβ by cells of the NVU leads to Aβ accumulation in the brain parenchyma and around cerebral blood vessels (Bateman et al., 2006, Zlokovic, 2008). Indeed, when comparing sporadic AD patients and cognitively normal controls, Mawuenyega and colleagues (2010) recently demonstrated impaired Aβ1-40 and Aβ1-42 clearance but no differences in Aβ production in the brains of AD patients. The seminal work of Berislav Zlokovic and others has elucidated the importance of CNS endothelial cells in determining brain Aβ concentrations via the trafficking of Aβ between the blood and the brain.

1.4.1. RAGE and LRPI: the ‘yin’ and ‘yang’ of transcellular Aβ transport at the BBB

Both brain-derived and peripheral Aβ is transported across the BBB via receptor-mediated transeptosis (Fig. 1.11). Highly expressed in brain endothelial cells of the BBB,
the efflux transporter P-glycoprotein (Pgp) has been implicated in Aβ transport out of the brain as APP/Pgp-null transgenic mice show increased brain Aβ levels (Cirrito et al., 2005). More recently, genetic knockout of the cellular prion protein (PrP*) in brain endothelial cells in vitro was also found to reduce Aβ transcytosis (Pflanzner et al., 2012). However much attention has focused on the receptor for advanced glycation endproducts (RAGE) and low-density lipoprotein receptor-related protein 1 (LRP1) as the central Aβ transporters at the BBB. Elucidating these transcellular pathways of Aβ movement has revealed how disrupting their normal activity can directly cause or indirectly accelerate Aβ buildup and AD pathogenesis.

1.4.1.1. RAGE ('yin')
The systemic circulation is a known source of soluble Aβ peptides and studies in both rodent and non-human primate models have highlighted the contribution of peripheral Aβ to the development of cerebral Aβ accumulation (Eisele et al., 2010, Sutcliffe et al., 2011, Ujiie et al., 2003, Mackic et al., 2002). RAGE is an Aβ receptor found on the surface of neurons and microglia and is also the major endothelial receptor for Aβ influx from the blood to the brain (Fig. 1.11; Deane et al., 2003). RAGE expression at the BBB is increased in the brain endothelium of many AD patients as well as in AD transgenic mouse models, likely due to accumulation of its ligand Aβ (Yan et al., 1996; Zlokovic, 2008). The Aβ/RAGE interaction at the luminal membrane provides a mechanism for circulating Aβ entry into the brain and its subsequent binding to neurons. This interaction can also stimulate the NF-κB pathway and cause pro-inflammatory cytokine release from activated endothelia. Furthermore, Aβ/RAGE interactions result in production of endothelin-1 which restricts cerebral blood flow (CBF) (Deane et al., 2003, Zlokovic, 2008). RAGE-specific inhibitors have been shown to reduce Aβ-mediated neurotoxicity, lower neuroinflammation, elevate CBF and improve cognitive outcome in APP transgenic mice (Deane et al., 2012).

1.4.1.2. LRP1 ('yang')
Working in parallel with RAGE, LRP1 is the major Aβ efflux transporter and binding of LRP1 and Aβ at the abluminal side of the BBB mediates Aβ clearance from brain to blood (Fig. 1.11; Shibata et al., 2000). LRP1 receptor mediates cellular uptake and internalization of Aβ for lysosomal degradation in neurons, astrocytes, microglia and vascular smooth muscle cells (Kanekiyo et al., 2011, Koistinaho et al., 2004, Bell et al.,
2009; Kanekiyo et al., 2012). However, LRPI plays a separate role in brain endothelial cells, mediating endocytosis and transport of its ligands including Aβ across the BBB (Deane et al., 2004). Vasculotropic mutations in the APP gene that predispose for vascular-associated Aβ accumulation (CAA) and dementia produce Aβ peptides that have low binding affinity for LRPI and are thus poorly removed from the brain (Zlokovic, 2011). Once in the plasma, about 70-80% of Aβ binds to soluble LRPI (sLRP1, cleaved from transmembrane LRPI by β-secretase) (Sagare et al., 2007, Zlokovic, 2011). sLRP1 acts as a peripheral 'sink' by pulling Aβ from brain to blood and transporting it to the liver for degradation, a process also mediated by cell surface LRPI (Sagare et al., 2007). Decreased LRPI levels have been detected in brain microvessels in aged rodents, non-human primates and humans as well as in AD mouse models and AD patients (Bell et al., 2009, Deane et al., 2004, Zlokovic, 2011). Furthermore, elevated plasma levels of free Aβ and associated reductions in sLRP1/Aβ binding have been recorded in MCI and AD patients (Sagare et al., 2007, Sagare et al., 2011). Interestingly, the major AD risk factor APOE4 seems to block LRPI-mediated Aβ clearance across the BBB (Deane et al., 2008). The authors here suggested that APOE4/Aβ complexes are not cleared as efficiently as APOE3/Aβ and APOE2/Aβ complexes (Deane et al., 2008). However the recent finding that APOE and Aβ do not strongly bind and instead compete for binding to LRPI on astrocytes suggests a similar mechanism may account for APOE interference in Aβ transcellular clearance at the BBB (Verghese et al., 2013). Future AD therapies will likely focus on modulating levels of these transcellular Aβ receptors to enhance Aβ clearance across the BBB and pre-clinical studies are already beginning to identify such compounds (see section 1.6).

1.4.2. Vascular abnormalities and BBB dysfunction in AD

Almost all of the well-described environmental risk factors for AD - atherosclerosis, stroke, diabetes, hypertension and smoking - have a vascular component (Bell and Zlokovic, 2009). Indeed, the risk of developing AD or vascular dementia is increased threefold in people with severe atherosclerosis (Hofman et al., 1997). Almost two decades ago, Schiebel and colleagues first proposed that gross vascular changes and a “failing blood-brain barrier” may be important in the development of AD (Schiebel et al., 1989). Insights from clinical imaging, post-mortem tissue analyses and mouse models have revealed the structural and molecular alterations to the brain vasculature in AD.
1.4.2.1. Hypoperfusion and vascular degeneration

Cerebral blood flow (CBF) is tightly coupled to the local neuronal activity and metabolism in distinct CNS regions. This link between local synaptic activity and local CBF rates is known as ‘functional hyperemia’ and forms the basis for functional magnetic resonance imaging (fMRI) imaging where blood oxygenation level-dependent (BOLD) contrast is used to measure resting CBF or CBF rates during a test of episodic memory (memory of autobiographical events) (Bell and Zlokovic, 2009). These fMRI studies have revealed reduced or delayed CBF responses in preclinical AD (specifically patients with MCI) that worsen in clinically-diagnosed AD patients (Hirao et al., 2005, Rombouts et al., 2005). Cerebral hypoperfusion and the resulting hypoxia (reduced oxygen supply) has serious effects on neuronal function such as reducing protein synthesis and ATP synthesis which in turn disrupts synaptic signaling and plasticity. Severe CBF reductions also mirror the effects of ischemic stroke – altered electrolyte and water gradients can cause oedema (brain swelling) and CBF levels below 20% lead to rapid neuronal cell death (Moskowitz et al., 2010, Zlokovic, 2011). Hypoperfusion/hypoxia can also induce or accelerate Aβ accumulation and plaque pathology in mice (Koike et al., 2010). Two molecular components – the transcription factors myocardin (MYOCD) and serum response factor (SRF) – have recently been implicated in causing cerebral hypoperfusion/hypoxia. MYOCD and SRF are expressed in the arterial vascular smooth muscle cell layer (VSMC) where they promote expression of contractile proteins (Bell and Zlokovic, 2009). In AD patients and AD mouse models, increased MYOCD and SRF levels in small arteries are associated with chronic arterial hypercontractibility, hypoperfusion and vessel regression (Chow et al., 2007). This vessel constriction reduces arterial pulsation which likely has a knock-on effect in clearing Aβ along paravascular pathways of the ‘lymphatic system’ as previously outlined. Furthermore, increased MYOCD/SRF enhances expression of sterol response element binding protein 2 (SREBP2), a transcriptional repressor of the Aβ clearance receptor LRPI (Bell et al., 2009). These effects combine to suppress Aβ clearance and exacerbate CAA.

In response to the hypoxic neural environment in AD, levels of the hypoxia-inducible angiogenic factor VEGF are elevated in microvessels and astrocytes of AD patients (Grammas, 2011). In addition, activated endothelial cells in AD brains secrete a number of pro-inflammatory cytokines and angiogenic factors like IL-1β, IL-8, TNF-α, ANG-II and integrins, likely in response to Aβ buildup on cerebral microvessels (Grammas,
However apart from one study indicating neural angiogenesis in AD, several independent groups have reported capillary regression and atrophy and reduced total microvascular density in AD patients and AD mouse models (Biron et al., 2011, Wu et al., 2005, Paris et al., 2004, Zlokovic, 2011). So why is angiogenesis lacking or difficult to identify in AD brains that have increased levels of angiogenic factors? One clue comes from genome-wide transcriptional profiling that reveals low levels of mesenchyme homeobox 2 gene (MEOX2, also known as GAX) expression in AD brain endothelium (Wu et al., 2005). Restoring MEOX2 expression in brain endothelial cells isolated from AD patients stimulated angiogenesis, reduced apoptosis and increased LRPI receptor expression (Wu et al., 2005). This suggests that low MEOX2 levels in concert with anti-angiogenic Aβ inhibits normal angiogenic responses to VEGF and hypoxia in AD and contributes to capillary degeneration (Wu et al., 2005, Paris et al., 2004).

1.4.2.2. Cerebral amyloid angiopathy (CAA)

While deposition of Aβ in the brain parenchyma results in the formation of senile plaques, neurovascular accumulation of Aβ around cerebral blood vessels leads to cerebral amyloid angiopathy (CAA; Fig. 1.6C). CAA is found in over 80% of AD patients and approximately 30% of elderly patients (Attems et al., 2005, Love et al., 2009). CAA with Aβ deposits can affect cerebral arteries and/or capillaries with Aβ buildup in the VSMC layer of cerebral arteries leading to vessel wall rupturing and cerebral hemorrhage (Fig. 1.12; Biffi and Greenberg, 2011). Considerable overlap exists between the genetic factors that underlie both AD and CAA. Certain point mutations in the APP gene such as the Dutch, Iowa, Arctic and Flemish mutations (named after geographical location of affected families) predispose to AD with severe CAA pathology and these individuals have accelerated VSMC degeneration and increased incidence of hemorrhagic stroke (Thal et al., 2008). Furthermore, in cases of sporadic AD, APOE ε4 carriers have more severe capillary-associated CAA compared to ε3 and ε2 carriers (Richard et al., 2010). In general, Aβ deposition in the cerebral vasculature causes significant damage to CNS endothelial cells and contributes to a range of characteristic CAA-associated neurovascular injuries including lobar hemorrhage, cerebral microbleeds, ischemic stroke and chronic vascular inflammation (Bell and Zlokovic, 2009). This acts to exacerbate CBF dysfunction, neurodegeneration and cognitive impairment in AD.
Aβ1-40 is the major Aβ species surrounding cerebral blood vessels in CAA where it triggers reductions in CBF, impairs functional hyperaemia, causes vascular oxidative stress and other symptoms of cerebrovascular dysfunction that often occur before plaque formation (Niwa et al., 2000; Ladecola, 2004). Recent studies have implicated the scavenging receptor CD36 in the mechanism linking Aβ1-40 and reactive oxygen species (ROS) production in CAA-affected vessels. CD36 is an innate immunity receptor found in microglia/macrophages and CNS endothelial cells that binds Aβ (Park et al., 2011). Park and colleagues (2013) found that deletion of CD36 in APP-Swe (Tg2576) mice reduces Aβ1-40 load and CAA pathology. This was accompanied by elevated endothelial LRP-1 expression, increased CBF and improved cognitive function (Park et al., 2013).

1.4.2.3. BBB and TJ dysfunction in AD

Anatomical defects in the brain vasculature have been recognized in AD patients and can occur prior to cognitive decline in patients with high risk of developing AD. Hypoperfusion/hypoxia, preceding or following Aβ accumulation on cerebral blood vessels in CAA, are also central hallmarks of cerebrovascular dysfunction in AD. At the molecular level, disruption and breakdown of the NVU and BBB in AD is a major area of focus in AD/neurovascular research at present. Whether BBB permeability is increased in AD remains controversial with histologic studies of blood protein infiltration into the CNS giving varying results (Erickson and Banks, 2013). In general though, increased BBB permeability and disruption appears more pronounced in AD patients with vascular (CAA) involvement (Akiyama et al., 1992, Zipser et al., 2007). As outlined already, perturbation of the endothelial transcellular Aβ receptors and transporters at the BBB in AD patients (loss of LRP1/elevated RAGE) interferes with normal Aβ clearance and likely contributes to AD pathogenesis. Other molecular alterations at the BBB include decreased expression of the glucose transporter GLUT-1 (Mooradian et al., 1997). This correlates with reduced cerebral glucose uptake in MCI individuals and pre-clinical AD patients that precedes brain atrophy in PET imaging studies (Hunt et al., 2007, Samuraki et al., 2007). Pathological changes among cells of the NVU also contribute to BBB dysfunction in AD. Sagare and colleagues (2013a) have recently reported that pericyte loss in AD mouse models increases BBB permeability, elevates brain Aβ levels and accelerates progression of CAA and neuronal loss. The AD risk factor APOE also influences BBB integrity in an isoform-dependent manner – wild-type astrocyte-derived APOE enhances TJ integrity by activating the PKCη pathway and Thr-phosphorylation of
occludin (Nishitsuji et al., 2011). APOE ε4 carriers may be more susceptible to BBB breakdown as APOE4 is less efficient at activating this PKCη pathway. Bell and colleagues (2012) also found that neurovascular leakage and BBB breakdown in APOE4-expressing knock-in mice is caused by activation of the proinflammatory cytokine cyclophilin A (CypA) in pericytes (Fig. 1.13). This leads to nuclear translocation of NF-κB and increased expression of the TJ-degrading enzyme MMP9 (matrix metalloproteinase 9) (Fig. 1.13, Bell et al., 2012). In contrast, APOE3 interaction with LRP1 on pericytes suppressed this inflammatory pathway and promoted TJ assembly (Bell et al., 2012). While endothelial cells and other components of the NVU may respond to initial extracellular Aβ accumulation by enhancing clearance mechanisms, severe cerebrovascular dysregulation in response to chronic Aβ exposure (and combined with underlying genetic risk factors for BBB disruption) is likely to compromise the BBB and exacerbate neurodegeneration and cognitive impairment in AD.

TJ-associated BBB dysfunction is well established in a host of neurological disorders such as multiple sclerosis, stroke and cerebral oedema. In relation to AD, Aβ-mediated alterations in TJ expression or localization and concomitant increases in BBB permeability in cell culture models have been reported by several groups. For example, Gonzalez-Velasquez et al. (2008) found that Aβ1-40 treatment triggered ZO-1 relocation from the plasma membrane in human brain endothelial cells while Tai et al. (2010) reported decreased occludin expression and increased permeability following Aβ1-40 addition to endothelial cells. Some studies have also assessed BBB and TJ integrity in vivo. Abnormal TJ protein distribution and morphology have been observed in CAA patients and in APP transgenic mice (Hartz et al., 2011; Carrano et al., 2011; Biron et al., 2011). However questions remain as to whether BBB and TJ dysfunction is observed throughout the AD brain vasculature and whether it is specific to AD or a common feature of all neurodegenerative disorders. In addition, while much attention has focused on understanding transcellular movement of Aβ, relatively little is known with regard to potential paracellular flux of Aβ in normal, presymptomatic or AD-affected individuals. To this end, previous work in the host laboratory using RNA interference (RNAi) to modulate TJ protein levels provides a means to test potential paracellular involvement in BBB transport of Aβ in homeostatic and AD neural environments.
1.5. RNA interference (RNAi): therapeutic modulation of TJs at the BBB

RNA interference (RNAi) is the process by which exogenous or endogenous small RNA molecules facilitate suppression of sequence-specific mRNA targets and prevent translation. The RNA interference (RNAi) phenomenon was first discovered by Fire and Mello (1998) over a decade ago and following the first in vivo proof-of-principle of siRNA-mediated gene silencing in mice infected with hepatitis C virus, a vast array of publications have validated the ability of RNAi to target disease-causing genes and suppress negative phenotypes in a host of in vitro and preclinical animal models of cancer, neurodegenerative disorders and other viral diseases (McCaffrey et al., 2002, Keaney et al., 2011). The advancing RNAi field has focused predominantly on applying RNAi to functional genomics and suppression of harmful or pathogenic proteins for therapeutic benefit. However a new approach has emerged in which RNAi is not used as the primary therapeutic agent, but instead targets the TJ protein claudin-5 to modulate BBB/iBRB paracellular permeability. This has been shown to facilitate water efflux across the BBB and the delivery of small molecule drugs across the BBB or iBRB to treat a host of neuro- and retinal-degenerative disorders.

1.5.1. Mechanism of RNAi

The three main RNAi effector molecules that induce post-transcriptional gene silencing (PTGS) are small interfering RNA (siRNA), short hairpin RNA (shRNA), and micro RNA (miRNA) (Fig. 1.14, Wang et al., 2010). Unlike synthetic siRNAs, miRNAs are imperfectly paired hairpin RNA structures that are encoded in the genome to control development and differentiation (Kim and Rossi, 2007). These dsRNAs are expressed as long primary miRNAs or pri-miRNAs in the nucleus, cleaved by the endoribonuclease Drosha into a stem-loop pre-miRNA of around 70nt and then transported to the cytoplasm by exportin-5 where a second endoribonuclease Dicer produces the mature ~22nt miRNA. Exogenous introduction of synthetic siRNA triggers activation of the endogenous PTGS mechanism by mimicking the product of Drosha and Dicer processing of pri-miRNAs (Kim and Rossi, 2007). Both pathways converge at the cytoplasmic RNA-induced silencing complex (RISC) where the antisense or guide strand of short RNA duplexes is incorporated into this multi-protein enzymatic scaffold (Fig. 1.14). RISC activation involves cleavage of the sense or passenger strand by its catalytic component Argonaute 2 (Ago2), generating the single-stranded antisense sequence that directs RISC
to complementary sequences in target mRNA (Grimm, 2009). This can induce gene silencing in one of two ways: completely complementary sequence-specific cleavage of the target mRNA in the case of siRNA or translational repression and RNA degradation due to imperfect matching of miRNA to its target mRNA 3' untranslated region (3' UTR) (Grimm, 2009). Ago2 cleaves target mRNA between bases 10 and 11 relative to the 5' end of the guide siRNA and the cleaved mRNA is degraded by cellular exonucleases (Meister et al., 2004, Keaney et al., 2011). Since the intracellular concentration of siRNA is diluted out as the cell undergoes division following transfection, siRNAs only transiently suppress gene expression. Maintaining stable siRNA expression can be achieved by engineering modified siRNAs to increase the stability and longevity of suppression or alternatively through the expression of shRNAs under the control of RNA polymerase promoters in DNA vectors (Brummelkamp et al., 2002). The resulting hairpin structure is processed intracellularly by Dicer to form functional siRNA. Since miRNAs are only partially complementary to the target mRNA, a single miRNA is likely able to interact and regulate the expression of hundreds of genes. However due to the potent knockdown of gene expression that follows the perfect match of siRNA and target mRNA, synthetic siRNA duplexes are primarily used to trigger PTGS in therapeutic applications of RNAi (Wang et al., 2010).

1.5.2. RNAi-mediated neuronal barrier modulation

It has been estimated that up to 98% of low-molecular weight drugs with known chemotherapeutic, neuroprotective, antiapoptotic or antineovascular potency are rendered almost useless as they cannot cross the BBB (Pardridge, 2009). This is due to a number of properties intrinsic to CNS endothelial cells as already highlighted: (1) a lack of fenestrae or small pores that allow rapid passage of molecules in peripheral endothelium; (2) few transcytotic vesicles thereby limiting the transcellular flow of molecules from the blood to the brain; (3) the presence of p-glycoprotein and other efflux pumps; (4) low rates of paracellular diffusion due to the high electrical resistance TJs. By restricting the paracellular passage of damaging blood-borne agents into neuronal tissue, TJs confer an essential protection at the interface between the blood and CNS but also limit the delivery of many systemically-deliverable small molecule therapeutic drugs. The finding of BBB size-selectivity in mice lacking claudin-5 highlighted the possibility of using the RNAi platform to target claudin-5 more transiently and exploit potential size-selectivity to deliver low-molecular weight drugs to the CNS.
I.5.2.1. Drug delivery

Using systemically injected siRNA targeting claudin-5, the first demonstration of RNAi-mediated modulation of the BBB was reported in mice by Campbell et al. (2008). Levels of claudin-5 mRNA showed significant decreases between 24 and 48 hours post-siRNA delivery with maximum claudin-5 protein suppression occurring 48 h after injection compared to uninjected, phosphate buffered saline (PBS), and nontargeting (NT) control siRNA-injected mice (Fig. 1.15A). This time window allowed for increased permeability of the BBB to a range of molecules up to 1 kDa in size including the contrasting agent gadolinium (Gd-DTPA, 742 Da). However, a molecule of 4.4 kDa in size (fluorescein isothiocyanate-labeled dextran, FD-4) was excluded at this time point. Crucially, this RNAi-mediated BBB permeability was not only size-selective but also reversible. Levels of claudin-5 returned to normal 72h post-siRNA injection and accordingly, BBB integrity was restored with Hoechst stain being unable to passively diffuse across the BBB. This technique was also applied to successfully deliver a low-molecular weight drug (thyrotropin releasing hormone) to neuronal tissue. In light of the ability of RNAi to transiently and size-selectively remodel the BBB, the same approach was used to modulate the molecularly-similar iBRB in animal models of retinopathies (Campbell et al., 2009; Tam et al., 2010). After establishing that the kinetics of claudin-5 suppression at the iBRB mirrored that of the BBB and initiated the same pattern of permeability, visual function was improved in mouse models of retinitis pigmentosa (RP) and in a light-induced retinal degeneration model following iBRB modulation and systemic delivery of relevant small molecule drugs. Since chronic diseases such as RP and age-related macular degeneration (AMD) would require long-term RNAi responses and drug applications but also short-lived remodeling of the functionally critical BBB and iBRB, Campbell and colleagues (2011) also developed a doxycycline-inducible shRNA system for transient knockdown of claudin-5 levels (Fig. 1.15B). The claudin-5 shRNA sequence under the control of a doxycycline-sensitive promoter was incorporated into the genome of an adeno-associated virus (AAV)-2/9 vector that can stably persist in retinal or brain endothelial cells following a single localized injection and therapeutic benefit was achieved in a laser-induced model of choroidal neovascularization (CNV), a hallmark of wet AMD. Using two well characterized and clinically approved drugs, 17-AAG (585 Da) and Sunitinib malate (532 Da), confocal scanning laser microscopy confirmed significant reductions in CNV volumes following systemic drug treatment compared to the NT shRNA-treated contralateral eye (Fig. 1.15C).
1.5.2.2. Water efflux

While RNAi-mediated barrier modulation has been shown to enhance the delivery of intravenously-applied low-molecular weight therapeutics into neuronal tissue, recent work in the host laboratory has found that temporary and size-selective TJ modulation can also be used to enhance the movement of water in the opposite direction, from the brain to the blood, in a mouse model of traumatic brain injury (TBI) (Campbell et al., 2012). TBI is marked by a build-up of water within the brain as a result of shifts in water diffusion across the BBB and the resulting cerebral oedema leads to dangerous increases in intracranial pressure and other secondary complications (Fig. 1.15D). In a cold-induced model of cerebral oedema, systemic administration of claudin-5 siRNA post-injury increases the paracellular diffusion of extra-neural water out of the brain for a period of between 24 and 72 hours and decreases lesion volume (Fig. 1.15E). This was accompanied by improvements in cognitive function in claudin-5 siRNA-injected mice as assessed by T-maze and neurological severity scores.

Increased paracellular drainage of water out of the brain following RNAi-mediated BBB modulation in a model of cerebral oedema indicated a potential role for TJ modulation in other settings. As outlined, the small Aβ peptide (approximately 4.4 kDa in size) is the major pathogenic component of AD where it builds up in the brain parenchyma and around the cerebral blood vessels. Regulating TJ levels using RNAi provides a tool to assess potential paracellular movement of Aβ across the BBB.

1.6. Experimental treatments for AD

In light of combined findings from AD genetics, amyloid/tau biochemistry, AD mouse models and patient brain imaging, proponents of the amyloid cascade hypothesis have proposed that targeting Aβ represents the most logical and promising therapeutic course in AD treatment. To date, methods based on decreasing Aβ production, increasing its degradation or enhancing its clearance from the brain have already been clinically tested or are presently at pre-clinical or human clinical trial stages. What is becoming clear is that rigorous clinical trial design will need to address a number of issues – a drug’s specificity for Aβ, its ability to target various Aβ species, early targeting of Aβ in the disease course and reliable biomarker and clinical measurements of outcome such as amyloid imaging. Recent insights from early-onset familial AD patients indicate that Aβ
accumulation, synaptic dysfunction and brain atrophy occur years before cognitive symptoms appear or are diagnosed (Bateman et al., 2012).

1.6.1. Approved drugs for treatment of AD
While glutamatergic neurons of the hippocampus and entorhinal cortex are the major neuronal populations first affected in AD, dysfunction and death of cholinergic neurons in the basal forebrain also occurs in AD (Holtzman et al., 2011). Acetylcholine is a neurotransmitter thought to be involved in plasticity, memory and attention in the CNS. This ‘cholinergic hypothesis’ of AD was first proposed in the early 1980s and spawned the development of drugs that inhibit cholinesterase, the enzyme that breaks down acetylcholine into inactive metabolites (Coyle et al., 1983). Of the 5 FDA-approved drugs currently available for AD treatment, four are cholinesterase inhibitors (donepezil, galantamine, rivastigmine, tacrine) (Table 1.2). However these small molecule drugs only provide a modest and short-lived benefit to AD cognitive symptoms and do not address the underlying pathophysiology (Holtzman et al., 2011). The other FDA-approved drug is memantine, a noncompetitive antagonist of NMDA glutamate receptors, which likely acts by blocking the effects of glutamate excitotoxicity found in AD (Areosa et al., 2005).

1.6.2. Current AD clinical trials
Based on the amyloid hypothesis of AD etiology and neuronal damage, researchers and pharmaceutical companies began to pursue novel therapeutics designed to inhibit Aβ production or enhance its clearance. Therapies based on reducing Aβ production by inhibiting the γ- or β-secretases that cleave the APP protein have had mixed results. Preclinical data for the γ-secretase inhibitor (GSI) semagacestat showed promising results with reduced Aβ deposition in transgenic human APP mice (Ness et al., 2004). However having reached phase III human clinical trials, semagacestat was abandoned as treated patients had increased cognitive deterioration and other complications compared to placebo-treated patients (Doody et al., 2013). This worsening cognitive impairment has been attributed in part to inhibition of cleavage of other γ-secretase substrates such as Notch, a protein involved in cell fate decisions in the brain (Holtzman et al., 2011). Nevertheless, specific γ-secretase modulators (GSM) that do not affect Notch cleavage are still being developed and have been shown to shift APP cleavage away from toxic Aβ1-42 to generate shorter Aβ species (Kounas et al., 2010). One such GSM, EVP-0962, is currently in phase II trials in patients with MCI. β-secretase (BACE) inhibitors on the
other hand represent a more attractive target as BACE has fewer substrates and these compounds act by stopping the BACE-mediated generation of the C99 fragment from APP, upstream of γ-secretase cleavage, that eventually gives rise to toxic Aβ peptides (Karren et al., 2011). MK-8931, a small molecule inhibitor of BACE1 and BACE2 developed by Merck, is currently in phase III trials in prodromal AD patients (MCI due to AD) (Table 1.2).

Enhancing Aβ clearance via passive immunization of AD patients with intravenous infusions of monoclonal antibodies (mAb) targeting Aβ is another common strategy that has also provided mixed outcomes thus far. The mechanism of action for anti-Aβ mAbs is still not fully understood but likely involves three non-mutually exclusive modes of action: (1) disruption of Aβ aggregation/neutralisation of Aβ monomers, oligomers, and other species; (2) Fc-receptor-mediated phagocytosis of Aβ by microglia with or without cytokine production and (3) sequestration of circulating plasma Aβ that enhances Aβ efflux from the brain to the plasma - the 'peripheral sink hypothesis' (Weiner and Frenkel, 2006, DeMattos et al., 2001). In two large phase III trials of bapinezumab (Johnson & Johnson/Pfizer), a mAb directed against the N-terminus of Aβ that binds soluble and fibrillar Aβ and activates microglial phagocytosis and cytokine release, there was no improvement in cognitive tests in mild-to-moderate AD. Side-effects included amyloid-related imaging changes such as vasogenic oedema likely due to inflammatory cytokine release (Salloway et al., 2014). Also this year, two phase III trials of solanezumab (Eli Lilly), a mAb directed against the mid-domain of Aβ and which binds monomeric but not fibrillar Aβ, had no effect on cognition or functional ability in moderate AD but showed improved cognition in mild AD (Doody et al., 2014).

The problems encountered with targeting Aβ thus far have largely arisen because the clinical trials have involved mid-to-end stage AD patients. Huge levels of synaptic and neuronal loss have already occurred before the cognitive symptoms in these patients at which point eliminating Aβ is probably too late (Goldsworthy and Vallence, 2013). The genetic and environmental background, for example the presence of mitigating APOE ε4 alleles in certain patients, also affects study design and outcome. Elderly patient populations in AD trials mean increased numbers of co-morbidities and thus the potential for interference with the experimental drug from other ongoing drug treatments. Moreover, elderly patients are more prone to side-effects due to aging-related changes in
metabolism (Huang and Mucke, 2012). There are also questions regarding the predictive value of pre-clinical drug testing based on transgenic APP mouse models where human APP and Aβ are overexpressed. These mouse models do not reflect the majority of sporadic AD cases where Aβ is inefficiently cleared rather than over-produced. In addition, neuronal loss is absent in the majority of human APP mouse lines (Ashe and Zahs, 2010). Therefore, targeting a single factor like Aβ may provide therapeutic benefit in APP mouse models whereas the multifactorial pathophysiology in human AD (Aβ, tau, neuroinflammation, neurotransmitter disruption, synaptic/neuronal degeneration) may require a combinatorial drug therapy approach (Huang and Mucke, 2012).

New clinical trials are already being established to test the amyloid cascade hypothesis in pre-symptomatic AD patients (either familial or sporadic cases), a goal that will incorporate and depend heavily on better diagnostic techniques including MRI scans, new amyloid-binding radiotracers for PET imaging and CSF biomarkers of Aβ and tau for earlier AD diagnoses (Goldsworthy and Vallence, 2013). The cohort of participants recruited to the Dominantly Inherited Alzheimer’s Network (DIAN) and Alzheimer’s Prevention Initiative (API) studies are symptom-free offspring of parents that developed familial AD. DIAN will test solanezumab (following the small cognitive benefit in mild AD and good safety record) along with another anti-Aβ monoclonal gantenerumab (Roche) that interacts with both parenchymal and vascular Aβ aggregates. API will use crenezumab (Genentech), an anti-Aβ mAb that binds to oligomeric and fibrillar Aβ with high affinity (Corbyn, 2013). In contrast, the A4 trial is recruiting elderly but clinically normal participants who have Aβ plaques based on PET imaging and will treat patients with solanezumab (Corbyn, 2013). Early PET imaging and biomarker data from these studies supports the concept that AD has a long pre-symptomatic phase with changes in Aβ, tau and neuronal function detected 10-20 years prior to expected disease onset (Bateman et al., 2012, Fagan et al., 2014). Nevertheless, the clinical results for the anti-Aβ mAbs in these preventative trials will likely decide the future of Aβ as the primary pathogen in AD.

1.6.3. Pre-clinical BBB-based therapies for AD

Over $1 billion has already been spent on clinical trials of monoclonal antibodies against Aβ that have ultimately failed to halt the progression of AD to date (Yu and Watts, 2013). An often overlooked but fundamental reason for failure in immunotherapy trials in AD is
the poor penetration of therapeutic antibodies across the BBB. Indeed, it has been
estimated that only 0.1-1% of circulating antibodies actually cross the BBB and enter the
brain parenchyma or CSF at steady-state concentrations (Podulso et al., 1994). This has
necessitated high doses of monoclonal antibodies in AD clinical trials as well as the need
for experimental mAbs to have high affinities to their target. Improved antibody uptake in
the brain would increase clinical efficacy of mAbs and allow reduced doses to limit side-
effects. This has fueled the development of BBB ‘Trojan horse’ approaches that take
advantage of receptor-mediated transcytosis (RMT) at the BBB (Yu and Watts, 2013). To
this end, much attention has focused on the transferrin receptor (TfR) that is highly
expressed in the CNS endothelium. Two groups working together in Genentech recently
reported the design of a bispecific antibody that binds TfR with one arm and binds
BACE1 with the other arm (Atwal et al., 2011, Yu et al., 2011). This bivalent anti-
TfR/BACE1 showed increased brain uptake and significantly reduced brain Aβ levels in
mice after a single intravenous injection compared to a monospecific BACE1 antibody
without a TfR-binding site. Interestingly, antibodies with low-affinity to TfR crossed the
BBB more efficiently (Yu et al., 2011). The authors found that high-affinity TfR
antibodies cannot readily detach from TfR once inside the endothelial cells and this
reduces the payload release on the brain side. This molecular ‘hitchhiking’ on TfR was
also recently adopted by Niewoehner and colleagues (2014) who designed an anti-TfR Fab
fused to the C-terminus of an anti-Aβ antibody. This approach uses only the specific
antigen-binding fragment (Fab) of anti-TfR and intravenous administration of this anti-
TfR(Fab)/Aβ antibody resulted in a 55-fold increase in amyloid plaque engagement
compared to the unmodified Aβ antibody (Niewoehner et al., 2014). If proven safe and
tolerable in primate models, applying these BBB ‘Trojan horse’ strategies to AD trials
may allow improved anti-Aβ delivery to the CNS and better clinical outcomes.

Altered levels of Aβ receptors and transporters in AD microvessels has also highlighted
potential therapeutic targets for ‘kick-starting’ the neurovascular clearance of Aβ in AD
patients. As described, the cell surface receptor RAGE mediates Aβ influx across the
BBB and RAGE expression is increased in brain endothelium and neurons of AD patients
(Deane et al., 2003). Based on this knowledge, Deane et al. (2012) identified a high-
affinity small molecule inhibitor of RAGE called FPS-ZM1 that binds specifically to the
V domain of RAGE and disrupts RAGE-Aβ interactions. FPS-ZM1 was able to cross the
BBB and block RAGE-mediated influx of Aβ at the BBB and in neurons. As a result,
treatment of APP transgenic mice with this RAGE inhibitor markedly reduced Aβ load and improved CBF rates and cognitive performance (Deane et al., 2012). Conversely, levels of the abluminal cell surface receptor LRPI are decreased in AD microvessels (Bell et al., 2009). Furthermore, the N-terminus of transmembrane LRPI can be cleaved to release soluble LRPI (sLRPI) that binds about 70-80% of plasma Aβ (Sagare et al., 2007, Zlokovic, 2011). In MCI and AD patients, plasma sLRPI levels are reduced and also oxidized which increases the amount of freely circulating Aβ which can re-enter the brain (Sagare et al., 2007, Sagare et al., 2011). Having identified cluster IV as the region of sLRPI that binds to Aβ, Sagare and colleagues (2013b) created a library of recombinant sLRPI cluster IV (LRP-IV) fragments. One such LRP-IV fragment, D3674G, showed enhanced binding to Aβ relative to other sLRPI ligands and subcutaneous treatment of APP mice with LRP-IV(D3674G) reduced brain and CSF Aβ levels and enhanced CBF and hippocampal function (Sagare et al., 2013b). These preclinical Aβ clearance therapies represent novel AD strategies and could be deployed in combination with secretase inhibitors and anti-Aβ mAbs for clinical benefit.

1.7. Objectives of this study

TJ protein complexes of the BBB endothelium play a critical role in neuronal homeostasis by restricting the paracellular flux of ions, macromolecules and immune cells. However, insights from studies of innate immunity in the CNS are changing the long-held view that the BBB is a ‘brick-wall’ guarding the neuronal tissue. Dynamic and regulated changes in TJ levels and distribution allow the paracellular recruitment of leukocytes into the CNS to assist neural protection and repair (Singer et al., 2014, Lampron et al., 2013). In AD for example, bone marrow-derived microglia are known to be crucial for restricting senile plaque formation (Simard et al., 2006). In addition, TJ plasticity at the BBB is observed in neural tissue surrounding the site of TBI where TJ protein levels change in response to fluid accumulation in the brain extracellular space (Nag et al., 2007, Campbell et al., 2012). However, a plethora of research has also suggested that both acute and chronic TJ disruption is detrimental in neurological conditions such as stroke and multiple sclerosis, increasing paracellular permeability and exacerbating neuronal damage (Knowland et al., 2014, Luissint et al., 2012). Therefore, a balance seems to exist in neural disease between loosening the paracellular pathway at the BBB to deal with injury while preventing entry of potentially damaging blood-borne molecules. In this regard, the status and consequences of changes to the BBB, in particular the TJs, in Alzheimer’s disease (and
its accompanying neurovascular pathology CAA) are not well-understood. Pathological changes in the BBB transcellular receptors of Aβ in AD suggests that BBB disruption significantly contributes to the disease process but whether BBB permeability is increased in AD has not been fully resolved (Erickson and Banks, 2013).

Among the aims of this study was to characterize TJs at the BBB in post-mortem human brains of AD and CAA patients. TJ status in these patients was also compared to BBB TJs in other neurological and neurodegenerative conditions such as progressive supranuclear palsy (PSP) and ALS to determine whether TJ changes are specific to AD or a common feature of neurological disease generally. To further understand the molecular architecture of TJs in AD, primary brain endothelial cells and brain tissue sections were obtained from transgenic human APP-Swe mice, a model that mimics various aspects of AD and CAA such as Aβ accumulation, synaptic dysfunction and memory deficits (Hsiao et al., 1996). Previous studies have suggested that Aβ can alter TJ protein levels in vitro (Hartz et al., 2011, Gonzalez-Velasquez et al., 2008, Tai et al., 2010). However, the nature and kinetics of these TJ changes and identification of the precise Aβ species that alter TJ levels is not well studied. Soluble synthetic Aβ1-40 monomers and dimers, specifically designed so as not to aggregate, were screened in mouse brain endothelial cells to understand the role of specific Aβ species in TJ regulation. Based on these findings, the movement of Aβ across brain endothelial cells in vitro and in vivo was explored in a second aspect of this study. While much attention has focused on understanding transcellular movement of Aβ, relatively little is known with regard to potential paracellular flux of Aβ in normal, presymptomatic or AD-affected individuals. To this end, previous work in the host laboratory using RNA interference (RNAi) to modulate TJ protein levels provided a means to test paracellular involvement in BBB transport of Aβ in vitro and in homeostatic and AD neural environments in vivo.
Figure 1.1: Barriers of the CNS. (a) The blood-brain barrier (BBB) is located at the level of endothelial cells lining the cerebral microvessels and is the major site for blood-brain molecular exchange since a single neuron is never usually more than 20 μm away from a capillary. (b) The blood-cerebrospinal fluid barrier (BCSFB) is found along the epithelium of the choroid plexuses of the brain’s ventricular system. It helps to separate the CSF secreted by the choroid plexus epithelium from the brain vasculature and neural tissue. (c) The arachnoid barrier is formed by the epithelium of the arachnoid layer of the meninges and completely surrounds the brain. Arachnoid villi can make contact with the venous blood vessels of the sagittal sinuses positioned within the meninges but fluid exchange is limited to CSF movement out of the brain and into the blood. At all three cellular barriers, tight junction (TJ) complexes help to regulate and limit paracellular (intercellular cleft) permeability though it is the high electrical resistance TJs of the BBB endothelium that help form the tightest barrier in the brain. (Source: Abbott et al., 2010).
Figure 1.2: The BBB and neurovascular unit (NVU). (A) Goldmann's trypan blue experiments highlighted the existence of an impermeable barrier separating the blood from the brain and CSF. (Source: Zlokovic, 2008). (B) Cell associations of the NVU. Endothelial cells line the capillary lumen and are sealed together by TJ complexes that restrict paracellular diffusion between the cells. The finger-like processes of pericytes partially surround the microvessel endothelium while astrocyte end-feet also encircle the capillaries. Axonal projections from neurons can signal to nearby smooth muscle cells and modulate cerebral blood flow while microglia are the brain's resident macrophages and function to scavenge foreign material, damaged cells and plaques. (Source: Abbott et al., 2010).
Angiogenesis and barrier induction

Interactions of CNS endothelial cells with parenchymal cells to seal the barrier

Maturation and maintenance

Figure 1.3: The three main stages of BBB development and maturation. (A) Invasion of vascular sprouts into the neuroectoderm is driven by factors released by neural progenitor cells. To date, the VEGF and Wnt/β-catenin pathways along with the receptor GPR124 are recognized as the major driving forces in early CNS angiogenesis. (B) In combination with embryonic neural vascularization, new blood vessels very quickly gain characteristics of the BBB in response to cellular cross-talk with incoming pericytes (through PDGFβ and TGFβ signaling) and astrocytes (through SHH and ANG-I signaling). (C) BBB maturation and sealing of the interendothelial TJs is achieved by ongoing interactions between cells of the NVU. For example, ApoE and ANG-II secretion from astrocytes are important factors in TJ stability. The integrity of the adult BBB and hence vascular permeability can be disrupted if this NVU cross-talk breaks down as in disease. (Source: Obermeier et al., 2013).
Figure 1.4: Transport pathways across the BBB. (A) TJ protein complexes seal the paracellular gap between neighboring endothelial cells and restrict the movement of water-soluble molecules. (B) Lipid-soluble molecules with a molecular weight < 200 Da can passively diffuse across the phospholipid membranes of CNS endothelium. (C) Carrier-mediated movement of glucose, amino acids and nucleosides involves specific transport proteins and can be unidirectional or bidirectional depending on the substrate’s concentration gradient. (D) Receptor-mediated transcytosis (RMT) is used to transport a variety of macromolecules like insulin, transferrin, IgG and Aβ across the BBB. It involves ligand binding to a receptor, endocytosis and transport of the receptor-macromolecule complex within a vesicle followed by dissociation of the complex and exocytosis of the ligand. (E) Similar to RMT, adsorptive transcytosis involves the cationization of a plasma protein such as albumin and binding of this positively charged molecule to sites along the negatively charged lipid membrane. This induces internalization and transcytosis of the ligand across the cell cytoplasm within a vesicle. (Source: Abbott et al., 2006).
Figure 1.5: Molecular organization of the BBB tight junctions. (A) Occludin and claudin-5 are the major transmembrane TJ components at the BBB and are linked to the actin cytoskeleton via the ZO family of intracellular scaffold proteins and other accessory proteins such as cingulin (not pictured here). Other postulated CNS TJ components including tricellulin and LSR are not represented here. (Design credit: Martin Keaney). (B) The structure of claudin proteins including the four transmembrane domains, two extracellular loops (ECLs) and one intracellular loop. The PDZ motif is highlighted in black at the C-terminus domain while the conserved and functionally important W-GLWC-C motif is highlighted in the first ECL. (Source: Van Itallie and Anderson, 2004).
Table 1.1: Pathways involved in tight junction formation and maintenance. (Adapted from Obermeier et al., 2013).

<table>
<thead>
<tr>
<th>NVU Cell Type</th>
<th>Pathway (Ligand/Receptor)</th>
<th>Effect on TJs</th>
</tr>
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<tbody>
<tr>
<td>Neural Progenitors/</td>
<td>Wnt/Frizzled</td>
<td>Up-regulation of claudin-3</td>
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<tr>
<td>Astrocytes/Pericytes</td>
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<tr>
<td>Pericytes</td>
<td>PDGFβ/PDGFRβ</td>
<td>Up-regulation of occludin and</td>
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<td>claudin-5</td>
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<tr>
<td>Pericytes</td>
<td>TGFβ/TGFβ</td>
<td>Induction of claudin-5 expression</td>
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<td>(in vitro)</td>
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<tr>
<td>Astrocytes</td>
<td>SHH/PTC1</td>
<td>Induction of occludin and claudin-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>expression</td>
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<tr>
<td>Astrocytes</td>
<td>ANG-1/TIE-2</td>
<td>Upregulation and subcellular</td>
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<tr>
<td></td>
<td></td>
<td>localization of TJ proteins</td>
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<tr>
<td>Astrocytes</td>
<td>ANG-II/AT1</td>
<td>Post-translational modification of</td>
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<tr>
<td></td>
<td></td>
<td>occludin</td>
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<tr>
<td>Basement membrane</td>
<td>Laminin/β1 integrin</td>
<td>Maintenance of claudin-5 expression</td>
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<td>(in vitro)</td>
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* TJ phosphorylation pathways

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<tr>
<td>c-PKC, n-PKC</td>
<td>Ser/Thr-phosphorylation</td>
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<td>of claudin-5 and occludin</td>
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<td>and changes in paracellular</td>
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<td>permeability</td>
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<tr>
<td>Glutamate, VEGF</td>
<td>Tyr-phosphorylation of</td>
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<td></td>
<td>claudin-5 and occludin</td>
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<td>and increased</td>
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<td>paracellular permeability</td>
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<tr>
<td>RhoA</td>
<td>Actin cytoskeleton and TJ</td>
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<td>disassembly</td>
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Figure 1.6: Neuropathology of AD and APP processing. (A) Significant brain atrophy in post-mortem brain section from an AD patient compared to control brain. (B) Identification of a neuritic amyloid plaque (dashed lines) and NFT (large arrow) in a high-power photomicrograph. (C) Anti-Aβ staining revealing diffuse plaques (large arrow), compact plaques (medium) and CAA (small). (D) Anti-phospho-tau staining revealing tau accumulation in neuronal cell bodies (large arrow) and processes (small). (E) Thioflavin-S staining of plaques (large arrow) and NFTs (small) revealing their β-pleated sheet structures. (Source: Holtzmann et al., 2011) (F) Under physiological conditions, most APP is directed down the non-amyloidogenic pathway and is processed by α-secretase. In AD, APP is predominantly processed by β-secretase (BACE) and γ-secretase generating short neurotoxic Aβ peptides. (Source: LaFerla et al., 2007).
Figure 1.7: Sequence of Aβ and its position within the APP transmembrane protein (red). Shown along the top sequence is the β-secretase cleavage site (left) and the various positions at which γ-secretase can cut the β-C-terminal fragment (β-CTF) of APP, generating a pool of Aβ peptides. These peptide fragments vary in their length and propensity to aggregate. Along the bottom sequence are the various mutations in APP that alter Aβ production and have been identified to cause familial AD and CAA. (Source: Benilova et al., 2012).
**Figure 1.8:** The genetic landscape of AD. In the top left corner, rare disease-causing mutations in *APP*, *PS1* and *PS2* were among the first major genes to be implicated in AD. These mutations segregate in a Mendelian (autosomal dominant) fashion and cause AD in virtually all mutant carriers. Positioned towards the centre are gene variants that strongly increase the risk of AD by approximately 5-20% - *TREM2* (rare) and *APOE ε4* (more common). In the bottom right corner, GWAS studies have identified over 20 common variants in genetic loci that slightly increase AD risk (1-2%) at a population level. Future whole-genome and whole-exome sequencing analysis may go some way to identifying additional rare variants that increase AD risk. (Source: Guerrierio et al., 2013a).
Figure 1.9: Aβ polymerization and soluble Aβ oligomers in AD. (A) Interconversion of various Aβ species. In the nucleation phase, misfolded Aβ monomers can polymerize to form Aβ dimers and higher order oligomers. In the elongation phase, oligomer assemblies can act as seeds for further addition of monomers to form amyloid fibrils. These fibril structures consist of cross-β-sheet units of Aβ peptides and exist in a complex equilibrium with monomers and oligomers. (Source: Kumar and Walter, 2011). (B) At synapses, several proposed Aβ receptors are thought to bind Aβ oligomers and activate downstream signaling cascades that ultimately lead to loss of synapses and dendritic spines. Murine PirB (paired immunoglobulin-like receptor B) and its human ortholog LlrB2 (leukocyte immunoglobulin-like receptor B2) mediate Aβ oligomer-induced impairment of hippocampal long-term potentiation and loss of synaptic plasticity in the visual cortex. Other receptors like PrPC (cellular prion protein), EphB2 (ephrin type B receptor 2) and NMDA (N-methyl-D-aspartate receptor) mediate the effect of Aβ oligomers on calcium (Ca^{2+}) influx and NMDA receptor-mediated long-term potentiation. (Source: Benilova and de Strooper, 2013).
Figure 1.10: The ‘lymphatic’ system in the brain. (A) Pathways of CSF flow bathing the brain. CSF is produced in the choroid plexus of the lateral ventricles (i) and flows along the subarachnoid space surrounding the brain (ii). CSF can exit the subarachnoid space and enter the bloodstream through arachnoid villi that project into the venous sinuses (iii) located in the dura mater (the outermost layer of the meninges). In a recently discovered pathway, CSF can also enter the extracellular space in the brain through paravascular spaces surrounding large arteries (iv).

(B) CSF enters the brain parenchyma along Virchow-Robin spaces surrounding arteries (i). With the assistance of astrocytes, it moves through the extracellular space (ii) and mixes with the ISF containing extracellular solutes and metabolites like Aβ (iii). It can then be cleared along paravenous drainage pathways (iv). (Source: Strittmatter, 2013).
Figure 1.11: Transcellular Aβ movement across the BBB. Cell-surface LRP1 on the abluminal (brain) side of endothelial cells binds free Aβ species (monomers, oligomers) and possibly Aβ bound to APOE from the brain ISF. This initiates transcytosis and efflux of Aβ across the BBB into the systemic circulation where soluble LRP1 (sLRP1) binds over 70% of Aβ in the plasma and transports it to the liver for clearance. On the luminal (blood) side, any Aβ not bound to sLRP1 can interact with RAGE and this mediates influx of peripheral Aβ into the brain parenchyma. Aβ/RAGE interaction can also cause release of proinflammatory cytokines like TNF-α and IL-6 and upregulation of ICAM1 and VCAM adhesion molecules through NF-κB nuclear translocation. Down-regulation of LRP1 and up-regulation of RAGE have been reported in AD patients and AD mouse models and this faulty clearance/increased re-entry of Aβ across the BBB contributes to Aβ buildup around cerebral blood vessels (CAA) and in the brain parenchyma. (Source: Zlokovic, 2008).
Figure 1.12: CAA development in capillaries (top) and arteries (bottom). Capillary-associated CAA (top) is specific to type-1 CAA cases with Aβ deposits closely associated with the outer basement membrane. Severe capillary-associated CAA can cause capillary occlusion and reduce CBF. ‘Classical’ CAA involves Aβ accumulation around cerebral arteries and veins and is found in type-1 and type-2 (no capillary involvement) CAA. Aβ is deposited within the basement membrane and VSMC layer of these larger vessels and can cause VSMC degeneration and cerebral hemorrhage. (Source: Thal et al., 2008).
Figure 1.13: APOE contribution to BBB integrity. Astrocyte-derived APOE3 can signal through the LRP1 receptor on pericytes and inhibit the proinflammatory CypA/NF-κB/MMP9 pathway. For reasons not yet elucidated, APOE4 is less efficient in signaling through LRP1 to dampen this proinflammatory pathway. Elevated CypA cytokine levels cause nuclear translocation of NF-κB and this in turn leads to increased MMP9 expression. This proteinase acts on the basement membrane and endothelial TJs of the NVU causing TJ/basement membrane degradation and BBB breakdown. (Source: Bell et al., 2012).
Figure 1.14: Mechanism of RNAi induction by siRNA (left) or shRNA (right). Application of double-stranded siRNA molecules (left) or DNA-based shRNA expression vectors (right) converges at the cytoplasmic RNA-induced silencing complex (RISC). Here the antisense or guide strand of short RNA duplexes is incorporated into this multi-protein enzymatic scaffold and directs RISC to complementary sequences in target mRNA. Once Watson-Crick base pairing is established between the mRNA and siRNA, sequence-specific cleavage of the target mRNA occurs and results in decreased cellular protein levels of the target. (Source: Grimm and Kay, 2007).
Figure 1.15: RNAi-mediated BBB/iBRB modulation by targeting the claudin-5 TJ protein. (A) Systemic administration of siRNA targeting claudin-5 leads to downregulation of claudin-5 for a period of between 24 and 72 hours. (Source: Campbell et al., 2008) (B) Contrast-enhanced magnetic resonance imaging (MRI) showing extravasation of Gd-DTPA in the right eye injected with AAV-claudin-5 shRNA. (C) Reductions in CNV volume following systemic administration of Sunitinib in AAV-claudin-5 shRNA-injected mice. (Source: Campbell et al., 2011) (D) Cerebral oedema progression in axial computerized tomography (CT) scans of a 12-year old boy who suffered a severe TBI. (E) Decreases in lesion volume and improved BBB phenotype as shown by reduced Evans blue dye extravasation at the site of injury in claudin-5 siRNA-treated mice. (Source: Campbell et al., 2012).
Table 1.2: A list of AD therapeutics – FDA-approved, in current or discontinued clinical trials, or pre-clinical BBB-based drugs. (Source for clinical trial stages: Alzheimer’s Forum – www.alzforum.org/therapeutics).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
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<tbody>
<tr>
<td><strong>FDA Approved</strong></td>
<td></td>
</tr>
<tr>
<td>Donepezil</td>
<td>Cholinesterase inhibitor</td>
</tr>
<tr>
<td>Galantamine</td>
<td>Cholinesterase inhibitor</td>
</tr>
<tr>
<td>Rivastigmine</td>
<td>Cholinesterase inhibitor</td>
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<tr>
<td>Tacrine</td>
<td>Cholinesterase inhibitor</td>
</tr>
<tr>
<td>Memantine</td>
<td>NMDA glutamate receptors</td>
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<tr>
<td><strong>Current/Discontinued Clinical Trials (as of July 2014)</strong></td>
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<tr>
<td>Bapinezumab (discontinued)</td>
<td>Aβ mAb</td>
</tr>
<tr>
<td>Solanezumab (phase III)</td>
<td>Aβ mAb</td>
</tr>
<tr>
<td>Gantenerumab (phase III)</td>
<td>Aβ mAb</td>
</tr>
<tr>
<td>Crenezumab (phase II)</td>
<td>Aβ mAb</td>
</tr>
<tr>
<td>Semagacestat (discontinued)</td>
<td>γ-secretase inhibitor</td>
</tr>
<tr>
<td>EVP-0962 (phase II)</td>
<td>γ-secretase modulator</td>
</tr>
<tr>
<td>MK-8931 (phase III)</td>
<td>BACE inhibitor</td>
</tr>
<tr>
<td><strong>Pre-clinical BBB-based adjunct therapies</strong></td>
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<tr>
<td>Bivalent anti-TfR/BACE1</td>
<td>Binds to TfR at BBB to enhance brain uptake of BACE1 antibody</td>
</tr>
<tr>
<td>Monovalent anti-TfR(Fab)/Aβ</td>
<td>Binds to TfR at BBB to enhance brain uptake of Aβ antibody</td>
</tr>
<tr>
<td>FPS-ZM1</td>
<td>RAGE inhibitor</td>
</tr>
<tr>
<td>LRP-IV</td>
<td>Binds and sequesters circulating Aβ</td>
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Chapter 2:

Materials and Methods
2. Materials and Methods

2.1. Cell Culture Methods

2.1.1. bEnd.3 cell culture maintenance and passaging
Mouse brain endothelial (bEnd.3) cells were obtained from ATCC and this line was originally isolated from the cerebral cortex of 6-week old BALB/c mice. This immortalized cell line has been transformed by infection with the NKTmT virus that expresses the middle T antigen and which initiates cell cycle entry in transformed cells. bEnd.3 cells were cultured in T175 filter-capped flasks (Sarstedt) in 25ml of Dulbecco’s modified Eagle medium (DMEM, BioWhittaker) supplemented with 20% fetal calf serum (FCS) and 100ml sodium pyruvate (2mM) per 500ml bottle. Flasks were stored in a 5% CO₂ incubator (Hepa Class 100, Thermo Scientific) at 37°C. Cultured cells were passaged to maintain exponential cell growth and to minimise cell death. DMEM medium was aspirated off and cells were washed gently with 10ml of Dulbecco’s phosphate buffered saline (DPBS) at pH 7.2 for 1-2 min. The PBS was removed and 3ml of trypsin-EDTA (Gibco-BRL) was added to the flasks to dissociate cells. The flasks were incubated for 5 min at 37°C to allow for trypsinisation. 12ml of supplemented DMEM was added to each flask to terminate trypsinisation. Following centrifugation for 5 min at 1,000rpm, the supernatant was removed and the pellets resuspended in 2ml of supplemented DMEM. 1ml of the cell culture was transferred to each of two T175 flasks containing 25ml of supplemented DMEM and the flasks were placed in the 37°C incubator.

2.1.2. Haemocytometer cell counting and cryopreservation
Cells were trypsinised as described above and spun down for 5 min at 1,000 rpm. Pellets were resuspended in 1ml DMEM and the samples pooled to 20ml in the same medium. A 1:10 dilution in DMEM was made and 20μl of the diluted cell suspension was pipetted along the edge of the glass coverslip on top of the haemocytometer. Cells present within the four 16-square corner areas were counted and the total number of cells in the original bEnd.3 stock was calculated as: average number of cells from the corner grids X 10 (dilution) X 10⁴ (since each corner is 1mmx1mmx0.1mm=0.1mm³ and multiplying by 10⁴ gives the number of cells per ml or cm³). For bEnd.3 cryopreservation, approximately 1 x 10⁶ cells were resuspended in freezing medium (80% FCS, 10% DMEM, 10%...
DMSO) and slow-cooled in freezing containers to ensure a controlled rate of cell freezing of approximately -1°C/minute in a -80°C freezer.

2.1.3. General siRNA transfection protocol using Lipofectamine 2000 Reagent

Each well of a 24-well plate was seeded at a cell density of 1x10^5 cells in 500μl of DMEM. For each well of cells to be transfected, 20pmol of targeting or non-targeting siRNA was diluted in 50μl of Opti-MEM (Gibco-Invitrogen). For each well, 1μl of LF2000 was diluted in 50μl of Opti-MEM in a 1.5ml eppendorf tube and incubated for 5 min at room temperature. The diluted LF2000 was then combined with the siRNA and incubated for 20 min at room temperature. 100μl of the siRNA-LF2000 mixture was then added to each well and mixed well. The plates were then gently swirled and placed in the 37°C incubator for the required length of time. All transfections were performed in triplicate and repeated two more times. All siRNA sequences are provided in the appendix.

2.1.4. Fluorescein isothiocyanate (FITC)-Aβ1-40 permeability assays

bEnd.3 cells were seeded at a cell density of 5x10^4 cells in 100μl of DMEM and grown to confluence on 1% fibronectin-coated Corning® HTS 24-well Transwell® polyester inserts with a pore size of 0.4μm. For transwell experiments, only 50μl (10pmol) of the siRNA-LF2000 mixture was applied due to lower cell density and smaller membrane size. 72 hours post-transfection (the timepoint of maximal siRNA-mediated TJ protein suppression), 10μM of FITC-Aβ1-40 peptide (Bachem) in DMEM was added to the apical chamber of each well and the cells incubated at 37°C. At time “zero”, 100ul of DMEM was collected from the basolateral chamber and replaced with new medium. Sampling aliquots were taken every 15 mins for 120 mins and transferred to an aluminium foil-wrapped 96-well plate (Nunc). FITC-Aβ1-40 fluorescence was determined using a fluorescence spectrofluorometer (Optima Scientific) at an excitation wavelength of 485nm and an emission wavelength of 520nm. After subtracting background fluorescence of media taken from untreated cells, relative fluorescence units (RFU) were converted to ng/ml values using a FITC-Aβ1-40 standard curve and corrected for the serial dilutions over the course of the experiment. For permeability assays measuring basolateral to apical movement of FITC-Aβ1-40, the peptide was added to the basolateral chamber and sampling aliquots taken from the apical side. The apparent
permeability coefficient \( P_{\text{app}} \) of FITC-Aβ1-40 was calculated using the following equation:

\[
P_{\text{app}} \text{ (cm/s)} = \frac{(\text{d}Q/\text{d}T)}{(A \times C_0)}
\]

where \( \text{d}Q/\text{d}T \) (\( \mu \text{g/s} \)) is the rate of appearance of FITC-Aβ1-40 on the receiver side after application, \( A \) (cm\(^2\)) is the effective surface area of the insert size and \( C_0 \) (\( \mu \text{g/ml} \)) is the initial FITC-Aβ1-40 concentration on the donor side. \( \text{d}Q/\text{d}T \) is the slope \( m \) \( (y = mx + c) \) calculated by plotting the cumulative amount \( Q \) versus time \( s \).

2.1.5. Transendothelial electrical resistance (TEER) measurements

bEnd.3 cells were seeded at a cell density of 5 \( \times \) 10\(^4\) cells as described for the permeability assay. TEER was determined for bEnd.3 cells using an EVOM resistance meter with Endohm Chamber (World Precision Instruments) and a Millicell-Electrical Resistance System. Prior to measurements, both the apical and basolateral chambers were bathed in fresh media at 37°C. The probe anode and cathode were carefully immersed in the chambers so as not to touch the cell monolayer and a current was passed across the cell monolayer. Changes in electrical resistance are measured in triplicate in units of \( \Omega \cdot \text{cm}^2 \) and the inherent resistance of a blank filter coated with 1% fibronectin is subtracted from the values obtained.

2.1.6. MTS cell viability assay

bEnd.3 cells were seeded on 96-well plates at a density of 1.6 \( \times \) 10\(^3\) cells in 100\( \mu \)l of DMEM medium. 24 hours later, bEnd.3 cells were transfected with 4 pmol of targeting or non-targeting siRNA using the LF2000 protocol described previously (all volumes one-fifth of those for 24-well plate protocol). 48 hours post-transfection (time-point of significant target gene suppression in bEnd.3 cells), the medium was replaced and 20\( \mu \)l of CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega) was added to each well. The reagent was incubated with the cells for two hours and the concentration of the formazon product was measured in a spectrophotometer at 490nm. Optical density (OD) values for control wells (no cells/background absorbance) were subtracted from the sample well OD values.

2.1.7. RNA extraction

RNA was extracted from bEnd.3 cells using either reagents and instructions supplied in the Qiagen RNeasy™ Mini Kit or by Trizol extraction. For Trizol extraction, media was
discarded and each well of a 24-well plate washed with 500μl of D-PBS. Cells were then lysed by addition of 400μl of Trizol (Invitrogen) reagent (volume for 3 replicate wells) and cell lysates added to 1.5ml eppendorfs. Following incubation for 5 mins at room temperature (RT), 200μl of chloroform was added and mixed vigorously. This was further incubated for 5 mins at RT and centrifuged at 13,000rpm for 15 mins at 4°C. The aqueous phase was then transferred to a new eppendorf and 500μl of isopropanol added and mixed to precipitate RNA. This mixture was incubated for 10 mins at RT and spun at 13,000rpm for 8 mins. The RNA pellet was then washed in 1ml of 75% ethanol and spun at 8,000rpm for 5 mins. After removing the ethanol, the pellet was left to air dry. All RNA samples were also DNase-treated RNA by addition of 50μl reaction mix to the dried RNA pellet: 5μl RQ 10X Reaction Buffer (Promega), 3μl RQ 1 RNase-free DNase (Promega), and 42μl RNase-free water. This DNase mix was incubated with the RNA pellet at 37°C for 1 hour. Afterwards 5ul of Stop Solution was added and incubated at 65°C for 10 mins. RNA concentrations were measured using a NanoDrop Spectrophotometer ND-1000 (Labtech). Samples were then aliquoted, snap-frozen and stored at -80°C for future use.

2.1.8. Protein extraction

Each well of the 24-well plate was washed with 500μl of ice-cold PBS which was then carefully aspirated off from the side of each well. Lysis buffer was made comprising 62.5mM Tris, 2% SDS, 10mM dithiothreitol and 1 protease inhibitor tablet (Roche) per 10ml. Approximately 200μl of this lysis buffer was added to each well and mixed vigorously. Cell lysate samples in triplicates were then pooled in fresh 1.5ml eppendorf tubes. The homogenate was centrifuged at 12,000rpm for 20 mins at 4°C. The supernatant containing soluble protein was collected, snap-frozen and stored at -80°C for future tight junction protein analysis.

2.2. In Vivo Techniques

2.2.1. Animals

APP-Swe mice (also known as APP-Tg2576, C57/Bl6;SJL background, stock number 001349) carrying the human amyloid precursor protein (APP695) gene containing the double Swedish mutations (K670N and M671L) under the control of the hamster prion promoter were obtained (18 in total) from Taconic Europe. Wild-type C57/Bl6J mice
were sourced from Jackson Laboratories and bred on-site in the Smurfit Institute of Genetics in Trinity College Dublin (TCD). All studies carried out in the Smurfit Institute of Genetics in TCD adhere to the principles laid out by the internal ethics committee at TCD and all relevant national licences were obtained prior to commencement of work.

2.2.2. Preparation of in vivo-jetPEI-siRNA complexes and tail vein injection

20µg of siRNA (20µl of 1µg/µl solution) was diluted in 100µl of 10% glucose solution. The volume was adjusted to 200µl with nuclease-free water and the solution briefly vortexed and centrifuged. In a separate tube, 6.4µl of in vivo-jetPEI (Polyplus Transfection) was diluted in 100µl of 10% glucose solution. The volume was adjusted to 200µl with nuclease-free water, mixed and briefly pulsed. The 200µl of in vivo-jetPEI solution was then added to the siRNA solution and the contents briefly vortexed. The tubes were then incubated for 15 mins at room temperature to allow the in vivo-jetPEI-siRNA complexes to form and afterwards kept on ice until injection. RNase-free tips and RNase-free tubes were used in all siRNA preparations. To perform mouse tail vein injections, C57 or APP-Swe mice were restrained within a 60ml plastic tube and the in vivo-jetPEI-siRNA solution was injected into the warmed mouse tail vein using a 30 gauge needle (BD Microlance). All siRNA sequences are provided in the appendix.

2.2.3. Brain microvasculature fractionation

Half-brains were homogenized in 5ml of DMEM on ice using a dounce homogenizer and the homogenate was spun at 3,000rpm for 5 mins. The resulting pellet was transferred to a 9cm petri dish containing 0.005% dispase (Sigma) and incubated at 37°C for 2 hours with plates swirled every 15 mins to allow for tissue dissociation. Homogenates were centrifuged at 3,000rpm for 5 mins and pellets were re-suspended in 12% dextran solution (Mw 70,000 from Leuconostoc spp.; Sigma). After briefly vortexing, samples were then centrifuged at 3,000rpm for 10 mins at 4°C. A thin red-ringed pellet should be visible at the bottom of the tube (except when terminal perfusions are performed beforehand). Pellets were re-suspended with PBS and centrifuged at 2,000rpm for 5 minutes to wash the capillary fraction. Cells were re-suspended in either RLT lysis buffer for RNA extractions using the Qiagen RNeasy® Mini Kit or protein lysis buffer for protein extraction as outlined for bEnd.3 cells.
2.2.4. Plasma isolation from APP-Swe mice

Blood was collected from the tail veins of APP-Swe mice by firstly restraining and warming the tails as outlined previously. A 1ml syringe was filled with saline (240μl of 0.9% sodium chloride) and then using a 30G needle, approximately 80μl of blood (1:4 dilution) was collected from the tail vein and transferred to a cold EDTA-coated 1.5ml eppendorf tube. Excessive blood loss was stopped by lightly pressing on the tail. All blood samples were extracted from APP-Swe mice blind to treatment. Working quickly, blood samples were then centrifuged at 1,500rpm for 10 mins at 4°C to separate the plasma from erythrocytes and leukocytes. The upper clear-coloured plasma phase was transferred to a fresh 1.5ml eppendorf tube, snap-frozen and stored at -80°C for further ELISA analysis.

2.2.5. Perfusion of tracer molecules

The extent of BBB permeability was assessed by terminal perfusion of tracer molecules including 3 and 10 kDa biotinylated dextran (lysine-fixable, Invitrogen). Untreated, targeting siRNA- or non-targeting siRNA-treated animals were anaesthetized by an intraperitoneal (IP) injection of a ketamine (100mg/kg) and xylazine (20mg/kg) cocktail. Since this is a lethal anaesthetic dose, the thoracic cavity was quickly opened to reveal the heart. Immediately after severing the right atrium, a DPBS solution containing 1mg/ml of the tracer molecule was slowly perfused into the left ventricle for 3 mins using a peristaltic pump. Following tracer molecule perfusion, tissues including brain, liver, lung and heart were dissected (total procedure lasts approximately 15 mins per animal) and placed in 4% paraformaldehyde (PFA, pH 7.4) overnight at 4°C for cryosectioning.

2.2.6. Magnetic resonance imaging (MRI)

Following administration of targeting or non-targeting siRNAs, BBB integrity was assessed via MRI using a dedicated small rodent Bruker BioSpec 70/30 with an actively shielded USR magnet. Mice were anaesthetized with 5% isoﬂurane in 100% oxygen and placed on an MRI-compatible support cradle with 1-1.5% isoﬂurane in 100% oxygen for maintenance. This cradle has an in-built system for maintaining the animal’s body temperature at 37°C and a probe underneath the animal allows it to be physiologically monitored (electrocardiogram, respiration and temperature). To ensure accurate positioning of the animal, an initial rapid pilot image is recorded and is used to ensure correct geometry for all subsequent scans. The scale of BBB integrity was then visualized
in high-resolution T₁-weighted MR images (resolution, 0.156 X 0.156 X 5mm³; field of view: 20 X 20 X 17.9mm³; matrix: 128 X 128 X 30; TR/TE: 500/2.7ms; flip angle: 30°; number of averages: 3; acquisition time: 2 min, 24 sec; repetitions: 8) before and after tail vein administration of 100µl of a 1:3 dilution of Gd-DTPA (Gadolinium diethylene-triamine penta-acetic acid, 0.5mmol/ml stock solution, Bayer) which is monitored over a period of 20 mins post-injection. Animals were sacrificed and tissue collected after MRI scanning. MRI analysis was performed using ImageJ and MIPAV software and all data was analyzed blind to treatment.

2.3. Analytical Techniques

2.3.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

2.3.2.1. SYBR Green method

Isolated RNA from bEnd.3 cells and capillary fractionation samples was quantified using one-step real-time reverse transcriptase-PCR (RT-PCR) on a 7300 Real Time PCR System (Applied Biosystems) with Quantitect SYBR Green I as fluorescent dye (Qiagen). The Quantitect One-Step RT-PCR Kit was able to support reverse transcription and subsequent gene-specific amplification in a single well. 15µl of the following master mix was added to each well of a 96-well plate:

Table 2.1: The reaction components of a master mix for one-step RT-PCR showing volumes for a single well reaction. This was prepared in bulk depending on the number of wells being used.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x Quantitect SYBR Green</td>
<td>10µl</td>
</tr>
<tr>
<td>Quantitect RT Mix</td>
<td>0.15µl</td>
</tr>
<tr>
<td>Primer Mix</td>
<td>1µl</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>3.85µl</td>
</tr>
<tr>
<td><strong>Total Volume/Well</strong></td>
<td><strong>15µl</strong></td>
</tr>
</tbody>
</table>

The sequences of complementary DNA PCR primers (Sigma-Aldrich) for RT-PCR experiments were designed using Primer3. All primer sequences are provided in the appendix. Forward and reverse primers (10pmol/µl) for the sequence of interest were pooled and master mixes were then vortexed and spun down before addition to 96-well
plates. 5µl of sample RNA (0.4 ng/µl) or H2O was added to each well to give a total reaction volume of 20 µl. Plates were then covered with an acetate sheet, spun down and placed in the 7300 Real Time PCR machine. RT-PCR reaction conditions were as follows:

- **Stage 1** (reverse transcription): 50°C x 20 min
- **Stage 2** (activation of Taq DNA pol): 95°C x 15 min
- **Stage 3** (cDNA amplification): [95°C x 15 sec 60°C x 1 min] X 37 times
- **Stage 4** (dissociation stage): 95°C x 15 sec 60°C x 1 min 95°C x 15 sec

Post RT-PCR analysis was carried out on the 7300 System Software and relative gene expression levels were measured using the comparative C_t method (ΔΔC_T).

### 2.3.2.2. Taqman method

Levels of RNA isolated from mouse brain cortices or mouse brain capillary fractions were also quantified in a StepOne Plus machine (Applied Biosystems) using Taqman Gene Expression Assays (Applied Biosystems). Target genes used a FAM reporter. Reaction conditions were as follows: 50°C x 2 min, 95°C x 20 s, [95°C x 1 s, 60°C x 20 s] x 40. Transcript levels were standardized using β-actin with a VIC reporter in the same well and assessed using the ΔΔC_T method.

### 2.3.2.3. The comparative C_t method (ΔΔC_T)

In order to measure relative gene expression levels using the ΔΔC_T method, a validation experiment was first carried out for the target gene/primer set of interest to ensure that the efficiency of target amplification was approximately equal to the reference endogenous control (β-actin). A calibrator sample (in most cases RNA from an untreated sample) is also applied. In the ΔΔC_T method, ΔC_T is calculated as:

\[
(C_T \text{ target sample} - C_T \text{ endogenous control})
\]

ΔΔC_T is then calculated as:

\[
(ΔC_T \text{ standardized target sample} - ΔC_T \text{ standardized calibrator})
\]

To convert this ΔΔC_T value to a fold difference value, the amount of target is calculated as: \(2^{ΔΔC_T}\).
For all RT-PCR experiments, expression levels of target genes were standardized to the housekeeping gene, β-actin. To assess levels of RNAi-mediated tight junction suppression, results were also expressed as a percentage of the non-targeting siRNA control (normalized to β-actin), where applicable.

### 2.3.3. Western blot

#### 2.3.3.1. Bicinchoninic acid (BCA) assay

A bicinchoninic acid (BCA) assay was carried out to determine total protein concentration in bEnd.3 cell protein samples. All reagents used in this biochemical assay were supplied by the ThermoScientific BCA Protein Assay Kit. Bovine serum albumin (BSA) standards were prepared as follows:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Stock BSA (μl)</th>
<th>Deionised H₂O (μl)</th>
<th>BSA conc. (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3.75</td>
<td>26.25</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>7.50</td>
<td>22.50</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>11.25</td>
<td>18.75</td>
<td>750</td>
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<tr>
<td>5</td>
<td>15</td>
<td>15</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>22.50</td>
<td>7.50</td>
<td>1500</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>0</td>
<td>2000</td>
</tr>
</tbody>
</table>

The stock BCA reagent was prepared by mixing Reagents A and B in a 50:1 dilution ratio. This solution contains copper sulphate and BCA which form the basis of the colometric BCA assay. 10μl of each BSA standard, 10μl of each protein lysate sample (in a 1:5 dilution) and 10μl of protein lysis buffer (as blanks) was each added to three wells on a 96-well plate. 200μl of BCA reagent was then added to every well and the plate was incubated immediately for 30 min at 37°C. Following incubation, the plate was placed in a spectrophotometer (Rosys 2010 Anthos) and absorbances read at 595nm. A standard curve was generated from the BSA standards and this was used to estimate the protein concentration in the samples by following the equation: \( y = \text{slope}(x + y_k) \), where \( y = \text{OD} \), \( x = \text{concentration of protein (mg/ml)} \), with \( \text{slope} \) and \( y_k \) being determined from the standard curve.
2.3.3.2. SDS-PAGE electrophoresis

Recipes for stock solutions used in western blot experiments during these studies are provided in the appendix. For western blot analysis, resolving and stacking gels were prepared to separate the protein samples by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis. A 10% resolving gel was prepared using 4ml distilled H₂O, 2.5ml 1.5M Tris·HCl (pH 8.8), 3.33ml 30% acrylamide, 100µl 10% SDS, 100µl ammonium persulfate (APS), and 10µl TEMED. This was mixed gently and the gel was poured between the assembled glass plates to 1cm below the comb teeth using a 10ml pipette. The gel was then overlaid with ethanol (to provide air-free conditions for polymerization) and allowed to polymerize for 30 min at RT. The overlaying ethanol was poured off and on top of this was poured a 4% stacking gel consisting of 6.1ml distilled H₂O, 2.5ml 0.5M Tris·HCl (pH 6.8), 1.3ml 30% acrylamide, 100µl 10% SDS, 100µl APS, and 10µl TEMED. The comb was then carefully inserted into the stacking gel and the gel was allowed to polymerize for 15 min. The glass plate was inserted into the Mini-Protean 3 electrophoresis module which was filled with 1X running buffer and the comb was removed prior to sample loading. 6µl of loading buffer (Thermo Scientific) was added to 30µl of protein aliquots and heated for 5 mins at 100°C along with 10µl of a pre-stained protein ladder (New England BioLabs). Equal amounts of samples (as determined by BCA assay, generally 30µg) were carefully loaded into the lanes and the order of sample loading recorded. The SDS-PAGE gel was run at 60mA for 1 hour in 1X running buffer.

2.3.3.3. Electrophoretic transfer of protein onto PVDF membranes

Following SDS-PAGE electrophoresis, the gel was removed from the glass plates and transferred to a Mini Trans-Blot cell module. This cassette consisted of 4 filter papers and a polyvinylidene fluoride (PVDF) membrane onto which the gel was placed and 4 more filter papers stacked on top. A 40mA current was run through the module for 2 hours for electrophoretic transfer of proteins onto the PVDF membrane.

2.3.3.4. Blocking and antibody probing of immunoblots

The blot was then blocked with 20ml of blocking solution (5% skimmed milk in 1X TBS) incubated at RT for 30 mins with gentle rocking. The blot was then probed with a 1:500 to 1:1000 dilution of the primary antibody (dilutions for specific antibodies outlined in chapters), in 5% skimmed milk in 1X TBS and incubated overnight at 4°C on a rocker.
The next morning the blot was washed 3 x 15 mins in 1X TBS. This was followed by
detection of primary antibody complexes using a 1:2000 dilution of horseradish
peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Abcam) in 5%
skimmed milk in 1X TBS. The membrane was placed on the shaker for 2 hours and the
blot then washed 3 x 15 mins in 1X TBS.

2.3.3.5. Chemiluminescence detection of HRP
To detect HRP, the immunoblot was placed in SuperSignal Chemiluminescence Substrate
(Pierce) solution. To develop images of the protein bands, the membrane was exposed to
Fugi X-ray films for 3-10 mins. The X-ray film was added to developer solution until
bands became visible and transferred to H₂O to stop development and finally to fixing
solution before being washed and dried. After incubating in Stripping Buffer (Pierce) the
membrane was then re-blocked and immunostained with rabbit anti-β-actin primary
antibody (Abcam) and goat anti-rabbit secondary antibody as described above. The β-
actin loading control was then exposed and visualised as described. Protein band
intensities were quantified by scanning with Epson Stylus CX3200 and analysed using
ImageJ software.

2.3.4. Enzyme-linked immunosorbent assay (ELISA)
Plasma and brain amyloid-β 1-40 levels were quantified using the Human Aβ40 ELISA
kit (Invitrogen). The absorbance of each well was read at 450nm on a spectrophotometer
(Rosys 2010 Anthos) and Aβ1-40 concentrations of plasma samples determined from the
Aβ1-40 peptide standard curve (detection limits of 6–500pg/ml) after correcting for
background absorbance and dilution factors. were recorded ELISA preparation and
analysis was performed blind to treatment.

2.3.5. Immunohistochemistry

2.3.5.1. Cryosectioning
Following dissection of tissues including brain, liver, lung and heart, the organs were
placed in 4% PFA (pH 7.4) overnight at 4°C. The next day organs were washed four
times for 15 mins with PBS and were sequentially submerged in solutions of 10, 20 and
30% sucrose (for eye cups, lenses must be removed prior to the sucrose gradient). Organs
were then embedded in specimen blocks using optimum cutting temperature (OCT)
solution (Tissue Tek) and snap frozen by liquid nitrogen using isopropanol to slow cooling. Frozen organs were sectioned on a cryo-sectioner (Leica CM 1900) into 12 μm sections and sections collected on Polysine slides (Menzel-Glazer). Brains were cut along the sagittal plane.

2.3.5.2. Immunohistochemistry of frozen brain sections

Mouse brain cryosections (12 μm thick) were permeabilized with 0.5% Triton-X in PBS for 20 mins at room temperature. Cryosections were then blocked with 5% normal goat serum (NGS, Sigma) for 20 mins and incubated overnight with a 1:100 to 1:1000 dilution of primary antibody as outlined later in individual chapters. The next day cryosections were washed 3 x 15 mins with PBS and the sections were then incubated with Cy2- or Cy3-conjugated goat anti-rabbit IgG secondary antibody or goat-anti mouse (1:500, both Abcam) for 3 hours at RT. Sections were again washed 3 x 15 mins with PBS, counterstained with DAPI (1:10000), mounted using Polymount Aqua Medium and a coverslip added. Analysis of brain cryosections was performed using a Zeiss Axioplan 2 fluorescent microscope or Olympus FluoView FV1000 confocal microscope with integrated software.

2.3.5.3. bEnd.3 immunocytochemistry

bEnd.3 cells (5 x 10^5 cells) were seeded on 1% fibronectin-coated Nunc™ Lab-Tek™ II Chamber Slides (Thermo Scientific) in DMEM. Following treatment with 1μM of Aβ1-40 monomer or Aβ1-40 dimer peptides, cells were washed with ice-cold D-PBS and then fixed with 4% paraformaldehyde (PFA, pH 7.4) or ice-cold methanol. The cells were blocked with 5% normal goat serum (NGS) in PBS and incubated with polyclonal rabbit anti-claudin-5 (1:100), polyclonal rabbit anti-occludin (1:100) or polyclonal rabbit anti-ZO-1 (1:100, all Invitrogen) overnight at 4°C. Cells were then washed 3 x 10 mins the next day with PBS and incubated with Cy3-conjugated goat anti-rabbit secondary antibody (1:500, Abcam) for 3 hours at room temperature. After washing (3 x 10 mins), the cells were counterstained with DAPI (1:10000). Analysis of stained cells was performed using a Zeiss Axioplan 2 fluorescent microscope or Olympus FluoView FV1000 confocal microscope with integrated software.
2.3.6. Statistical analysis

For each data set, the mean (μ), standard deviation (SD) and standard error of the mean (SEM) was calculated. A two-tailed unpaired Student’s t-test was used to evaluate the significance of differences between each data set and corresponding non-targeting controls. Differences were considered statistically significant when P values were ≤ 0.05.

For multiple comparisons, as was the case for comparison of permeability coefficients (P_{app}) between siRNA treatments, ANOVA followed by a Bonferroni post-test for multiple comparisons was used with P ≤ 0.05 representing significance.
Chapter 3:

Characterization of brain endothelial tight junctions in human Alzheimer’s disease (AD) cases, in transgenic amyloid precursor protein (APP-Swe) mice and *in vitro* following treatment with amyloid-β (Aβ).
3.1. Abstract
BBB and tight junction (TJ) alterations have been identified and implicated in the pathology of multiple CNS diseases including TBI, stroke, multiple sclerosis and brain tumors. While features of vascular dysfunction such as changes in cerebral blood flow (CBF), hypoperfusion/hypoxia and cerebral microbleeds are reported in AD and CAA patients, dysregulation of the BBB at the molecular level is not well characterized in AD/CAA. In this section, levels of the major TJ proteins claudin-5 and occludin as well as amyloid-β (Aβ) pathology were examined in post-mortem human brain sections from a variety of neurodegenerative and non-neurodegenerative disorders. Reduced levels or aberrant localization of claudin-5 and occludin at the BBB was most profound in the brains of AD patients, specifically in vessels with Aβ accumulation/CAA. Furthermore, claudin-5 and occludin levels were decreased in brain endothelial cell fractions isolated from aged transgenic APP-Swe mice, a murine model of AD, compared to age-matched wild-type controls. Immunohistochemical analysis of aged APP-Swe brains showed that, as in human AD brains, these TJ changes segregate with CAA, with intact claudin-5 and occludin immunostaining in areas with no Aβ deposition. Finally, the effect of various soluble recombinant forms of Aβ on TJ complexes of mouse brain endothelial cells (bEnd.3) was assessed in vitro. Time- and dose-dependent decreases in claudin-5 and occludin but not ZO-1 were detected at the protein level following exposure to soluble Aβ1-40 monomers and dimers. These combined results suggest that altered claudin-5 and occludin TJ protein levels in AD/CAA patients are directly related to the effects of Aβ accumulation on cerebral blood vessels.

3.2. Introduction
Changes to the physiological architecture of the NVU and in particular the BBB of endothelial cells have been proposed as a cause and/or consequence of major CNS disorders (Zlokovic, 2008, Luissint et al., 2012). In acute settings, BBB disruption can occur after a defined sudden insult such as a traumatic brain injury (TBI) or disturbance of the brain’s blood supply during an ischemic (restricted blood flow) or hemorrhagic (bleeding into parenchymal tissue) stroke. With regards breakdown of the paracellular barrier, TJ protein levels change in response to fluid accumulation in the brain extracellular space after TBI with decreased claudin-5 and occludin levels at the site of injury 48 hours post-TBI (Nag et al., 2007). While this may exacerbate neuroinflammation and neuronal damage after a TBI, other work suggests loosening of
the TJ s around the site of injury may actually be protective and acts to control and relieve early brain oedema (Campbell et al., 2012). In stroke, reperfusion following the initial ischemic attack causes a biphasic increase in BBB permeability that worsens cerebral vasogenic edema and increases mortality (Knowland et al., 2014). A recent study proposes that TJ abnormalities 48 hours after stroke are responsible for this second peak of increased BBB permeability (Knowland et al., 2014). The status of TJ integrity in chronic neurodegenerative disease is not well-characterized and questions remain as to whether BBB damage is a cause or consequence of neuronal death in these disorders. In amyotrophic lateral sclerosis (ALS, also known as motor neurone disease), disease progression is defined by death of the upper and lower motor neurons in the motor cortex, the brainstem and the spinal cord. This in turn causes muscle weakness and atrophy throughout the body and is ultimately fatal (Kiernan et al., 2011). While many have proposed that TJ s of the BBB are modulated in ALS, the data is less clear (Zlokovic, 2008, Obermeier et al., 2013). Mice expressing mutant human SOD1 (Cu/Zn superoxide dismutase 1), an antioxidant enzyme linked to ALS, have leaky BBB and blood-spinal cord (BSCB) barriers (Zhong et al., 2008, Winkler et al., 2014). However, reduced expression of the TJ proteins occludin and ZO-1 was only detected at the BSCB in these animals (Garbuzova-Davis et al. 2007, Zhong et al., 2008). Furthermore, in sporadic ALS patients, occludin and claudin-5 expression was diminished only in the cervical spinal cord and medulla oblongata of the brainstem but not elsewhere (Garbuzova-Davis et al., 2012, Miyazaki et al., 2011).

Various aspects of BBB dysfunction have been implicated in exacerbating amyloid build-up and AD/CAA pathogenesis: infiltration of toxic blood components across the BBB, perturbation of Aβ transporters and release of inflammatory cytokines by the BBB endothelium (Erickson and Banks, 2013). TJ integrity in AD/CAA and the role of the paracellular pathway of the BBB in disease onset and progression are less well known. Working with Prof Michael Farrell in the Dublin Brain Bank, Beaumont Hospital (Dublin), post-mortem paraffin-embedded human brain tissue was obtained from 30 neurological disease donors for analysis of BBB TJ s in these conditions. While I was blind to the neuropathological diagnoses prior to the experimental work, I received the diagnostic information from the neuropathologist afterwards. As well as brain samples from AD/CAA and ALS patients, the cohort included brain tissue from patients with other neurodegenerative diseases including Lewy body dementia (LBD), progressive...
supranuclear palsy (PSP), frontotemporal dementia (FTD) and multiple systems atrophy (MSA). LBD is a progressive degenerative dementia that accounts for 10-15% of all diagnosed dementias (Huang and Halliday, 2013). It is characterized by the neuropathological presence of abnormal cytoplasmic protein inclusions called Lewy bodies throughout the brain. Fibrillar aggregates of α-synuclein are the main protein component of these Lewy bodies and as such LBD is known as a synucleinopathy (Huang and Halliday, 2013). LBD shares pathological characteristics with Parkinson’s disease with Lewy body pathology and loss of dopamine-producing neurons in the substantia nigra found in both conditions (Braak et al., 2007). In addition, features of AD pathogenesis such as loss of cholinergic neurons and diffuse amyloid plaques (but not tau neuritic involvement) are associated with LBD in some patients (Gomperts et al., 2008).

MSA is another neurodegenerative synucleinopathy and is characterized by a combination of parkinsonism (muscle tremor/rigidity), cerebellar ataxia and autonomic dysfunction (hypotension, urinary problems, abnormal breathing) (Ahmed et al., 2012). Cytoplasmic inclusions of α-synuclein in oligodendrocytes are the major pathological hallmark of MSA (Ahmed et al., 2012). PSP and FTD on the other hand are classified as tauopathies, that is the presence of abnormal hyperphosphorylation of the tau microtubule protein and intracellular clumps of tau at post-mortem. PSP is a progressive neurodegenerative disorder whose hallmark symptoms include ophthalmoplegia (problems with eye movement due to paralysis of extraocular muscles) and motor impairments such as loss of balance and neck dystonia (Rampello et al., 2005). FTD is the clinical presentation of frontotemporal lobar degeneration and is the second most common early onset (< 65 years) dementia after AD, accounting for 15-20% of cases (Pan and Chen, 2013). As suggested by its name, it is characterized by atrophy of the frontal and temporal lobes and three clinical FTD variants exist: behavioural variant FTD (behavioural and personality changes), semantic dementia (loss of meaning of words and objects) and progressive non-fluent aphasia (speech difficulties). In 50% of FTD cases, tau pathology is apparent whereas in another 40% of cases, elevated TDP-43 (TAR DNA-binding protein 43) levels are a feature (Pan and Chen, 2013). Mutations in MAPT (tau gene) cause FTD with parkinsonism while TDP-43 mutations or hexanucleotide repeat expansions in the promoter region of C9ORF72 result in FTD with motor neuron degeneration (ALS) (Pan and Chen, 2013, Renton et al., 2011, DeJesus-Hernandez et al., 2011).
Mouse models of AD re-capitulate important features of the disease process including Aβ accumulation, amyloid plaques, synaptic dysfunction and memory loss but other aspects of AD such as tau pathology and neuronal loss are mostly absent (Ashe and Zahs, 2012). These models are generally based on human APP and/or PS mutant genes and include various single transgenic (APP-Swe), double transgenic (APP/PS1) and triple transgenic (3x Tg-AD) creations that differ by rate of Aβ accumulation, plaque formation and onset of memory deficits (Webster et al., 2014). With regards the cerebrovascular phenotypes in AD/CAA, the well-characterized APP-Swe mouse was chosen for this study as it replicates features of CAA such as Aβ buildup around brain microvessels, disruption of the VSMC layer and impaired vasodilatory (CBF) responses (Christie et al., 2001, Domnitz et al., 2005, Han et al., 2008). APP-Swe mice overexpress a human APP gene containing the double ‘Swedish’ mutation (K670N/M671L) driven by a hamster prion promoter (Hsiao et al., 1996). Since their creation in the mid-90s, APP-Swe mice have become the most characterized and widely used AD mouse model. Increased levels of soluble Aβ1-40 and Aβ1-42 are first detected around 3-4 months of age in these mice and this is followed by an age-dependent increase in soluble and insoluble Aβ deposition with amyloid plaque pathology evident by 11-13 months (Kawarabayashi et al., 2001, Schaeffer et al., 2011). Previous studies have suggested that BBB dysfunction in APP-Swe mice begins as early as 4 months of age and precedes amyloid plaque formation (Ujiie et al., 2003). This claim is based on serum protein leakage and extravasation of systemically administered dyes into the brain parenchyma (Ujiie et al., 2003, Paul et al., 2007). While cerebrovascular and BBB phenotypes are apparent in young APP-Swe mice, these phenotypes worsen with animal age and also correlate with CAA severity (Han et al., 2008). In combination with the analysis of claudin-5 and occludin in human post-mortem AD/CAA brains, pure fractions of brain endothelial cells were isolated from aged APP-Swe (20 months) to further characterize levels of the major TJ proteins and Aβ transporters at the BBB in an Aβ-rich environment. This was accompanied by measurements of transendothelial electrical resistance (TEER) across APP-Swe brain endothelial monolayers compared to age-matched wild-type controls and immunohistochemical analysis of TJs in aged APP-Swe brains.

A number of in vitro studies have assessed the impact of various Aβ species on brain endothelial cell cultures. The first study of note by Marco and Skaper (2006) found that addition of Aβ1-42 (20 μM, undefined aggregate state) to rat brain endothelial cells
decreased occludin expression and caused re-location of ZO-1 and claudin-5 from the cell membrane. Gonzalez-Vasquez and colleagues (2008) showed that exposing human brain endothelial cells (ACBRI 376 primary HBMVECs) to soluble aggregates of Aβ1-40 (2.5-10 μM) triggered ZO-1 to disperse from the cell borders and this was accompanied by a decrease in TEER values. Tai and colleagues (2010) found that in immortalized human brain endothelial cells (hCMEC/D3), Aβ1-40 (10 μM, undefined aggregate size) increased paracellular permeability in vitro and induced occludin but not ZO-1 or claudin-5 down-regulation. In contrast, Hartz and colleagues (2011) reported that monomeric Aβ1-40 (100 nM, undefined aggregate size after preparation) reduces claudin-5 and claudin-1 but not occludin or ZO-1 levels in primary rat brain microvessel cultures. Finally, Kook and colleagues (2012) found that Aβ1-42 (1-5 μM, “mostly” monomers) disrupted claudin-5, occludin and ZO-1 expression and localization in immortalized mouse brain endothelial cells (bEnd.3). Clearly, comparable and contrasting results have emerged from these in vitro assays with regards the effect of Aβ on TJs. A major problem with the experimental design in a number of these studies is the poor characterization of the Aβ material used. The effects of Aβ on neuronal signaling and viability have been shown to vary depending on the species and solubility of Aβ employed and therefore knowing the precise species of Aβ being used is vital (Nature Neuroscience Editorial, 2011, Shankar et al., 2008). Aβ has a strong propensity to aggregate and chemically synthesized Aβ from manufacturers has certain limitations including impurities and differing aggregation states depending on supplier, batch and other external conditions (Finder et al., 2010). Working with Dr Tiernan O’Malley and Prof Dominic Walsh in Brigham and Women’s Hospital, Harvard University (Boston, USA), recombinant soluble Aβ1-40 monomers and dimers were provided that have been designed to prevent oligomerization and have been previously characterized (Walsh et al., 2009, O’Malley et al., 2014). These modified peptides were then applied to bEnd.3 cells in vitro and the integrity of the major TJ proteins assessed by RT-PCR, western blotting and immunocytochemistry.

3.3. Methods

3.3.1. bEnd.3 cell culture

Mouse brain endothelial (bEnd.3) cells (ATCC) were cultured in T175 filter-capped flasks (Sarstedt) in 25ml of Dulbecco’s modified Eagle medium (DMEM, BioWhittaker) supplemented with 20% fetal calf serum (FCS) and 100ml sodium pyruvate (2mM) per
500ml bottle. Flasks were stored in a 5% CO₂ incubator (Hepa Class 100, Thermo Scientific) at 37°C. Cultured cells were passaged to maintain exponential cell growth and to minimise cell death.

3.3.2. Treatment of bEnd.3 cells with synthetic Aβ1-40 monomers and dimers

Synthetic recombinant Aβ1-40 monomer (F19P) and dimer (dityrosine (diY)-cross-linked) peptides were kindly provided by the Walsh Laboratory, Brigham & Women's Hospital. These peptides were expressed and purified from an E. coli expression system (Walsh et al., 2009). An unmodified recombinant Aβ1-40 monomer resuspended in ammonium bicarbonate (50mM, pH 8.5) was also provided by the Walsh Laboratory. Scrambled Aβ1-40 peptide was obtained from Anaspec. All peptides were resuspended in nuclease-free (NF) H₂O and peptide stocks maintained at 1 mg/ml at -80°C. Prior to addition to bEnd.3 cells, the integrity and molecular weight of synthetic Aβ peptides was verified by SDS-PAGE and Coomassie Blue staining. Prior to Aβ treatment, bEnd.3 cells were seeded on 1% fibronectin-coated 24-well plates (1 x 10⁵ cells/well). Aβ peptides were added to bEnd.3 cells at concentrations of 0.1-1 μM in serum-free DMEM media. RNA was extracted from bEnd.3 cells using reagents and instructions supplied in the Qiagen RNeasy™ Mini Kit. Protein was isolated using lysis buffer (62.5 mM Tris, 2% SDS, 10 mM Dithiothreitol, 10 μl protease inhibitor cocktail/100 ml) followed by centrifugation at 12,000 rpm. for 20 min at 4°C and supernatant removed for TJ protein analysis. All transfections were performed in triplicate and repeated two more times.

3.3.3. bEnd.3 immunocytochemistry

bEnd.3 cells (5 x 10⁴ cells) were seeded on 1% fibronectin-coated Nunc™ Lab-Tek™ II Chamber Slides (Thermo Scientific) in DMEM. Following treatment with 1μM of Aβ1-40 monomer or Aβ1-40 dimer peptides, cells were washed with ice-cold D-PBS and then fixed with 4% paraformaldehyde (PFA, pH 7.4) or ice-cold methanol. The cells were blocked with 5% normal goat serum (NGS) in PBS and incubated with polyclonal rabbit anti-claudin-5 (1:100), polyclonal rabbit anti-occludin (1:100) or polyclonal rabbit anti-ZO-1 (1:100, all Invitrogen) overnight at 4°C. Cells were then washed 3 x 10 mins the next day with PBS and incubated with Cy3-conjugated goat anti-rabbit secondary antibody (1:500, Abcam) for 3 hours at room temperature. After washing (3 x 10 mins), the cells were counterstained with DAPI (1:10000). Analysis of stained cells was
performed using a Zeiss Axioplan 2 fluorescent microscope or Olympus FluoView FV1000 confocal microscope with integrated software.

3.3.4. bEnd.3 cell viability assays
bEnd.3 cells were seeded on 1% fibronectin-coated 96-well plates at a density of $1.6 \times 10^3$ cells in DMEM medium. 24 hours later, 0.5 to 5 μM of Aβ1-40 (F19P) monomer or Aβ1-40 (diY) dimer was applied to bEnd.3 cells. At the time-point of significant TJ protein suppression (12 h), the medium was replaced and 20μl of CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega) was added to each well. The reagent was incubated with the cells for two hours and the concentration of the formazon product was measured in a spectrophotometer at 490nm.

3.3.5. Isolation of primary mouse brain endothelial cells
In order to isolate and compare brain endothelial cells from APP-Swe transgenic mice and wild-type controls, brains were dissected from aged APP-Swe mice (20 months of age) and age-matched wild-type controls (5 animals per group). After removing the olfactory bulb, brain stem and cerebellum, brains were rolled on whatman paper to remove the meninges and chopped into 1-2 mm$^3$ pieces. Brains from each group were then pooled and placed in working buffer containing HBSS and BSA (recipes for primary isolation buffers and growth media are supplied in the appendix). Following homogenization of brains in a 2ml dounce homogenizer, samples were centrifuged at 1,800rpm for 5 mins at 4°C. The homogenates were then resuspended and incubated in 3 ml of collagenase/dispase-based digest medium for 60 mins at 37°C with occasional agitation. The digested tissue was then spun at 1,800rpm for 5 mins at 4°C and the pellet resuspended in 10 ml of 22% BSA in PBS. This mixture was then spun at 3,000rpm for 20 mins at 4°C to reveal a thick white plug at the top (containing myelin, neurons and astrocytes) and a red pellet containing the microvessels. After resuspending and washing the pellet in working buffer, the microvessel fraction was then resuspended in 2 ml of digest medium and incubated for 30 mins at 37°C with occasional agitation. The digested microvessel fraction was then pelleted at 1,800rpm for 5 mins at 4°C, washed in working buffer and resuspended in 3 ml of growth medium containing 5 μg/ml of puromycin. Since cultured endothelial cells of the BBB show strong multi-drug resistance and express high levels of drug efflux pumps, puromycin allows for selection of brain endothelial cells from other contaminants such as vessel-associated microglia, pericytes.
or smooth muscle cells. 200 μl of cells were then added to collagen IV/fibronectin-coated wells of a 24-well transwell plate (Corning) and incubated at 37°C in a 5% CO₂ incubator. After 3 days, the media was replaced with normal growth media (without puromycin) and incubated until cells reached confluence (approx. 5-6 days). TEER values were measured across confluent primary brain endothelial cell monolayers using a Millicell-Electrical Resistance System. Protein fractions were extracted from wild-type and APP-Swe brain endothelial monolayers using the following lysis buffer: 250mM Tris (pH 6.8), 8% SDS, 35% glycerol, 100mM DTT and bromophenol blue. Protein lysates were spun at 12,000rpm for 20 mins at 4°C and stored at -20°C.

3.3.6. Western blot analysis and Coomassie Blue staining
Antibodies used in this chapter were as follows: polyclonal rabbit anti-claudin-5, polyclonal rabbit anti-occludin, polyclonal rabbit anti-ZO-1, polyclonal rabbit anti-tricellulin (all 1:500, Invitrogen), polyclonal rabbit anti-low-density lipoprotein receptor-related protein 1 (LRP1) (C-terminal, 1:500, Sigma Aldrich), polyclonal rabbit anti-RAGE (1:500), and polyclonal rabbit anti-β-actin (1:2000, both Abcam). Briefly, membranes were incubated with primary antibody overnight at 4°C, washed with TBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2000, Abcam) for 2 hours at room temperature. To detect HRP, immunoblots were incubated with enhanced chemiluminescence (ECL) solution. To determine integrity and molecular weight of synthetic recombinant Aβ1-40 monomers and dimers, the peptides were run on a 16% SDS-PAGE gel. After electrophoresis, the gel was stained with 30 ml of Coomassie Blue Solution (Fermentas) and left to incubate overnight at room temperature with gentle shaking. The next day the Coomassie Blue was removed and the gel washed for 24 hours with dH₂O. The gel was then scanned using an Epson Stylus CX3200.

3.3.7. Immunohistochemistry and Thioflavin S staining of mouse brain cryosections
Mouse brain cryosections (12 μm thick) were permeabilized with 0.5% Triton-X, blocked with 5% NGS and incubated overnight with polyclonal rabbit anti-claudin-5, polyclonal rabbit anti-occludin (both 1:100, Invitrogen) or polyclonal rabbit anti-LRP1 (C-terminal, 1:100, Sigma Aldrich). Sections were then incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:500, Abcam) for 3 hours at room temperature. In order to visualize amyloid-β deposits (plaques and CAA), Thioflavin S (Sigma Aldrich) was
then incubated with mouse brain cryosections. Thioflavin S binds to beta-sheet rich structures such as amyloid aggregates and because of its fluorescence signal at 482nm (green), it was used to stain for Aβ aggregates in brain cryosections where Cy3-conjugated IgG was also applied. Briefly, cryosections were incubated with 0.05% Thioflavin S in 50% ethanol for 8 mins in the dark followed by 2 x 10 second washes with 80% ethanol and 3 x 5 mins washes with dH2O and counterstained with DAPI. Analysis of brain cryosections was performed using a Zeiss Axioplan 2 fluorescent microscope or Olympus FluoView FV1000 confocal microscope with integrated software.

### 3.3.8. Preparation and immunohistochemistry of paraffin-embedded human brain sections

Paraffin-embedded blocks of post-mortem human brain tissue from 30 deceased human subjects were obtained with ethical approval from the Dublin Brain Bank at Beaumont Hospital in Dublin, Ireland. Brain regions and neuropathological diagnoses for each subject are provided in Table 3.1. 10 μm sections were cut from paraffin-embedded blocks using a microtome (Leica) and following xylene deparaffinization and ethanol rehydration, antigen retrieval was performed by boiling sections for 2 x 5 min rounds in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0). Sections were incubated with Congo Red stain (Sigma Aldrich) as per manufacturer’s instructions. Briefly, sections were placed in an alkaline sodium chloride solution for 10 mins followed by incubation in Congo Red solution for 20 mins. Sections were then washed 3 x 30 seconds with 100% ethanol followed by 3 x 5 mins washes with PBS and counterstained with Hoescht (1:10000). For immunostaining of Aβ and claudin-5/occludin, sections were blocked with 5% NGS and double-stained by incubating with polyclonal rabbit anti-Aβ (AW7, 1:500) and monoclonal mouse anti-claudin-5 or anti-occludin (both 1:100, Invitrogen) overnight at 4°C. Polyclonal rabbit AW7 antibody raised against Aβ was kindly provided by the Walsh Laboratory, Brigham & Women’s Hospital. Brain sections were then incubated with Cy2-conjugated goat anti-mouse IgG (to detect Aβ) and Cy3-conjugated goat anti-rabbit (to detect claudin-5 or occludin) (both 1:500, Abcam) for 3 hours at room temperature and counterstained with Hoescht. Analysis of brain sections was performed using a Zeiss Axioplan 2 fluorescent microscope or Olympus FluoView FV1000 confocal microscope with integrated software.
software. Cutting of sections, staining and microscopy was performed blind to neuropathological diagnosis.

3.4. Results

3.4.1. Analysis of TJ protein integrity in post-mortem human brains from neurological disease patients including AD and CAA cases

In collaboration with the Dublin Brain Bank (Beaumont Hospital, Dublin), paraffin-embedded brain tissue from a neurological disease patient cohort (30 patients in total) was obtained with the aim of examining TJ protein integrity in the cerebral vasculature as well as amyloid pathology around blood vessels and in the brain parenchyma (Table 3.1). Blind to the neuropathological diagnosis of each donor tissue sample, 10 μm sections were cut from the paraffin-embedded blocks of brain tissue for Aβ and TJ protein immunohistochemistry. Two separate methods were used to examine Aβ pathology: Congo Red histological stain binds to insoluble protein aggregates with β-pleated sheet structures (namely ‘amyloids’ like Aβ but also α-synuclein and tau aggregates) while anti-Aβ immunohistochemistry using the AW7 antibody raised against human Aβ allows specific detection of Aβ in various forms including both diffuse and senile plaques (Wilcock et al., 2006). To first discern the characteristic staining pattern in AD brain tissue using the Congo Red stain, brain cryosections from a 90-year-old patient (not included in cohort) who was diagnosed with AD (and CAA post-mortem) were stained with Congo Red (Fig. 3.1). Large numbers of dense extracellular amyloid plaques were visualized throughout the brain parenchyma (Fig. 3.1A). Accumulation of amyloid fibrils could also be seen around the cerebral microvessels such as arterioles and capillaries (Fig. 3.1B). As a non-specific histological stain of β-sheet secondary structure in protein fibrils, Congo Red was also found to detect intracellular tau aggregates known as neurofibrillary tangles (NFTs) in AD brain tissue as recognized by their characteristic ‘teardrop’-shaped morphology (Fig. 3.1C).

Paraffin-embedded brain sections from each patient in the neurological disease cohort were analyzed using the Congo Red stain and separately, sections were dual-labelled for Aβ and the major TJ proteins, claudin-5 or occludin, in endothelial cells of the brain vasculature (Fig. 3.2, top panel). Quantification of the number of claudin-5- and occludin-positive microvessels in the donor cases was also performed based on a TJ protein scoring system (Appendix Fig. A1) with vessels having greater than 50% claudin-
5/occludin immunoreactivity being recorded as positive (Fig. 3.2, bottom panel). This was followed by confirmation of the neuropathological diagnoses from the Dublin Brain Bank. High levels of claudin-5 and occludin were observed throughout the brain sections analyzed from non-AD donors including non-neurodegenerative cases like TBI (contralateral to site of injury) and non-AD neurodegenerative cases like ALS (Fig. 3.2). Congo Red and anti-Aβ staining was negative in the non-neurodegenerative cases. In ALS, Aβ immunostaining was also negative although low amounts of small Congo-positive specks were observed which possibly corresponded to intracellular protein inclusions of TDP-43, SOD1 or ubiquitin often seen in ALS patient brains (Kiernan et al., 2011). Small amounts of positive Congo Red staining without obvious amyloid pathology (negative Aβ immunostaining) were recorded in other non-AD neurodegenerative cases like LBD, PSP, FTD and MSA (Appendix Fig. A2). Intact claudin-5 and occludin vessel immunoreactivity was observed throughout the brain regions analyzed in patient samples from these four non-AD neurodegenerative conditions. In contrast, the presence of Aβ in AD cases (verified by both Congo Red and anti-Aβ antibody) appeared to correlate strongly with a decrease in the levels of claudin-5 and occludin in brain microvessels in AD patient neural tissue (Fig. 3.2). CAA-affected microvessels in particular showed markedly reduced levels of claudin-5 and occludin and this finding was recorded in both familial (case number A05 - D23N Iowa mutation predisposing for AD with severe CAA) and sporadic forms of AD/CAA (Fig. 3.2). This trend was further supported by observations of intact claudin-5 and occludin TJ staining in non-CAA microvessels and in areas with no parenchymal Aβ deposition and interestingly in microvessels in close proximity but not in contact with amyloid plaques or CAA-affected vessels (Fig. 3.3). Analysis of the number of claudin-5- and occludin-positive vessels in the brain sections of this patient cohort confirmed a significant decrease in the number of TJ protein-positive vessels in AD cases compared to non-AD cases, a phenomenon which was more pronounced in AD cases with CAA pathology (Fig. 3.2 – bottom). It is worth noting that one case of LBD (case number A08) also showed CAA pathology and was excluded from the final analysis.

3.4.2. Isolation of primary mouse brain endothelial cells and characterization of BBB TJs in aged transgenic APP-Swe mice

Having observed a correlation between Aβ deposition around cerebral blood vessels in AD/CAA patients and reduced levels or aberrant localization of the major TJ proteins...
claudin-5 and occludin, further analysis of BBB and TJ integrity in an ‘Aβ-rich’ environment was carried out using the APP-Swe mouse model of AD/CAA. These mice show aged-dependent increases in levels of soluble and insoluble Aβ and using Thioflavin S, another histological stain that binds to β-sheet-rich protein aggregates, the presence of large and widespread amyloid plaques and CAA pathology was confirmed in brain cryosections from aged (20 month old) APP-Swe mice (Fig. 3.4). Following a dispase/collagenase-based digestion procedure, vascular fractions (endothelial cells, pericytes, smooth muscle cells) were obtained from aged APP-Swe mice and age-matched C57 wild-type (WT) controls and cultured with puromycin-containing media to select for endothelial cells. Western blot analysis of protein fractions from these brain endothelia showed decreased levels of claudin-5 and occludin in aged APP-Swe mice but no major changes in levels of other TJ proteins ZO-1 and tricellulin (Fig. 3.5 – top). Previous studies in AD mouse models have reported altered expression of the major Aβ transporters, LRPl and RAGE, at the BBB (Deane et al., 2004, Kook et al., 2012). In brain endothelial cells of aged APP-Swe mice analyzed here, levels of LRPl were decreased while RAGE expression was low in both APP-Swe and control animals with no obvious difference between the groups (Fig. 3.5 – top). In addition, isolated brain endothelial cells were grown on transwell inserts in order to measure TEER, an estimate of in vitro permeability levels. Measurements of TEER across confluent monolayers of brain endothelial cells showed a small but significant decrease in TEER values from cell monolayers derived from aged APP-Swe mouse brains compared to wild-type controls (Fig. 3.5 – bottom). Thus reduced levels of claudin-5 and occludin in the neuronal vasculature of aged APP-Swe mice associate with changes in BBB permeability.

3.4.3. Immunohistochemical analysis of BBB TJs in young and aged wild-type mice and aged transgenic APP-Swe mice

Results from the western blot and TEER analyses indicated that the major TJ protein components, claudin-5 and occludin, as well as the LRPl efflux transporter of Aβ are disrupted in aged APP-Swe brains and may contribute to a brain-wide loss of BBB integrity. However to further understand the spatial relationship between increased Aβ levels and TJ dysfunction, immunohistochemical analysis of Aβ aggregates and claudin-5/occludin in aged (20 months) APP-Swe mice and age-matched and young (3 months) WT controls was performed. Immunostaining of claudin-5 and occludin in brain microvessels of young wild-type animals showed strong and continuous TJ protein
staining whereas in aged wild-type animals, more punctate and discontinuous patterns of staining were observed (Fig. 3.6 – top). However in aged APP-Swe mice, claudin-5 and occludin immunoreactivity was almost absent in CAA-affected microvessels. In contrast, in non-CAA microvessels of the same animals, TJ protein signals were stronger and similar to patterns observed in aged WT animals (Fig. 3.6 – top). This was confirmed by quantifying the number of claudin-5- and occludin-positive vessels in all three groups with highest TJ protein levels found in young WT animals and the lowest in aged APP-Swe mice (Fig. 3.6 – bottom). Similarly, decreased levels of LRP1 in aged APP-Swe vasculature were confined specifically to CAA-affected vessels (Appendix Fig. A3).

3.4.4. TJ protein levels following treatment of mouse brain endothelial cells (bEnd.3) with soluble Aβ1-40 monomers and dimers

Characterization of claudin-5 and occludin at the BBB in AD/CAA patients and in the APP-Swe mouse model indicated that reductions in TJ protein levels are not spread evenly throughout the brain vasculature. Specifically, data from both human post-mortem and aged APP-Swe mouse brain sections highlight a spatial relationship between Aβ accumulation around microvessels and reduced claudin-5 and occludin. To test whether the build-up of Aβ itself may be responsible for the TJ changes seen in endothelial cells of CAA-affected vessels, recombinant Aβ1-40 monomers and dimers were obtained from the Walsh Laboratory (Brigham and Women’s Hospital, Boston). The shorter more soluble 1-40 isoform of Aβ was chosen since previous work suggested that Aβ1-40 is the predominant isoform in CAA-affected microvessels like arterioles (Thal et al., 2008). These synthetic peptides contain certain modifications to prevent oligomerization and have been previously characterized: a single amino acid change (F19P) has been incorporated into the Aβ1-40 monomer sequence while a dityrosine (diY) cross-link produces a stable Aβ1-40 dimer (Fig. 3.7; Walsh et al., 2009, O’Malley et al., 2014).

Coomassie Blue staining of these Aβ monomer and dimer preparations following separation by SDS-PAGE confirmed their approximate sizes of 4.4 kDa and 8.8 kDa, respectively (Fig. 3.7). A mouse brain endothelial cell line (bEnd.3) was then exposed to increasing doses (0.1-1 μM) of Aβ1-40 monomer or dimer and subsequent western blot analysis showed a dose-dependent decrease in claudin-5 and occludin protein levels whereas levels of ZO-1 intracellular TJ protein remained unchanged (Fig. 3.8 - top). Furthermore, this effect on TJs was not due to the peptide modifications since treatment of bEnd.3 cells with an unmodified Aβ1-40 monomer (without F19P) exhibited the same
pattern of TJ changes (Appendix Fig. A4). Given the implication that Aβ1-40 monomer and dimer were impacting major TJ components, the effect of these synthetic peptides directly on bEnd.3 cell viability was measured. However, no observable cell death was recorded when bEnd.3 were treated with up to 5 μM, a dose 5-times higher than previously used (Fig. 3.8 – bottom). To assess the kinetics of Aβ1-40 monomer- and dimer-mediated changes in claudin-5 and occludin, bEnd.3 cells were treated with the Aβ peptides (1 μM concentration) over the course of 48 hours. Subsequent western blot analysis showed a transient and reversible decrease in claudin-5 and occludin levels with reductions first observed 3-6 hours after treatment and levels returning to baseline 24-48 hours after treatment (Fig. 3.9). In contrast, ZO-1 levels remained more constant at earlier timepoints. Furthermore, an apparent rebound up-regulation of occludin and ZO-1 protein levels was observed 24-48 hours after treatment with Aβ1-40 monomer or dimer (Fig. 3.9).

3.4.5. Immunocytochemical analysis of TJs following treatment of bEnd.3 cells with soluble Aβ1-40 monomers and dimers

Having observed Aβ1-40-mediated effects on TJ components at the protein level, immunocytochemical analysis was carried out to assess whether localisation of claudin-5, occludin and ZO-1 at the TJs of bEnd.3 cells was also affected. After culturing bEnd.3 cells on chamber slides coated with fibronectin and treating them with Aβ1-40 monomer or dimer over the course of 48 hours, a decrease in the cell membrane staining of claudin-5 and occludin was visualized 6 and 12 hours post-treatment (Fig. 3.10). However this was a transient event with levels returning to those observed at baseline 48 hours post-treatment (Fig. 3.10). The localisation of ZO-1 at the TJs was not disturbed at any timepoint post-treatment and similar to the Western blot analysis, there appeared to be an overt up-regulation in the levels of ZO-1 at the cell periphery (Fig. 3.10).

3.4.6. Transcript levels of TJ components following treatment of bEnd.3 cells with soluble Aβ1-40 monomers and dimers

Given the dynamic dose- and time-dependent effects that Aβ1-40 monomer and dimer displayed on levels of TJ components as assessed by western blot and immunocytochemistry, these results suggested that Aβ peptides might impact transcriptional regulation of TJs components. To this end, RT-PCR assays of TJ mRNA levels over the course of 48 hours in response to 1 μM of Aβ1-40 monomer or dimer were
performed (Fig. 3.11). Interestingly, transcripts of claudin-5, occludin and ZO-1 did not show significant down-regulation at any timepoint. With regards to bEnd.3 exposure to Aβ1-40 monomer, levels of occludin mRNA were up-regulated 48 hours post-treatment while ZO-1 transcripts were significantly increased 6, 12, 24 and 48 hours post-treatment, reflecting the increase in these components seen at the protein level (Fig. 3.11 – top). ZO-1 transcript levels were up-regulated after treatment with Aβ1-40 dimer but only at 12 and 24 hours. Both claudin-5 and occludin mRNAs were up-regulated 24 and 48 hours post-treatment with Aβ1-40 dimer with no observable difference in expression at other timepoints (Fig. 3.11 – bottom). These RT-PCR results suggest that while transcriptional changes may account for TJ protein up-regulation at later timepoints, loss of TJ proteins observed in the early stages of Aβ1-40 exposure are not preceded by transcriptional changes. Instead, exposure of brain endothelial cells to Aβ1-40 may result in post-translational modifications of claudin-5 and occludin that ultimately lead to their degradation.

3.5. Discussion

Dysregulation of TJ complexes bridging the paracellular cleft at the BBB is a contributing factor to poor prognosis and disease pathogenesis in neurological conditions like stroke and multiple sclerosis (MS) (Knowland et al., 2014, Wolburg et al., 2003). In MS, loss of BBB integrity and subsequent transendothelial entry of autoreactive leukocytes into the brain causes the destruction of myelin-producing oligodendrocytes (Compston and Coles, 2008). To this end, selective loss of claudin-3 at the BBB in a mouse model of MS (experimental autoimmune encephalomyelitis) and mislocalization of occludin and ZO-1 in CNS vessels of MS patients suggests that alterations of brain endothelial TJ in MS increases BBB permeability and may facilitate leukocyte transmigration (Wolburg et al., 2003, Kirk et al., 2003, Leech et al., 2007). In this study, levels of the major transmembrane TJ proteins claudin-5 and occludin were found to be significantly lower in brains of AD and AD/CAA patients compared to other neurodegenerative diseases cases without Aβ involvement like ALS, LBD and PSP. In AD/CAA patients, these TJ alterations were specifically confined to CNS vessels in direct contact with Aβ (CAA-affected) with normal TJ protein immunostaining observed in areas without Aβ deposition. These findings are in accordance with a previous study conducted by Carrano and colleagues (2011) that reported loss of claudin-5, occludin and ZO-1 in Aβ-laden capillaries (type I CAA) of six AD patients. Since the majority of the CAA-affected
microvessels examined in this study were arterioles (type II CAA), it is possible that loss of claudin-5 and occludin seen in AD/CAA patients here is due to disruption of the VSMC layer in arterioles that are absent in capillaries. However, reduced levels of capillary claudin-5 and occludin were also observed in a familial AD/CAA case (A05 – Iowa mutation) showing significant capillary CAA pathology. Nevertheless, the rigor of this study would be improved by using a VSMC marker like smooth muscle actin and also an endothelial marker like lectin or CD31 to deduce whether TJ alterations in CAA vessels could be the result of VSMC or endothelial degeneration. Furthermore, ZO-1 in human donor cases was not examined here due to a failure to observe clear vascular ZO-1 localization in paraffin-embedded brain sections with an anti-ZO-1 polyclonal antibody (Invitrogen). Understanding ZO-1 integrity at the BBB in CAA-affected vessels in humans would help to interpret data from aged APP-Swe mice and Aβ-treated bEnd.3 cells which showed that ZO-1 levels were not markedly reduced.

It is also worth addressing certain pathological observations from this neurological disease patient cohort and caveats to the analysis of TJ integrity in non-AD neurodegenerative cases in this study. One patient (case number A25) had a post-mortem diagnosis of AD with CAA pathology but had no clinical symptoms of dementia. This presence of Aβ pathology without clinical dementia has been reported previously and is accompanied by larger brain and hippocampal volumes (Erten-Lyons et al., 2009). Why exactly synaptic/neuronal dysfunction and ultimately cognitive deficits are absent despite the presence of major AD features post-mortem like amyloid plaques and NFTs is unknown. However, a recent GWAS study in non-demented elderly subjects with AD pathology has identified variants in the RELN gene (encoding Reelin) as a possible protective factor (Kramer et al., 2011). These variants promote up-regulation of Reelin, an extracellular matrix glycoprotein that enhances synaptic plasticity, inhibits Aβ-mediated synaptic dysfunction and modulates tau phosphorylation (Durakoglugil et al., 2009, Kramer et al., 2011). With regards to the non-AD neurodegenerative donor cases in the patient cohort examined, no literature exists with regards to BBB or TJ dysfunction in the tauopathies or synucleinopathies analyzed here. Although no major TJ disturbances (in comparison to AD/CAA donors) were observed in the ALS, PSP, LBD, MSA and other donor cases, it is still possible that BBB and TJ alterations are a pathological feature of these CNS disorders in brain regions not analyzed in this study. For example, the brain regions of the three PSP donor cases examined in this study were the calcarine cortex
(occipital lobe), striatum and hippocampus, however PSP principally affects the basal ganglia, brainstem, frontal cortex and cerebellum (Rampello et al., 2005). With regards ALS where motor neurons of the motor cortex and brainstem degenerate, tissue from the frontal lobe (which contains the motor cortex) was analyzed in the three ALS donor cases but the precise cortical region was not provided. Therefore, brain-wide analyses of TJ integrity at the BBB in these disorders (and at different stages of disease progression) would be required to confidently discount TJ protein changes as a pathological feature of tau- and/or α-synuclein-associated conditions.

The APP-Swe mouse model of AD and CAA provided a means of assessing the integrity of TJs and the BBB at the protein level in an Aβ-rich environment. Brain endothelial-enriched protein fractions obtained from 20 month-old APP-Swe mice contained lower amounts of claudin-5 and occludin and approximately equal ZO-1 and tricellulin levels when compared to C57 WT animals of similar age. These results partially agree with a previous characterization of TJ protein components in younger APP-Swe mouse brains (Hartz et al., 2011). In that study, Hartz and colleagues (2011) reported decreased claudin-5 and claudin-1 protein levels in brain microvessel isolations from 9 month-old APP-Swe mice whereas occludin and ZO-1 remained unchanged. However, since the authors did not isolate endothelial cells per se but rather microvessel fractions (likely containing pericytes, astrocytes and smooth muscle cells), it is possible that changes in endothelial-specific occludin were masked by pericyte- and astrocyte-derived occludin (Shimizu et al., 2008, Bauer et al., 1999). Nevertheless, the work from Hartz et al. along with this present study suggest that TJ alterations in APP-Swe mice may begin at earlier stages around the period of CAA onset and worsen as Aβ accumulates with age. Such reductions in transmembrane TJ proteins likely contribute to increased BBB permeability as measured by TEER readings across brain endothelial monolayers in this study. These findings could also be followed-up in other AD mouse models in particular the APP-SweDI line harboring the Swedish mutation and also the vasculotropic Dutch/Iowa (E693Q/D694N) mutations that cause early-onset cerebrovascular Aβ deposition and severe CAA pathology in these animals (Davis et al., 2004). As well as analysis of BBB TJs in aged APP-Swe mice, levels of the major Aβ receptors/transporters at the BBB were measured. In agreement with previous findings from AD mouse models and AD patients, levels of LRPI (which mediates Aβ clearance across the BBB) were reduced in brain endothelial cells from APP-Swe mice. However, while increased expression of RAGE
(which mediates brain entry of Aβ from the circulation) has been documented in AD mouse models and AD patients, RAGE levels were low and unchanged in the APP-Swe mouse-derived brain endothelial cells examined in this study (Deane et al., 2003, Kook et al., 2012, Donahue et al., 2006). The reason for this discrepancy is unclear though previous studies of BBB-associated RAGE in AD mouse models were conducted in younger animals (8-9 months) (Deane et al., 2003, Kook et al., 2012).

In light of the decreased levels of claudin-5 and occludin observed in CAA-affected vessels of both AD patients and aged APP-Swe mice, the direct effects of Aβ on TJ integrity were assessed via treatment of bEnd.3 cell cultures with recombinant Aβ1-40 monomers and dimers. These peptides were used as previous in vitro studies of TJ responses to Aβ have used poorly characterized Aβ material that may be prone to aggregation. Treatment of bEnd.3 cells with soluble Aβ1-40 monomers and dimers caused a dose-dependent reduction in claudin-5 and occludin without altering ZO-1. However these changes were temporary with TJ protein levels returning after 48 hours. Furthermore, an apparent rebound up-regulation of the intracellular TJ protein ZO-1 at both the mRNA and protein levels suggests that transcriptionally increasing ZO-1 may be a cellular response to rescue paracellular barrier tightness after loss of claudin-5 and occludin transmembrane proteins. However since claudin-5 and occludin transcript levels did not mirror the protein loss, the exact mechanism by which Aβ mediates reduced TJ protein levels is unclear. Possible mechanisms that remain unexplored include TJ protein modifications like phosphorylation, cross-talk between transcellular Aβ receptors and TJ components and even the possible direct interaction of Aβ peptides with TJ proteins. In addition, the polarized nature of brain endothelial cells suggests Aβ may exert different effects depending on its apical or basolateral addition. Other studies have proposed potential pathways by which Aβ causes TJ degradation: MAPK (mitogen-activated protein kinase) activation (Tai et al., 2010); Aβ-RAGE-mediated induction of reactive oxygen species (Carrano et al., 2011); increased MMP (matrix metalloprotease) activity (Hartz et al., 2011, Kook et al., 2012) and Aβ-RAGE-mediated activation of the Ca^{2+}/calcineurin pathway (Kook et al., 2012). However as outlined previously, substantial differences exist among these studies with regards to cell types, Aβ assemblies and concentrations of Aβ applied, making comparisons between them difficult. One caveat to the experimental design used here is that while soluble Aβ assemblies are likely to be located around blood vessels in AD and CAA (particularly in early stages), Aβ
aggregates and fibrils are the predominant $\alpha\beta$ species in end-stage AD/CAA (Thal et al., 2008). It would therefore be important to compare the effects of insoluble $\alpha\beta$ aggregates on TJ integrity with those of soluble $\alpha\beta$ monomers/dimers/oligomers.

The combined findings presented here from human AD/CAA patients, aged APP-Swe mice and $\alpha\beta$-treated bEnd.3 cells indicate a potential role for claudin-5 and occludin in AD pathogenesis. These results suggest that $\alpha\beta$-mediated reductions in claudin-5 and occludin can increase BBB permeability (as measured by TEER) and that chronic loss of these TJ proteins as in AD/CAA may contribute to CAA-associated abnormalities like cerebral microhemorrhages. In the next chapter, the contributions of claudin-5 and occludin TJ proteins in sealing the paracellular barrier in vitro will be explored using RNAi as a molecular tool to modulate TJ protein levels. In the context of AD, this allows for the study of $\alpha\beta$ movement across brain endothelial cells in the absence of claudin-5 and occludin.
Table 3.1: Brain regions and pathological diagnoses for neurological disease patient cohort examined in this study. Post-mortem brain tissue from 30 separate donors was analyzed in total.

<table>
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<td>A07</td>
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</tr>
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<td>Frontal</td>
<td>AD</td>
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<td>AD + CAA</td>
</tr>
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</tr>
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<td>AD + CAA; Subclinical PD</td>
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</tr>
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<td>CAA + Path of AD</td>
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Figure 3.1: Characteristic pathologies in human AD brain sections using Congo Red stain. (A) Widespread amyloid plaques in superior frontal gyrus (frontal lobe). (B) Cerebral amyloid angiopathy (CAA) – amyloid accumulation around both an arteriole (top arrow) and capillary (bottom arrow) in posterior cingulate cortex. (C) Intracellular neurofibrillary tangles (NFTs) indicated by arrows in posterior cingulate cortex. Scale bar = 50 μm.
Figure 3.2: Characterization of claudin-5 and occludin TJ proteins in brain microvessels and assessment of amyloid pathology in paraffin-embedded brain sections from a neurological disease patient cohort (30 cases in total). Top - Representative images from a non-neurodegenerative (TBI, contralateral region from injury site) donor, non-AD neurodegenerative (ALS) donor, early-onset (EO) familial AD (APP-Iowa mutation) and late-onset (LO) sporadic AD cases. Congo Red staining for protein aggregation revealed significant amyloid plaques and CAA in AD cases and low to background positivity in ALS cases. Immunohistochemical analysis of Aβ (green), claudin-5 (red) and occludin (red) showed strong claudin-5 and occludin immunoreactivity in brain microvessels (arterioles) of non-AD/CAA cases. In contrast, the presence of Aβ and CAA in brain microvessels of AD cases correlated strongly with a reduction in levels of claudin-5 and occludin immunoreactivity. Scale bar in all images = 50 μm. Bottom – Quantification of

<table>
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<th>Condition</th>
<th>No. of CLDN5+/Occ⁻ vessels (per mm)</th>
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<tbody>
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<td>TBI (contralateral)</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>PSP</td>
<td>90 ± 15</td>
</tr>
<tr>
<td>ALS</td>
<td>100 ± 20</td>
</tr>
<tr>
<td>LBD</td>
<td>120 ± 25</td>
</tr>
<tr>
<td>AD</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>AD + CAA</td>
<td>50 ± 15</td>
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</tbody>
</table>
claudin-5- and occludin-positive vessels (per mm²) in paraffin-embedded brain sections from the neurological disease patient cohort. These measurements were based on the scoring system outlined in Appendix Fig. A1. There was a significant decrease in the number of claudin-5-positive vessels in AD cases compared to non-AD cases and a significant decrease in the number of claudin-5- and occludin-positive vessels in AD cases with CAA involvement compared to non-AD cases. (**P ≤ 0.01 for claudin-5 in AD versus PSP, ALS and LBD, ***P ≤ 0.001 for claudin-5 in AD/CAA versus PSP, ALS and LBD, ***P ≤ 0.001 for occludin in AD/CAA versus PSP, ALS and LBD; one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons. TBI: n = 1, PSP: n = 3, ALS: n = 3, LBD: n = 2, AD: n = 4, AD + CAA: n = 11. Note: one TBI case, SD here represents deviation between multiple sections from the same region of the same patient).
Figure 3.3: Intact TJ protein staining in AD/CAA brains. *Left* – Strong microvascular claudin-5 (red) and occludin (red) immunoreactivity can be observed in areas close to Aβ deposition/amyloid plaques (green - detected by anti-Aβ immunohistochemistry). *Right* - Strong claudin-5 and occludin staining can also be observed in non-CAA microvessels (no Aβ immunoreactivity, shown by arrows) in close proximity (50-200 μm) to CAA-affected vessels where claudin-5 and occludin staining is absent. Scale bar = 50 μm.
Figure 3.4: Amyloid plaque and CAA pathology in brain cryosections of aged (20 months) APP-Swe transgenic mice. (a) Thioflavin S (green) staining reveals abundant amyloid plaque cores in cortical regions. (b) CAA pathology in a leptomeningeal artery. (c) CAA pathology in cortical microvessels (capillaries, arterioles). Scale bar = 100 μm.
Figure 3.5: Characterization of the major TJ proteins and Aβ transporters and transendothelial electrical resistance (TEER) measurements of BBB integrity in brain endothelial cell fractions. Top – Western blot analysis of TJ proteins (claudin-5, occludin, ZO-1 and tricellulin) and Aβ transporters (LRP1, RAGE) in brain endothelial cell fractions isolated from aged (20 months) APP-Swe mice and age-matched WT controls. Decreased levels of claudin-5, occludin and LRP1 were observed in the APP endothelial cell fractions. (n = 5 animals per group – brains pooled for endothelial cell isolation). Bottom – Measurements of TEER across confluent monolayers of pure isolated brain endothelial cells showed decreased TEER values in cell monolayers derived from APP-Swe mouse brains. (*P ≤ 0.05, Student’s t-test; n = 8 transwells per group. Data are means ± SD).
Figure 3.6: Immunohistochemical analysis of the TJ proteins claudin-5 and occludin in aged (20 months) APP-Swe mice and age-matched and young (3 months) wild-type (WT) controls. Top – Immunostaining of claudin-5 (red) and occludin (red) in brain microvessels shows strong and continuous TJ protein staining in young WT animals but more punctate and discontinuous patterns in aged WT animals. In CAA vessels (thioflavin S staining of Aβ aggregates in green) of aged APP-Swe mice, levels of claudin-5 and occludin were very low. In contrast, in non-CAA vessels of the same animals, TJ protein staining was more pronounced and similar to patterns observed in aged WT animals. Scale bar = 50 μm. Bottom – Quantification of claudin-5- and occludin-positive vessels in young WT, aged WT and aged APP-Swe animals. (n = 4 animals per group. Data are means ± SD).
Figure 3.7: Synthetic recombinant Aβ1-40 (F19P) monomer and Aβ1-40 dityrosine (diY) cross-linked dimer peptides. Top - Molecular model of Aβ1-40 monomer and dimer indicating their approximate molecular weights in kiloDaltons (kDa). Bottom - SDS-PAGE analysis and Coomassie Blue staining of Aβ1-40 (F19P) monomer and Aβ1-40 (diY) dimer peptides. MW – molecular weight.
Figure 3.8: Treatment of mouse brain endothelial cells (bEnd.3) with synthetic recombinant Aβ1-40 monomer and dimer peptides (0.1-1 μM). Top - Western blot of TJ protein levels 12 hours after treatment of bEnd.3 cells with increasing concentrations of scrambled Aβ1-40 peptide, Aβ1-40 (F19P) monomer or Aβ1-40 (diY) dimer. A dose-dependent decrease in claudin-5 and occludin but not ZO-1 protein levels was observed in cells treated with Aβ1-40 monomer or Aβ1-40 dimer. (n = 3 separate cell transfections per concentration). Bottom - MTS-based cell viability assay measured 12 hours after treatment of bEnd.3 cells with increasing concentrations of Aβ1-40 (F19P) monomer or Aβ1-40 (diY) dimer (n = 3 separate cell transfections. Data are means ± standard error of the mean (SEM)).
Figure 3.9: Western blot analysis of TJ protein levels over the course of 48 hours following treatment of bEnd.3 cells with 1 μM of Aβ1-40 (F19P) monomer or Aβ1-40 (diY) dimer. Reduced levels of claudin-5 and occludin were observed 3-6 hours after treatment with Aβ1-40 monomer or dimer with levels returning to baseline 24-48 hours after treatment. In contrast, levels of ZO-1 remained constant at earlier timepoints. Furthermore, an apparent rebound up-regulation of occludin and ZO-1 protein levels was observed 24-48 hours after treatment with Aβ1-40 monomer or dimer. (n = 3 separate cell transfections per timepoint).
Figure 3.10: Immunocytochemistry analysis of claudin-5, occludin and ZO-1 in response to treatment of bEnd.3 cells with 1 μM of Aβ1-40 (F19P) monomer (top panel) or Aβ1-40 (diY) dimer (bottom panel). Localisation of claudin-5 and occludin at the TJs of bEnd.3 cells showed a decrease in the punctate staining of these TJ proteins 6 and 12 hours post treatment with Aβ1-40 monomer or dimer with levels returning to those observed at baseline 48 hours post treatment. The localisation of ZO-1 at the TJs did not decline at any timepoint post-treatment with up-regulation in the levels of ZO-1 at the cell periphery 48 hours post-treatment. Scale bars = 40 μm. (Images representative of 3 separate cell transfections per timepoint).
Figure 3.11: RT-PCR analysis of TJ mRNA levels over the course of 48 hours in response to 1 μM of Aβ1-40 (F19P) monomer (top panel) or Aβ1-40 (dIY) dimer (bottom panel). Levels of claudin-5, occludin and ZO-1 mRNA did not show significant down-regulation at any timepoint. Levels of occludin transcript were up-regulated 48 hours post-treatment with Aβ1-40 monomers while ZO-1 transcripts were significantly increased 6, 12, 24 and 48 hours post-treatment. ZO-1 transcript levels were up-regulated after treatment with Aβ1-40 dimer but only at 12 and 24 hours. Both claudin-5 and occludin transcripts were up-regulated 24 and 48 hours post-treatment with Aβ1-40 dimer with no observable difference in expression at other time points. (n = 3 separate cell transfections per timepoint. Data are means ± SEM).
Chapter 4:
Paracellular movement of amyloid-β (Aβ) across brain endothelial cells \textit{in vitro} following RNAi-mediated down-regulation of claudin-5 and occludin.
4.1. Abstract
Aβ- and CAA-associated reductions in the vascular levels of transmembrane TJ proteins claudin-5 and occludin were observed in AD/CAA donor brains, aged APP-Swe mice and in brain endothelia in vitro as outlined in the previous chapter. While reduced levels of claudin-5 and occludin were associated with decreased TEER values across brain endothelial monolayers from aged APP-Swe mice, changes to BBB paracellular permeability following loss of claudin-5 and occludin and its impact on AD progression is unknown. In this section, RNA interference (RNAi) was used to determine the effects of co-suppression of these two TJ proteins on the physiological integrity of TJs in vitro. Firstly, small interfering RNAs (siRNAs) designed to target claudin-5 and occludin transcripts induced potent and prolonged target suppression in bEnd.3 cells at mRNA and protein levels and this TJ protein down-regulation was accompanied by a significant decrease in TEER values. Furthermore, transfection of bEnd.3 cell monolayers with TJ-directed siRNAs increased bEnd.3 permeability to a synthetic FITC-labeled Aβ1-40 peptide. To assess the extent of bEnd.3 permeability to Aβ1-40 following down-regulation of claudin-5 and occludin, recombinant modified Aβ1-40 monomers and dimers were used as commercially available FITC-labeled Aβ1-40 has the potential to oligomerize. Interestingly, while diffusion of Aβ1-40 monomer across bEnd.3 monolayers was increased in the absence of claudin-5 and occludin, Aβ1-40 dimer could not diffuse to the same extent. This suggests the presence of a size-selective TJ to molecules of approximately 4.4 kDa but not 8.8 kDa when claudin-5 and occludin levels in brain endothelial cells are simultaneously decreased.

4.2. Introduction
Since claudin-5 and occludin are major regulators of paracellular permeability between endothelial cells of the brain vasculature, loss of these TJ proteins in chronic conditions like AD may contribute to BBB disruption and influx of serum proteins and immune cells that exacerbate neuronal damage (Erickson and Banks, 2013). However, decreased levels of claudin-5 and occludin in response to Aβ in vessels of human AD/CAA and murine APP-Swe brains also indicate a potential involvement in Aβ transport across the BBB. Just as changes in the transcellular Aβ transporters at the BBB, LRP1 and RAGE, alter brain Aβ levels and are associated with AD pathogenesis, changes in the paracellular TJ proteins may also impact Aβ movement between the brain and the blood (Deane et al., 2003, Deane et al., 2004, Bell et al., 2009). In the previous chapter, TEER analysis of
brain endothelial monolayers from APP-Swe mice suggests that an increase in BBB permeability is associated with reduced claudin-5 and occludin. To assess the extent of endothelial permeability to Aβ in the absence of claudin-5 and occludin, siRNAs directed against these TJ components were used to engage the RNAi pathway in bEnd.3 cells in vitro and modulate TJ protein levels. Subsequently, growth of siRNA-treated bEnd.3 cells on porous membranes of transwell inserts allowed for measurement of paracellular Aβ1-40 movement across bEnd.3 monolayers. bEnd.3 permeability to a range of various Aβ1-40 assemblies was examined: commercially available FITC-labeled Aβ1-40, recombinant Aβ1-40 (F19P) monomer and recombinant Aβ1-40 (diY) dimer. These recombinant peptides, modified to prevent oligomerization, were tested to assess TJ size-selectivity in the absence of claudin-5 and occludin and also because commercially available Aβ monomers may aggregate depending on preparation and storage conditions (Finder et al., 2010).

While no previous reports of brain endothelial cell permeability assays of paracellular Aβ movement could be found in the literature, some studies have assessed the effect of Aβ-mediated TJ modulation on paracellular permeability to a range of tracer molecules. Tai and colleagues (2010) found that Aβ1-40-induced down-regulation of occludin in the human brain endothelial cell line CMEC/D3 increases paracellular permeability to a 70-kDa FITC-dextran molecule. Furthermore, Kook et al. (2012) reported an increase in bEnd.3 permeability to a 40-kDa FITC-dextran molecule following Aβ1-42-mediated perturbation of claudin-5, occludin and ZO-1. In this study, RNAi was used to decrease claudin-5 and occludin levels in bEnd.3 cells. Aβ1-40 was not used to modulate TJs as this would have required two separate additions of Aβ1-40 to bEnd.3 monolayers - Aβ1-40 treatment to modulate TJ proteins and Aβ1-40 addition for transwell permeability measurements. As a result, there would be a strong chance of the initial Aβ1-40 treatment cross-reacting and affecting quantitative measurements of Aβ1-40 movement in the permeability assay. Activation of the RNAi pathway in vitro via delivery of siRNA molecules allows for controlled and timed regulation of specific target genes and has been used previously in the host laboratory to target the claudin-5 TJ protein in vitro and in vivo (Campbell et al., 2009, Campbell et al., 2012).
4.3. Methods

4.3.1. siRNA transfection of bEnd.3 cells using Lipofectamine 2000 Reagent

bEnd.3 cells were cultured as described previously and prior to cell transfections, each well of a 24-well plate was seeded at a cell density of 1x10^5 cells in 500µl of DMEM. For each well of cells to be transfected, 20pmol of targeting or non-targeting (NT) siRNA was diluted in 50µl of Opti-MEM (Gibco-Invitrogen). For each well, 1µl of LF2000 was diluted in 50µl of Opti-MEM in a 1.5ml eppendorf tube and incubated for 5 min at room temperature. The diluted LF2000 was then combined with the siRNA and incubated for 20 min at room temperature. 100µl of the siRNA-LF2000 mixture was then added to each well and mixed well. The plates were then gently swirled and placed in the 37°C incubator for the required length of time. RNA was extracted from bEnd.3 cells using reagents and instructions supplied in the Qiagen RNeasy® Mini Kit or by Trizol extraction. Protein was isolated using lysis buffer (62.5 mM Tris, 2% SDS, 10 mM Dithiothreitol, 10 µl protease inhibitor cocktail/100 ml) followed by centrifugation at 12,000 rpm for 20 min at 4°C and supernatant removed for TJ protein analysis. All transfections were performed in triplicate. All siRNA sequences are provided in the appendix. For Cy3-siRNA transfections, Silencer® Cy3-labelled NT siRNA was supplied by Invitrogen.

4.3.2. Transwell permeability assays

bEnd.3 cells (5 x 10^4 cells/well) were grown to confluence on 1% fibronectin-coated Corning® HTS 24-well Transwell® polyester inserts with a pore size of 0.4µm and transfected with 10 pmol of targeting or non-targeting siRNAs using LF2000 as described. 72 hours post-transfection (the timepoint of maximal siRNA-mediated tight junction protein suppression), 10 µM of fluorescein isothiocyanate-amyloid-β (FITC-Aβ) 1-40 or 1-42 peptide (Bachem) in DMEM was added to the apical chamber of each well and the cells incubated at 37°C. Sampling aliquots were taken from the basolateral chamber and replaced with fresh media every 15 min for 2 hours and transferred to 96-well plates (Nunc). FITC-Aβ fluorescence was determined using a spectrofluorometer (Optima Scientific) at an excitation wavelength of 485nm and an emission wavelength of 520nm. Relative fluorescence units (RFU) were converted to ng/ml values using FITC-Aβ standard curves and corrected for background fluorescence and serial dilutions over the course of the experiment. For permeability assays measuring basolateral to apical movement of FITC-Aβ, the peptide was added to the basolateral chamber and sampling
aliquots taken from the apical side. After subtracting background fluorescence of media taken from untreated cells, relative fluorescence units (RFU) were converted to ng/ml values using a FITC-\(\text{A}\beta1-40\) standard curve and corrected for the serial dilutions over the course of the experiment. For transwell experiments involving synthetic \(\text{A}\beta1-40\) (F19P) monomer and (diY) dimer, 10 \(\mu\text{M}\) of either \(\text{A}\beta1-40\) monomer or dimer was applied to the apical chamber and its movement across \(\text{bEnd.3}\) monolayers measured using the Human \(\text{A}\beta40\) ELISA kit (Invitrogen). The apparent permeability coefficient \(P_{\text{app}}\) for each treatment was calculated using the following equation:

\[
P_{\text{app}} \text{ (cm/s)} = \frac{(dQ/dT)}{(A \times C_0)},
\]

where \(dQ/dT\) (\(\mu\text{g/s}\)) is the rate of appearance of \(\text{A}\beta\) peptide on the receiver side after application, \(A\) (\(\text{cm}^2\)) is the effective surface area of the insert size and \(C_0\) (\(\mu\text{g/ml}\)) is the initial \(\text{A}\beta\) peptide concentration on the donor side. \(dQ/dT\) is the slope \(m\) (\(y = mx+c\)) calculated by plotting the cumulative amount (Q) versus time (s).

4.3.3. RT-PCR and Western blot analysis
Claudin-5 and occludin mRNA levels in \(\text{bEnd.3}\) cells following siRNA treatment were measured by SYBR Green RT-PCR. Antibodies used for western blot analysis in this chapter were as follows: polyclonal rabbit anti-claudin-5, polyclonal rabbit anti-occludin (both 1:500, both Invitrogen) and polyclonal rabbit anti-\(\beta\)-actin (1:2000, Abcam).

4.3.4. Half-maximal inhibitory concentration (IC\(50\)) measurements of claudin-5 and occludin siRNAs
IC\(50\) measurements indicate how much of a particular drug is needed to inhibit the biological function of the drug’s target by half. For assessing siRNA inhibitory potential, \(\text{bEnd.3}\) cells were seeded onto wells of a 24-well plate as described and transfected with 10 – 200 nM of NT siRNA, claudin-5 siRNA, occludin siRNA or claudin-5 and occludin siRNAs (using LF2000 as outlined previously). 72 hours post-transfection (the timepoint of maximal siRNA-mediated tight junction protein suppression), RNA was extracted from \(\text{bEnd.3}\) cells using the RNeasy Mini Kit (Qiagen) and claudin-5 and occludin mRNA levels quantified by SYBR Green RT-PCR. IC\(50\) was measured for claudin-5 siRNA, occludin siRNA or claudin-5 and occludin siRNAs by plotting percentage expression levels (as a means of inhibition levels) against log siRNA concentration on a sigmoidal dose-response curve – IC\(50\) is the siRNA concentration at which the curve passes through the 50% expression (inhibition) level.
4.4. Results

4.4.1. siRNA-mediated suppression of claudin-5 and occludin and measurement of TEER in bEnd.3 cells

To determine the effects of co-suppression of claudin-5 and occludin transmembrane TJ proteins on the physiological integrity and permeability of brain endothelial cells, bEnd.3 cells were transfected using the LF2000 reagent with siRNAs designed to target claudin-5 and occludin. The ability of siRNA-LF2000 complexes to transfect bEnd.3 cells was assessed using a Cy3-conjugated NT siRNA which allows for visualization of siRNA-LF2000 complexes by fluorescent microscopy (Fig. 4.1). This showed that siRNA-LF2000 complexes begin to transfect bEnd.3 cells approximately 2 hours post-treatment with maximal transfection observed 6-24 hours post-treatment. The efficacy of both claudin-5 and occludin siRNAs in mediating target gene suppression was assessed at 24, 48, 72, 96 hours and 1 week after administration. bEnd.3 cell transfection with the individual siRNAs (claudin-5 siRNA only or occludin siRNA only) potently down-regulated claudin-5 and occludin respectively at the mRNA level as measured by RT-PCR (Fig. 4.2). TJ transcript suppression lasted for up to a week post-transfection with mRNA levels starting to rise again at the 1-week time-point. Co-administration of both claudin-5 and occludin siRNAs was found to efficiently down-regulate claudin-5 and occludin concomitantly with percentage suppression levels equivalent to those measured following single siRNA transfection (Fig. 4.2). This siRNA-mediated down-regulation of claudin-5 and/or occludin was also shown to be dose-dependent as assessed using IC50 measurements of claudin-5 and occludin siRNA potency (Appendix Figure A5). Changes in transcript levels of TJ components were also reflected at the protein level as measured by western blot (Fig. 4.3). To assess the effect of siRNA-mediated claudin-5 and/or occludin down-regulation on barrier integrity in bEnd.3 cells, TEER recordings were taken across bEnd.3 monolayers. Reductions in TEER were observed following siRNA-mediated TJ suppression (25-30 Ω.cm² at 72 hours post-transfection) compared to NT siRNA-treated cells (48-50 Ω.cm² at 72 hours) (Fig. 4.4). Furthermore, this decrease in TEER was not due to siRNA transfection-associated cell death. Treatment of bEnd.3 cells with single or double siRNAs (complexed with LF2000) had no effect on bEnd.3 cell viability as confirmed via an MTS-based cell viability assay (Fig. 4.4).
4.4.2. Paracellular flux of FITC-labeled A\(\beta\)1-40 across bEnd.3 monolayers following claudin-5 and occludin suppression

Activation of the RNAi pathway to suppress claudin-5 and occludin in bEnd.3 cells elicited a similar transient regulation of TJ protein levels as seen previously with A\(\beta\)1-40 monomers and dimers. Since this siRNA-mediated TJ protein down-regulation was also accompanied by decreases in TEER, the next step involved assessing potential changes in paracellular transport of A\(\beta\) across brain endothelial cell monolayers with decreased TJ integrity. To this end, the movement of a synthetic FITC-conjugated A\(\beta\)1-40 molecule across bEnd.3 cell monolayers was measured using transwell permeability assays. In these assays, endothelial cells are grown on a porous membrane and the apical and basolateral chambers represent the luminal (blood) and abluminal (brain) sides of endothelial cells, respectively (Fig. 4.5). 72 hours after siRNA transfection (at the timepoint of maximal TJ protein suppression), FITC-A\(\beta\)1-40 was added to either the apical or basolateral chamber and its movement across the porous membrane was measured using a spectrofluorometer (Fig. 4.5). Addition of FITC-A\(\beta\)1-40 to the apical aspect of cells showed that the molecule could diffuse along its own concentration gradient and its rate of diffusion was enhanced when both claudin-5 and occludin were suppressed alone or in tandem (Fig. 4.6). Similarly, diffusion of FITC-A\(\beta\)1-40 when applied to the basolateral aspect of cells could be enhanced when both claudin-5 and occludin were suppressed using siRNAs (Fig 4.6). The apparent permeability coefficient (P\(_{\text{app}}\)), a measure of the rate of FITC-A\(\beta\)1-40 movement to the receiver side after application, was highest for bEnd.3 cells treated with both occludin and claudin-5 siRNAs when the peptide was applied apically (8.66 x 10\(^{-14}\) cm/s) or basolaterally (41.74 x 10\(^{-14}\) cm/s). Similarly, enhanced diffusion of the other major A\(\beta\) isoform, A\(\beta\)1-42 (also conjugated to FITC), was observed when claudin-5 and occludin levels were modulated (Appendix Fig. A6).

4.4.3. Paracellular flux of recombinant A\(\beta\)1-40 monomers and dimers across bEnd.3 monolayers following claudin-5 and occludin suppression

While commercially available FITC-labeled A\(\beta\)1-40 is soluble, it still has the potential to oligomerize (Finder et al., 2010). Therefore, the ability of recombinant A\(\beta\)1-40 (F19P) monomer and A\(\beta\)1-40 (d\(\beta\)Y) dimer (modified to prevent their oligomerization) to passively diffuse across bEnd.3 monolayers was examined. Furthermore, these modified peptides enable potential TJ size-selectivity to different A\(\beta\) assemblies of different

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molecular weights to be assessed. Since recombinant Aβ1-40 monomer and dimer do not have a fluorescent label, detection of peptide movement across bEnd.3 monolayers was carried out using ELISA assays. Aβ1-40 monomer was applied to the apical aspect of transwell chambers and its movement across bEnd.3 monolayers measured over the course of 90 mins. Transfection of bEnd.3 cells with TJ-directed siRNAs increased paracellular Aβ1-40 monomer flux across bEnd.3 monolayers to the basolateral chamber compared to NT siRNA-transfected cells and untransfected cells (Fig. 4.7). However when Aβ1-40 dimer was applied to the apical aspect of transwell chambers, no significant differences in the rate of Aβ1-40 dimer movement across bEnd.3 monolayers was observed between NT siRNA- or TJ-directed siRNA-treated cells (Fig. 4.7). While the Aβ1-40 monomer could diffuse across the paracellular pathway in a process regulated by the levels of claudin-5 and occludin, the dimer could not diffuse to the same extent, suggesting the presence of a size-selective TJ to molecules of approximately 4.4 kDa (Aβ1-40 monomer) but not 8.8 kDa (Aβ1-40 dimer) when claudin-5 and occludin levels were down-regulated separately or concomitantly (Fig. 4.8).

4.5. Discussion

Decreased TJ integrity following claudin-5 and occludin down-regulation is associated with Aβ accumulation in brains of AD patients and the APP-Swe murine model of AD and in brain endothelial cells in vivo. In this chapter, the effects of claudin-5 and occludin loss on the rates of paracellular Aβ movement were examined in vivo via siRNA-mediated TJ protein suppression in mouse bEnd.3 cells and various Aβ assemblies: commercial FITC-Aβ1-40, recombinant Aβ1-40 (F19P) monomer and recombinant Aβ1-40 (diY) dimer. Firstly, treatment of bEnd.3 cells with siRNAs directed against claudin-5 and occludin showed robust decreases in these components at both mRNA and protein levels and associated reductions in TEER. Secondly, siRNA-mediated down-regulation of claudin-5 and/or occludin resulted in increased movement of FITC-Aβ1-40 across bEnd.3 monolayers along its own concentration gradient when applied apically or basolaterally. Furthermore, this paracellular Aβ flux was found to be dependent on Aβ1-40 size as diffusion of recombinant Aβ1-40 monomer but not dimer was increased when claudin-5 and occludin were down-regulated separately or in combination. This finding points towards a defined size-selectivity along the paracellular pathway of brain endothelial cells following siRNA-mediated claudin and/or occludin suppression. While addition of Aβ1-40 to apical or basolateral compartments may also directly impact on TJ protein levels
during the permeability assays, the duration of these assays (2 hours) is less than the time it takes for Aβ1-40 to affect TJ protein levels (~ 3-6 hours, as assessed in Fig. 3.9 in Chapter 3).

The results presented here suggest that down-regulation of claudin-5 and occludin may allow enhanced paracellular flux of soluble Aβ1-40 monomers across the BBB. However TEER measurements are much higher across the in vivo BBB (~1800 Ω.cm²) compared to bEnd.3 cells as in vitro brain endothelial cell culture does not accurately mimic the complex neurovascular interactions between endothelial cells, pericytes, astrocytes, microglia and neurons (Abbott et al., 2010). Therefore, as outlined in the next chapter, it was necessary to assess the BBB permeability phenotype in vivo in the absence of claudin-5 and occludin. To this end, activation of the RNAi pathway by systemic siRNA administration and the use of defined molecular weight tracer molecules provide tools in which to assess TJ integrity and BBB permeability in vivo.
Figure 4.1: Administration of Cy3-conjugated non-targeting (NT) siRNA complexed with LF2000 efficiently transfected bEnd.3 cells with maximal transfection observed 6-24 hours post-siRNA treatment (DAPI: blue, Cy3-NT siRNA: red). Scale bar = 10 μm.
Figure 4.2: Down-regulation of claudin-5 and occludin transcripts in bEnd.3 cells following transfection of siRNAs targeting claudin-5 and occludin. RT-PCR analyses of claudin-5 (top – after claudin-5 siRNA treatment), occludin (middle – after occludin siRNA treatment), and claudin-5 and occludin (bottom – after claudin-5 and occludin siRNA co-treatment) mRNA levels at 24, 48, 72, 96 hours and 1 week post-siRNA transfection (blue bars = NT siRNA, green = occludin siRNA, red = claudin-5 siRNA). Claudin-5 and occludin transcript levels were significantly decreased following treatment with claudin-5 and/or occludin siRNAs. (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons; n = 3 separate cell transfections. Data are means ± SEM).
Figure 4.3: Down-regulation of claudin-5 and occludin proteins in bEnd.3 cells following transfection of siRNAs targeting claudin-5 and occludin. Western blot analyses of claudin-5 (top - after claudin-5 siRNA treatment), occludin (middle - after occludin siRNA treatment), and claudin-5 and occludin (bottom - after claudin-5 and occludin siRNA co-treatment) protein levels at 24, 48, 72, 96 hours and 1 week post-siRNA transfection (NT - non-targeting siRNA, CLDN5 - claudin-5 siRNA, Occ - occludin siRNA, C/O - claudin-5 and occludin siRNAs). Claudin-5 and occludin protein levels were significantly decreased for up to a week following treatment with claudin-5 and/or occludin siRNAs.
Figure 4.4: Down-regulation of claudin-5 and occludin reduces transendothelial electrical resistance (TEER) without affecting bEnd.3 cell viability. Top - TEER measurements across bEnd.3 cell monolayers were reduced following treatment with claudin-5 siRNA only, occludin siRNA only or co-treatment with both claudin-5 and occludin siRNAs (n = 3 cell transfections per treatment. Data are means ± SEM). Bottom - MTS-based cell viability assay showed no effects of siRNA treatment on bEnd.3 cell survival. Increasing cell amounts (1 x 10^5 to 1.6 x 10^5) were used as assay controls. (n = 3 cell transfections per treatment. Data are means ± SEM).
Figure 4.5: Schematic diagram of transwell permeability assay design using FITC-labeled Aβ1-40 (yellow dots). bEnd.3 cells were grown on fibronectin-coated transwell inserts and following siRNA transfection, FITC-Aβ1-40 was added to either the apical (top) or basolateral (bottom) and its movement tracked across bEnd.3 cell monolayers over time by means of spectrofluorometry.
**Figure 4.6:** Paracellular movement of FITC-AB1-40 across bEnd.3 monolayers. *Top* - Application of FITC-AB1-40 to the apical chamber of the transwell. Transfection of bEnd.3 cells with TJ-directed siRNAs increases paracellular FITC-AB1-40 movement across the cell monolayer to the basolateral chamber compared to NT siRNA-transfected cells and untransfected cells. The apparent permeability coefficient ($P_{app}$ - right) was highest for bEnd.3 cells treated with claudin-5 and occludin siRNAs. *Bottom* - Application of FITC-AB1-40 to the basolateral chamber of the transwell. In the reverse assay, bEnd.3 cell transfection with TJ-directed siRNAs also increased paracellular FITC-AB1-40 movement across the cell monolayer to the apical chamber. The apparent permeability coefficient ($P_{app}$ - right) was again highest for bEnd.3 cells treated with claudin-5 and occludin siRNAs. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons, $n = 3$ separate cell transfections. Data are means ± SEM).
Figure 4.7: Paracellular movement of Aβ1-40 (F19P) monomer and Aβ1-40 (diY) dimer across bEnd.3 monolayers. Top - Aβ1-40 (F19P) monomer was applied to the apical aspect of transwell chambers and its movement across bEnd.3 monolayers over the course of 90 mins measured by ELISA. Transfection of bEnd.3 cells with TJ-directed siRNAs increases paracellular Aβ1-40 (F19P) monomer movement across the cell monolayer to the basolateral chamber compared to NT siRNA-transfected cells and untransfected cells. The apparent permeability coefficient ($P_{app}$ - right) was highest for bEnd.3 cells treated with claudin-5 siRNA alone or both claudin-5 and occludin siRNAs. Bottom - Aβ1-40 (diY) dimer was applied to the apical aspect of transwell chambers and its movement across bEnd.3 monolayers over the course of 90 mins measured by ELISA. No significant differences in the rate of Aβ1-40 (diY) dimer movement across bEnd.3 monolayers were observed between NT siRNA- or TJ-directed siRNA-treated cells. ($^*P < 0.05$, $^{**}P < 0.01$, one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons, $n = 3$ separate cell transfections. Data are means ± SEM).
Figure 4.8: Comparison of apparent permeability coefficients ($P_{\text{app}}$) for synthetic $\text{A}\beta 1-40$ (F19P) monomer and $\text{A}\beta 1-40$ (diY) dimer following apical application and movement across bEnd.3 monolayers. bEnd.3 cells treated with TJ-directed siRNAs show increased $P_{\text{app}}$ values for $\text{A}\beta 1-40$ monomer but not dimer compared to NT siRNA-treated or untreated cells. This suggests that down-regulation of claudin-5, occludin or both proteins confers a size-selective TJ to molecules ~ 4.4 kDa but not ~ 8.8 kDa in size. (Data are means ± SEM).
Chapter 5:

Assessment of blood-brain barrier (BBB) permeability in wild-type mice *in vivo* following RNAi-mediated down-regulation of claudin-5 and occludin.
5.1. Abstract

Previous findings had shown that soluble recombinant Aβ1-40 monomers and dimers could regulate the levels of the transmembrane TJ proteins, claudin-5 and occludin, in an in vitro system. Furthermore, down-regulation of claudin-5 and occludin using siRNAs reduced TJ integrity as measured by TEER and could size-selectively increase the paracellular permeability of brain endothelial cells to Aβ1-40 monomers (~ 4.4 kDa in size) in vitro. In this section, the role of these two TJ proteins in regulating paracellular permeability at the BBB was assessed in vivo in wild-type (WT) mice. Firstly, systemic administration via the tail vein of siRNAs targeting claudin-5 and occludin (injections separated by 24 hours) was employed to mediate co-suppression of claudin-5 and occludin in the brain vasculature. This was confirmed by RT-PCR and western blot analyses on isolated brain capillary fractions and immunohistochemistry of mouse brain cryosections. Having established a means of controlled and timed co-suppression of claudin-5 and occludin, the extent of BBB permeability following loss of claudin-5 and occludin was examined using a gadolinium contrast agent (Gd-DTPA - 742 Da) detected via magnetic resonance imaging (MRI) and trans-cardial perfusions of two different molecular weight biotin-dextran molecules: 3 kDa and 10 kDa. These BBB permeability assays revealed increased brain entry of Gd-DTPA and 3 kDa biotin-dextran, but not 10 kDa biotin-dextran, when claudin-5 and occludin were down-regulated concomitantly at the BBB. Furthermore, down-regulation of claudin-5 and occludin at the murine BBB allowed for increased diffusion of human Aβ (following intravenous delivery of soluble Aβ-containing hippocampal extracts from an AD patient) from the blood to the brain parenchyma. Similar to the findings from bEnd.3 cells in vitro, these results suggest the presence of a size-selective TJ in the absence of claudin-5 and occludin at the BBB in vivo and which may play a role in enhancing Aβ transport across the BBB.

5.2. Introduction

In vitro models of the BBB based on culture of primary or immortalized brain endothelial cells have been used to study various aspects of BBB development, regulation and drug permeability (reviewed by Wilhelm and Krizbai, 2014). While progress is being made in establishing stem cell-based BBB models using induced pluripotent stem cell (iPSC)-derived brain endothelial cells and co-culture systems involving astrocytes and/or pericytes (Lippmann et al., 2012, Lippmann et al., 2014), it is extremely difficult to model all aspects of the NVU in vitro. This includes interactions between multiple cell
types of the NVU as well as with ECM protein-containing basement membranes and features of the systemic circulation such as pulsatile flow. As a result, in vitro BBB models to date have been able to re-capitulate some but not all of the most important BBB features such as high TEER, strong adherens and tight junction protein expression and polarized expression of various transporters (Wilhelm and Krizbai, 2014). On the other hand, while assays of BBB permeability in vivo using rodent models can be expensive and time-consuming, they remain the most reliable method of assessing BBB permeability to prospective drugs and under pathological conditions (Mensch et al., 2009). Accurate measurements of in vivo BBB permeability include (i) magnetic resonance imaging (MRI) of BBB integrity in live animals via intravenous bolus of Gd-DTPA (gadolinium diethylene-triamine penta-acetic acid, 742 Da in size) contrast agent (Larsson et al., 1990, Harris et al., 2002), (ii) trans-cardial perfusions in anaesthetized mice or intravenous administration in live animals of inert tracers (eg. Evans blue dye) or defined molecular weight tracers (eg. fluorescent or biotin dextran conjugates) for analysis of BBB penetration by microscopy post-sacrifice (Moos and Møllgård, 1993, Boje, 2001), and (iii) the use of trans-cranial windows for real-time imaging of surface cortical vessels and parenchymal tissue following intravenous injection of fluorescent tracers (Yang et al., 2010).

As previously discussed, claudin-5 and occludin are major TJ components of both the rodent and human BBB and are highly enriched in the brain vascular endothelium compared to peripheral organ vasculature (Daneman et al., 2010a). RNAi has previously been used in the host laboratory to down-regulate claudin-5 at the BBB and iBRB in vivo with siRNA-mediated suppression of claudin-5 size-selectively and reversibly modulating these barriers to molecules < 1 kDa in size which would otherwise be unable to traverse the BBB/iBRB (Campbell et al., 2008, Campbell et al., 2009). This finding came from a combination of BBB/iBRB permeability assays, such as MRI using Gd-DTPA and trans-cardial perfusion of EZ-Link Sulfo-NHS-Biotin (443 Da), and measures of visual and cognitive function in animal models following intravenous delivery of low molecular weight drugs (Campbell et al., 2008, Campbell et al., 2009, Tam et al., 2010). As shown in Chapters 3 and 4 here, down-regulation of occludin is associated with decreased TEER across brain endothelial monolayers isolated from APP-Swe mice and with increased movement of Aβ across bEnd.3 monolayers in vitro. Previous studies have also shown increased BBB permeability following decreased occludin expression (Wosik et al., 2007,
Argaw et al., 2009, Carrano et al., 2011). Following the observation in Chapter 4 that loss of claudin-5 and occludin in bEnd.3 cells causes a size-selective increase in brain endothelial permeability to Aβ1-40 monomers and dimers in vitro, systemic administration of siRNAs targeting claudin-5 and occludin were used to examine BBB permeability in vivo when claudin-5 and occludin are simultaneously decreased in the brain vasculature.

5.3. Methods

5.3.1. Magnetic resonance imaging (MRI)

Following administration of targeting or non-targeting siRNAs, BBB integrity was assessed via MRI using a dedicated small rodent Bruker BioSpec 70/30 with an actively shielded USR magnet. Mice were anaesthetized with 5% isoflurane in oxygen and placed on an MRI-compatible support cradle with 0.5-1.5% isoflurane in oxygen for maintenance. This cradle has an in-built system for maintaining the animal’s body temperature at 37°C and a probe underneath the animal allows it to be physiologically monitored (electrocardiogram, respiration and temperature). To ensure accurate positioning of the animal, an initial rapid pilot image is recorded and is used to ensure correct geometry for all subsequent scans. The scale of BBB integrity was then visualized in high-resolution T1-weighted MR images (resolution, 0.156 X 0.156 X 5mm³; field of view: 20 X 20 X 17.9mm³; matrix: 128 X 128 X 30; TR/TE: 500/2.7ms; flip angle: 30°; number of averages: 3; acquisition time: 2 min, 24 sec; repetitions: 8) before and after tail vein administration of 100μl of a 1:3 dilution of Gd-DTPA (Gadolinium diethylenetriamine penta-acetic acid, 0.5mmol/ml stock solution, Bayer) which is monitored over a period of 20 mins post-injection. MRI analysis was performed using ImageJ and MIPAV software and all data was analyzed blind to treatment.

5.3.2. In vivo BBB permeability assays using tracer molecules

The extent of BBB permeability was assessed by terminal perfusion of tracer molecules including 3 kDa and 10 kDa biotin-dextran (lysine-fixable, Invitrogen). Following systemic siRNA administration and down-regulation of TJ proteins, a PBS solution containing 1mg/ml of the tracer molecule was slowly perfused into the left ventricle of anaesthetized mice for 3 min using a peristaltic pump. Following tracer molecule perfusion, tissues including brain, liver, lung and heart were dissected and placed in 4% paraformaldehyde (PFA, pH 7.4) overnight at 4°C. Tissues were then cryoprotected using
a 10%, 20% and 30% sucrose gradient and 12 µm frozen sections cut and incubated overnight with Cy3-conjugated streptavidin (1:100, Sigma Aldrich) at 4°C. The following day sections were washed 3 x 15 mins with PBS and counterstained with DAPI. Analysis of tissue cryosections was performed on the same day using constant exposure settings on a Zeiss Axioplan 2 fluorescent microscope and streptavidin-Cy3 fluorescence in tissue sections was quantified and compared using ImageJ software. Fluorescence values for tissue sections obtained from PBS-perfused animals without tracer were also measured as background controls. All perfusions and tissue analyses were performed blind to treatment.

5.3.3. Assessing murine BBB permeability to human brain-derived amyloid-β

Frozen post-mortem hippocampal tissue from a 90-year-old Alzheimer’s disease patient was obtained with ethical approval from the Dublin Brain Bank. Soluble fractions were processed from hippocampal tissue using previous protocols (Shankar et al., 2008). Briefly, tissue was homogenized in tris-buffered saline (TBS, 1 ml per 0.2 g tissue) using a Dounce homogenizer and centrifuged at 175,000 g in a TLA100.2 rotor on a Beckman TL 100 centrifuge for 30 min at 4°C. Supernatant was aliquoted, snap-frozen and stored at -80°C. Following down-regulation of claudin-5 and occludin by systemic administration of target siRNAs complexed to in vivo-jetPEI polymer, 100 µg/ml (final volume of 200 µl) of TBS extract was injected into the tail vein of siRNA-treated mice. 5 min post-injection, animals were sacrificed and brains fixed in 4% PFA for cryosectioning and Aβ immunostaining.

5.3.4. RT-PCR

Levels of RNA isolated from mouse brain capillary fractions were quantified in a StepOne Plus machine (Applied Biosystems) using Taqman Gene Expression Assays (Applied Biosystems) with the following IDs: claudin-5 (Mm00727012_s1), occludin (Mm00500912_m1). Target genes used a FAM reporter. Reaction conditions were as follows: 50°C×2 min, 95°C×20 s, [95°C×1 s, 60°C×20 s]×40. Transcript levels were standardized using β-actin with a VIC reporter in the same well and assessed using the ΔΔC_T method. Cell marker analysis of capillary fractions was carried out using SYBR Green RT-PCR. Sequences of primers used to amplify neuronal, astrocyte, microglial, pericyte, endothelial and myelin markers are provided in the appendix.
5.3.5. Western blot and immunohistochemistry analysis

Antibodies used for western blot analysis in this chapter were as follows: polyclonal rabbit anti-claudin-5, polyclonal rabbit anti-occludin (both 1:500, both Invitrogen) and polyclonal rabbit anti-β-actin (1:2000, Abcam). Mouse brain cryosections (12 μm thick) were permeabilized with 0.5% Triton-X, blocked with 5% NGS and incubated overnight with polyclonal rabbit anti-claudin-5, polyclonal rabbit anti-occludin (both 1:500, both Invitrogen), polyclonal rabbit anti-amyloid-β AW7 antibody (1:1000) or isolectin IB4-Alexa Fluor 488 (1:300, Invitrogen). Sections were then incubated with Cy2- or Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:500, Abcam) for 3 h at room temperature and counterstained with DAPI. Analysis of brain cryosections was performed using a Zeiss Axioplan 2 fluorescent microscope or Olympus FluoView FV1000 confocal microscope with integrated software.

5.4. Results

5.4.1. siRNA-mediated co-suppression of claudin-5 and occludin at the BBB in vivo

Having established that co-administration of siRNAs directed to claudin-5 and occludin mediates target gene silencing in bEnd.3 cells in vitro, we sought to suppress both claudin-5 and occludin simultaneously in vivo by tail vein administration of claudin-5 siRNA and occludin siRNA complexed separately to the cationic polymer in vivo-jetPEI in C57 WT mice. Following co-administration of claudin-5 and occludin siRNAs, brain capillary protein fractions were isolated 24, 48 and 72 hours post-injection using a dispase/dextran fractionation method for pulling down brain microvessels (Fig. 5.1). No significant down-regulation of claudin-5 was observed at 24, 48 or 72 hours post-injection compared to NT siRNA-treated animals (Fig. 5.2A). While occludin was also not significantly suppressed at 24 or 48 hours post-injection, 2 of 3 animals showed occludin suppression at 72 hours post-injection (Fig. 5.2A). This suggested that occludin and claudin-5 may have different protein half-lives since previous studies have shown that maximum claudin-5 protein suppression occurs 48 hours after injection (Campbell et al., 2008; Campbell et al., 2009). Thus the siRNA-in vivo-jetPEI injections were then separated by 24 hours, occludin siRNA first (time = 0) followed by claudin-5 siRNA (t = 24 hours), to create a time period of simultaneous claudin-5 and occludin down-regulation. Western blot analyses of brain capillary protein fractions isolated 72 hours after the first siRNA injection showed vast improvements in the efficacy of occludin siRNA and claudin-5 siRNA to down-regulate occludin and claudin-5, respectively (Fig.
5.2B). Decreased occludin and claudin-5 mRNA levels were also observed by RT-PCR in brain capillary RNA fractions isolated from mice treated with occludin siRNA (t = 0) and claudin-5 siRNA (t = 24 hours) (Fig. 5.3). Furthermore, immunostaining of occludin and claudin-5 in brain sections from siRNA-treated animals showed decreased vascular claudin-5 and occludin immunoreactivity in hippocampal and cortical microvessels of claudin-5 and occludin siRNA-treated mice (Fig. 5.4).

5.4.2. Assessment of the extent of BBB permeability following down-regulation of claudin-5 and occludin in vivo

In vivo suppression of claudin-5 at the BBB in mice was previously found to cause a size-selective increase in BBB permeability to molecules < 1 kDa in size (Campbell et al., 2008, Campbell et al., 2009). Various BBB permeability assays were conducted here to assess the extent of BBB permeability following co-suppression of claudin-5 and occludin. Firstly, magnetic resonance imaging (MRI) was used to assess the BBB phenotype in mice receiving staggered systemic injections of both occludin and claudin-5 siRNAs. Following tail vein injection of the contrasting agent Gd-DTPA, bolus chase analysis showed distinct differences in Gd-DTPA extravasation patterns in hippocampal regions between NT siRNA- and claudin-5 and occludin siRNA-treated mice (Fig. 5.5). In T-1 weighted contrast MR images, high concentrations of Gd-DTPA interfere with the signal intensity and present as dark contrasting. In claudin-5 and occludin siRNA-treated mice, T-1 longitudinal relaxation rates are initially decreased and then take a longer time to return to baseline levels compared to NT siRNA-treated mice indicating persistence of Gd-DTPA in neural tissue (Fig. 5.5). The return to baseline levels by repetition 8 in claudin-5 and occludin siRNA-treated mice suggests that the majority of the Gd-DTPA is cleared out of the brain soon after administration. Nevertheless, the pattern of increased Gd-DTPA extravasation evident in the first repetitions after the bolus in claudin-5 and occludin siRNA-treated mice confirmed BBB modulation to a molecule of 742 Da in these mice. However to assess the extent of BBB permeability in claudin-5 and occludin siRNA-treated mice, it was necessary to test BBB integrity to larger molecular weight compounds. In this regard, TJ siRNA-treated mice were anaesthetized and perfused transcardially with a 3 kDa or 10 kDa biotin dextran tracer molecule and tissue sections subsequently stained with streptavidin-Cy3 for examination by fluorescent microscopy. Claudin-5 and occludin siRNA-treated mice showed increased brain vascular permeability to 3-kDa biotin dextran compared to animals treated with claudin-5 siRNA.
only, occludin siRNA only or NT siRNA as observed by increased tracer staining in the brain parenchyma (Fig. 5.6). However, perfusion of 10 kDa biotin-dextran did not show any signs of extravasation with no significant differences between any of the treatment groups (Fig. 5.6). In addition, co-suppression of these two TJ components did not appear to have a deleterious effect on peripheral organ permeability post-perfusion with biotin dextrans (Appendix Fig. A7). Thus, simultaneous down-regulation of occludin and claudin-5 using siRNAs can modulate the BBB to compounds of at least 3 kDa but less than 10 kDa in size.

5.4.3. Movement of human Aβ from blood-to-brain following down-regulation of claudin-5 and occludin at the murine BBB

*In vitro* studies in Chapter 3 had shown that soluble Aβ1-40 monomer but not dimer could passively diffuse at increased rates across bEnd.3 cells in a manner dependent on occludin and claudin-5 levels at the TJ. Furthermore, since co-suppression of claudin-5 and occludin *in vivo* increases BBB permeability to molecules of ~3 kDa but not greater than 10 kDa in size, this suggested that soluble Aβ1-40 monomers (~4.4 kDa) could potentially diffuse across a claudin-5- and occludin-deficient BBB. To examine the ability of soluble Aβ to traverse the BBB *in vivo*, soluble Aβ-containing fractions were isolated from post-mortem hippocampal tissue of an end-stage AD patient (Fig. 5.7). Soluble Aβ-containing AD hippocampal extracts were then injected intravenously in WT mice following co-suppression of claudin-5 and occludin. Double labelling for Aβ and cerebral microvessels (IB4) showed the presence of human Aβ in the extraneuronal space of hippocampal regions in claudin-5 and occludin siRNA-treated mice but not in NT siRNA-treated animals (Fig. 5.8). Extravasation of soluble Aβ was also seen in cortical regions of claudin-5 and occludin siRNA-treated mice following intravenous administration of Aβ-containing AD hippocampal extracts (Appendix Fig. A8). This finding suggests that soluble Aβ, likely in monomeric form, can diffuse across the BBB along its own concentration gradient (from blood-to-brain) when claudin-5 and occludin are down-regulated in the brain vasculature.

5.5. Discussion

Similar to findings from the *in vitro* bEnd.3 permeability assays, down-regulation of claudin-5 and occludin in brain endothelial cells *in vivo* increases BBB permeability to Aβ. Together with results from the biotin dextran permeability assays, this indicates the
presence of a size-selective TJ at the BBB (to molecules of ~ 3-4 kDa but not greater than 10 kDa in size) following reductions in claudin-5 and occludin. This size-selective increase in paracellular BBB permeability also suggests that monomeric forms of Aβ (~ 4.4 kDa) are the primary Aβ species diffusing along the paracellular pathway. In contrast to in vitro results where suppression of claudin-5 alone or occludin alone could also enhance Aβ monomer diffusion, down-regulation of these components separately did not seem to enhance BBB diffusion of a 3 kDa biotin dextran molecule in vivo. This likely reflects the differences between the barrier ‘tightness’ of in vitro brain endothelial cell cultures (TEERs – 30-100 Ω.cm²) and the in vivo BBB (~ 1800 Ω.cm²) and is in line with previous studies showing that molecules greater than 1 kDa in size cannot cross the BBB after claudin-5 down-regulation (Nitta et al., 2003, Campbell et al., 2008, Campbell et al., 2009).

To achieve simultaneous down-regulation of claudin-5 and occludin at the BBB in vivo, systemic intravenous injections of occludin siRNA and claudin-5 siRNA had to be separated by 24 hours. While this may help prevent saturation of the RNAi pathway, it may also reflect different protein half-lives for these transmembrane TJ components in brain endothelial cells: approximately 30 hours for claudin-5 (Campbell et al., 2008, Campbell et al., 2009) and approximately 60 hours for occludin. The half-life of a TJ protein likely depends on various overlapping factors including the number of protein molecules needed to maintain barrier function and interact with other TJ components in a homeostatic environment and linked to that, the protein’s rates of synthesis and degradation (Steed et al., 2010). While relatively little is known with regards to TJ dynamics in brain endothelial cells, the potential for different half-lives among different components of TJ complexes should be considered in the study of TJ dysfunction and in the design of therapies to restore TJ integrity in neurological disease.

Intravenous administration of soluble Aβ-containing hippocampal extracts resulted in Aβ diffusion from blood-to-brain following claudin-5 and occludin suppression in WT mice. However in relation to AD, where Aβ accumulates in the brain parenchyma and the neural side of blood vessels in CAA, potential movement of Aβ from brain-to-blood would represent a more relevant measure of paracellular Aβ transport. As such, the results from WT mice presented here highlighted a potential role for occludin and claudin-5
siRNA-mediated BBB modulation in APP-Swe mice, a transgenic mouse model of AD in which Aβ builds-up over time in the brain parenchyma and around the blood vessels and leads to cognitive defects.
Figure 5.1: Relative purity of mouse brain capillary fractions isolated using a dispase/dextran method. Levels of neuronal (NeuN, synaptotagmin), astrocyte (GFAP, S100β), pericyte (PDGFRβ), endothelial (claudin-5, Tie2), myelin (MBP, MOBP) and microglial (CD68, PTPRC) markers were assessed in brain capillary mRNA samples by RT-PCR and values expressed relative to brain parenchyma mRNA levels. (n = 3 separate brain capillary fractions. Data are means ± SEM).
Figure 5.2: siRNA-mediated co-suppression of claudin-5 and occludin in WT mice. (a) Western blot analyses of isolated brain microvasculature protein fractions following intravenous co-administration of occludin and claudin-5 siRNAs (C/O). Apart from occludin down-regulation 72 hour post-injection, there was minimal suppression of claudin-5 or occludin at 24, 48 or 72 hours post-injection compared to NT siRNA-treated animals. (b) Staggered injections of occludin (t = 0) and claudin-5 siRNAs (t = 24h later) enhanced the efficacy of occludin and claudin-5 siRNAs in mediating target gene suppression.
Figure 5.3: Transcript levels of claudin-5 and occludin as measured by RT-PCR in brain capillary fractions isolated from animals treated with non-targeting (NT) siRNA, claudin-5 (CLDN5) siRNA, occludin (Occ) siRNA or claudin-5 and occludin (C/O) siRNAs. Systemic administration of claudin-5 siRNA or occludin siRNA specifically down-regulates their target gene while co-treatment with both claudin-5 and occludin siRNAs suppresses both claudin-5 and occludin. (n = 3-4 animals per treatment. Data are means ± SEM).
Figure 5.4: Immunostaining of claudin-5 and occludin in cortical and hippocampal microvessels of untreated, NT siRNA-treated and claudin-5 and occludin (C/O) siRNA-treated animals. Decreased claudin-5 and occludin vascular staining was observed in C/O siRNA-treated animals compared to untreated and NT siRNA-treated control animals. (Images representative of 4-5 animals per experimental group, scale bar = 40 µm).
Figure 5.5: MRI analysis of the BBB permeability phenotype in claudin-5 and occludin siRNA-treated WT mice. Top - The area of interest in hippocampal regions is highlighted in the T-1 weighted MR image by the red square. Bottom - Bolus chase analyses of Gd-DTPA (742 Da) extravasation following tail vein injection of the contrasting agent was performed for hippocampal regions. In T-1 weighted contrast MR images, high concentrations of Gd-DTPA interfere with the signal intensity and present as dark contrasting. For each hippocampal region analyzed, a slower clearance rate of Gd-DTPA was observed in claudin-5 and occludin siRNA-treated mice compared to NT siRNA-treated mice indicating persistence of Gd-DTPA in neural tissue and hence modulation of the BBB to a molecule less than 1 kDa in size. (**P ≤ 0.01, Student’s t-test, n = 3-4 animals. Data are means ± SD).
Figure 5.6: BBB permeability to 3 kDa and 10 kDa biotin-dextran in claudin-5 and occludin siRNA-treated WT mice. (A) Animals treated with NT siRNA, claudin-5 (CLDN5) siRNA, occludin (Occ) siRNA or claudin-5 and occludin (C/O) siRNAs were given a trans-cardial perfusion of 3 kDa or 10 kDa biotin-dextran and brain sections stained with streptavidin-Cy3. Extravasation of 3 kDa biotin-dextran from the blood into the brain parenchyma was observed in C/O siRNA-treated mice only. No apparent extravasation of 10 kDa biotin-dextran was observed in any of the treatment groups. (Scale bar = 100 μm). (B) Higher magnification of cerebral blood vessels in siRNA-treated mice showing 3 kDa biotin-dextran extravasation in C/O siRNA mice. (C) The intensity of biotin-dextran/streptavidin-Cy3 fluorescence in brain sections was measured.
using ImageJ and permeability relative to NT siRNA-treated control was graphed. (*P ≤ 0.05, one-way ANOVA with Bonferroni’s post-hoc test, n = 3-4 animals. Data are means ± SEM)

![Image](image.png)

**Figure 5.7:** SDS-PAGE/Coomassie Blue analysis of tris-buffered saline (TBS)-soluble fraction extracted from post-mortem hippocampal tissue of an Alzheimer disease (AD) patient. MW - molecular weight.
Figure 5.8: Systemic administration of amyloid-β (Aβ)-containing AD-TBS extracts and analysis of Aβ movement from the blood into the brain parenchyma in claudin-5 and occludin (C/O) siRNA-treated WT mice. **Top** - Immunostaining of Aβ and microvessels (via isolectin IB4-Alexa Fluor 488) in brain hippocampal regions of untreated, NT siRNA-treated or C/O siRNA-treated animals following tail vein injection of AD-TBS extracts. (Scale bar = 80 μm). **Bottom** - Quantification of Aβ extravasation from vessels into parenchyma of hippocampal regions. (**P ≤ 0.01, Student’s t-test, n = 4-5 animals per treatment. Data are means ± SEM).
Chapter 6:

Paracellular movement of amyloid-β (Aβ) across the blood-brain barrier (BBB) in amyloid precursor protein (APP-Swe) mice following RNAi-mediated down-regulation of claudin-5 and occludin.
6.1. Abstract

Previous chapters have described enhanced rates of Aβ flux across brain endothelial cell monolayers in vitro and a size-selective increase in BBB permeability in vitro and in WT mice following siRNA-mediated co-suppression of claudin-5 and occludin. Furthermore, brain entry of soluble Aβ following intravenous administration of human AD hippocampal extracts was more pronounced in claudin-5 and occludin siRNA-treated WT mice compared to NT siRNA controls. These findings are directly applicable to AD and CAA where failure to clear neurotoxic Aβ leads to its accumulation in the brain parenchyma and around cerebral blood vessels, triggering a host of downstream events such as neurovascular alterations, synaptic dysfunction and inflammatory cytokine release that ultimately result in neurodegeneration. In this chapter, the effects of simultaneous down-regulation of claudin-5 and occludin on Aβ movement across the BBB was explored in a murine model of AD, namely the APP-Swe mouse that over-expresses a double mutant form of human APP leading to increased brain Aβ levels and impaired cognitive function. Co-suppression of claudin-5 and occludin at the BBB in young (3-4 months) APP-Swe mice via intravenous siRNA administration corresponded with an increase in plasma levels of Aβ1-40. Dosing APP-Swe mice with claudin-5 and occludin siRNAs every 3 weeks for up to a year resulted in sustained and consistent increases in plasma Aβ1-40 levels and decreased brain/plasma ratios of Aβ1-40. Furthermore, claudin-5 and occludin siRNA-treated APP-Swe mice performed better on hippocampal-linked spatial memory tests (T maze trials) suggesting an improvement in cognitive function following Aβ movement out of the brain. Levels of the major transcellular Aβ transporters/receptors in the brain and liver were also unaffected by claudin-5 and occludin co-suppression, pointing towards the paracellular pathway of the BBB as the principal route of Aβ exit into the blood following claudin-5 and occludin down-regulation. Since previous findings demonstrated a size-selective BBB to molecules of 3-4 kDa and less than 10 kDa in size when claudin-5 and occludin are co-suppressed, this indicates that Aβ monomers (~ 4.4 kDa) are likely the principal Aβ species being drained paracellularly across the claudin-5 and occludin-deficient BBB. Targeting TJ components of the BBB paracellular pathway in early-stage AD in a controlled, reversible and size-selective manner may therefore offer a potential avenue to clear small Aβ species and prevent downstream Aβ aggregation, plaque formation and Aβ-mediated synaptic/neuronal damage.
6.2. Introduction

AD mouse models based on over-expression of mutant human transgenes associated with familial forms of AD re-capitulate important aspects of AD pathogenesis and provide a valuable tool to assess Aβ accumulation, degradation and clearance across the BBB (Ashe and Zahs, 2012, Schaeffer et al., 2011). Over the last 15 years, research on Aβ transport across the BBB has focused predominantly on transcellular receptors and transporters of Aβ in brain endothelial cells. As outlined previously, this has identified mechanisms of receptor-mediated transcytosis involving LRPI (for Aβ efflux from the brain) and RAGE (for Aβ influx into the brain) (Bell and Zlokovic, 2009). Results presented in the previous chapters showed that following down-regulation of transmembrane TJ components and subsequent loosening of the paracellular pathway, Aβ movement is increased across brain endothelial cell monolayers and across the BBB of WT mice from blood-to-brain along its own concentration gradient. To assess whether potential paracellular movement of Aβ represents a second pathway (in addition to transcellular LRPI) of Aβ clearance across the BBB, the well-characterized APP-Swe mouse model of AD was used in which Aβ levels rise in the brain parenchyma from 3 months of age (Hsiao et al., 1996, Kawarabayashi et al., 2001). Results presented in Chapter 3 found that aged APP-Swe animals (20 months of age) show CAA-associated reductions in levels of the transmembrane TJ proteins claudin-5 and occludin. While such TJ ‘dysfunction’ has been proposed to exacerbate synaptic and neuronal damage in AD due to the resulting increases in BBB permeability and potential influx of neurotoxic blood-borne agents, the effects of TJ protein down-regulation on Aβ levels has not been explored in young or old APP-Swe mice.

In addition to probing brain and plasma levels of Aβ, APP-Swe mice also enable the effects of altered Aβ metabolism/transport on neuroinflammation, synaptic function and cognition/memory to be tested. Production and release of pro-inflammatory cytokines (eg. IL-1β and TNF-α) in glial cells surrounding Aβ deposits has previously been described in APP-Swe mice (Benzing et al., 1999, Mehlhorn et al., 2000) and therapies aimed at relieving Aβ load in APP-Swe mice can also help alleviate neuroinflammatory responses (Deane et al., 2012). Beginning at approximately 5-6 months of age, progressive impairments in cognitive function as a result of Aβ-mediated synaptic dysfunction have been reported in APP-Swe mice (Spires-Jones and Knafo, 2012). This is based on behavioural tests of spatial memory (Morris water maze, T-/Y-maze).
associative learning (fear conditioning, passive avoidance) and recognition memory (novel object recognition tests) (Webster et al., 2014). One of these tasks, the T maze test of hippocampal-linked spatial memory, was used here to assess how changes in Aβ transport across the BBB impact cognitive function in siRNA-treated APP-Swe mice.

6.3. Methods

6.3.1. Plasma/brain tissue isolation from APP-Swe mice
Blood was collected from the tail veins of APP-Swe mice using a 30G needle and transferred to cold EDTA-coated tubes. Samples were centrifuged at 1,500 rpm for 10 min at 4°C and the plasma phase stored at -80°C. Mouse brain hippocampus was homogenized in ice-cold tris-buffered saline (TBS) and centrifuged at 15,000 rpm for 30 min at 4°C. Supernatants were collected for analysis of soluble amyloid-β 1-40 (Aβ1-40) levels and pellets were re-suspended in ice-cold guanidine buffer (5M guanidine hydrochloride/50mM TrisCl, pH 8.0) for analysis of insoluble Aβ 1-42 levels.

6.3.2. ELISA analysis of plasma and brain levels of Aβ1-40 and Aβ1-42
Plasma and brain Aβ1-40 levels were quantified using the Human Aβ40 ELISA kit (Invitrogen). Brain Aβ1-42 levels were quantified using the Human Aβ42 ELISA kit (Invitrogen). In these solid phase sandwich ELISA assays, each well of the 96-well plate is already coated with a monoclonal antibody specific to the N-terminus of human Aβ (common to both Aβ1-40 and Aβ1-42). 50μl of samples and standards were added to the wells and then incubated with rabbit antibody specific to the C-terminus of human Aβ1-40 or Aβ1-42. After washing, the bound rabbit antibody was then detected by adding an HRP-conjugated anti-rabbit secondary antibody and after additional washing, a substrate solution is added to produce a colored product. Absorbances (proportional to the intensity of the colored product) were read at 450nm on a spectrophotometer (Rosys 2010 Anthos) and Aβ concentrations determined from the Aβ peptide standard curves after correcting for background absorbance and dilution factors. ELISA preparation and analysis was performed blind to treatment.

6.3.3. Hippocampal-linked spontaneous alternation task (T-maze test)
T-maze trials were performed using a T-shaped apparatus made of wood and Perspex glass consisting of three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° with respect to the other two. The
protocol for spontaneous T-maze alternation as previously described by Deacon and Rawlins (2006) was followed. All trials were performed blind to treatment. Briefly, the animal being tested was first placed at the start position located at the end of the stem arm. The door of the start position is then released to allow the animal to choose one of the goal arms. Once inside one of the goal arms, the door of that arm is closed and the animal is confined there for 30 seconds. The animal is then placed back at the start position facing away from the goal arms for 15 seconds, the stem door re-opened and the animal given 2 mins to choose one of the goal arms again. If the second choice of arm is different from the first, the animal is scored as “alternating”. Mice were tested 3 times per day for a period of 7 days.

6.3.4. RT-PCR using Taqman assays
Levels of RNA isolated from cortical regions of siRNA-treated APP-Swe mice were quantified in a StepOne Plus machine (Applied Biosystems) using Taqman Gene Expression Assays (Applied Biosystems) with the following IDs: nerve growth factor (NGF: Mm00443039_m1), transforming growth factor beta-1 (TGFβ-1: Mm00441726_m1), glial-derived neurotrophic factor (GDNF: Mm00599849_m1), intercellular adhesion molecule-1 (ICAM-1: Mm00516023_m1), tumor necrosis factor alpha (TNFa: Mm99999068_m1), CCL-5 (Mm01302427_m1), interleukin-1 beta (IL-1β: Mm00434228_m1), brain-derived neurotrophic factor (BDNF: Mm04230607_s1), vascular cell adhesion molecule-1 (VCAM-1: Mm01320970_m1), C-X-C-motif chemokine 10 (CXCL10: Mm00445235_m1), C-C-motif chemokine 11 (CCL-11: Mm00441238_m1). Target genes used a FAM reporter. Reaction conditions were as follows: 50°Cx2 min, 95°Cx20 s, [95°Cx1 s, 60°Cx20 s]x40. Transcript levels were standardized using β-actin with a VIC reporter in the same well and assessed using the ΔΔCT method.

6.3.5. Post-mortem histopathological analysis
Post-mortem examinations were undertaken following euthanasia of siRNA-treated APP-Swe mice. To assess multiple organs for gross pathological changes, samples of the brain, liver, lung, heart, kidneys and spleen were fixed in formalin. Tissues were then paraffin-embedded, sectioned at 5 μm and stained by haematoxylin and eosin (H&E). Images were obtained using an Olympus BX51 Upright epi-fluorescence microscope.
6.3.6. Western blot analysis
Antibodies used in this chapter were as follows: polyclonal rabbit anti-claudin-5 (1:500), polyclonal rabbit anti-occludin (1:500, both Invitrogen), polyclonal rabbit anti-LRP1 (C-terminal, 1:500, Sigma Aldrich), polyclonal rabbit anti-ApoE (1:500), polyclonal rabbit anti-RAGE (1:500) and polyclonal rabbit anti-β-actin (1:2000, all Abcam). Polyclonal rabbit AW7 antibody raised against amyloid-β was kindly provided by the Walsh Laboratory, Brigham & Women’s Hospital and used to probe for the amyloid precursor protein (APP). Briefly, membranes were incubated with primary antibody overnight at 4°C, washed with TBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2000, Abcam) for 2 h at room temperature. To detect HRP, immunoblots were incubated with enhanced chemiluminescence (ECL) solution.

6.3.7. Immunohistochemistry of mouse brain cryosections
Mouse brain cryosections (12 μm thick) were permeabilized with 0.5% Triton-X, blocked with 5% NGS and incubated overnight with polyclonal rabbit anti-claudin-5 or polyclonal rabbit anti-occludin (both 1:100, both Invitrogen). Sections were then incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody (1:500, Abcam) for 3 hours at room temperature and counterstained with DAPI. Analysis of brain cryosections was performed using a Zeiss Axioplan 2 fluorescent microscope or Olympus FluoView FV1000 confocal microscope with integrated software. The number of claudin-5-positive and occludin-positive blood vessels in cortical and hippocampal regions was measured using ImageJ software.

6.4. Results
6.4.1. Levels of plasma Aβ1-40 in APP-Swe mice following siRNA-mediated co-suppression of claudin-5 and occludin at the BBB
As decreased claudin-5 and occludin levels had been observed in CAA-affected vessels of AD patients and an increase in Aβ flux measured when these TJ components were suppressed in vitro or in WT mice, this stimulated the role of claudin-5 and occludin at the BBB to be explored in a murine model of AD, namely the well-characterized APP-Swe mouse that over-produces Aβ with age in the brain which in turn creates an Aβ concentration gradient between the brain and the blood (Hsiao et al., 1996, Kawarabayashi et al., 2001). Having established the kinetics of simultaneous siRNA-
mediated claudin-5 and occludin suppression in WT mice and characterized the resulting BBB phenotype in these animals, the same siRNA administration protocol was applied to APP-Swe mice. Immunohistochemical analysis showed that intravenous injection of claudin-5 and occludin siRNAs (separated by 24 hours) could achieve co-suppression of claudin-5 and occludin in cerebral blood vessels of APP-Swe mice (Fig. 6.1). In human AD brains, Aβ1-40 is the most abundant Aβ species and is the more soluble Aβ isoform while Aβ1-42 is more fibrillogenic and is prominent in the plaques of the brain parenchyma (Biffi and Greenberg, 2011, Iadecola, 2004). Aβ1-40 is also the major Aβ species surrounding cerebral blood vessels in CAA where it triggers reductions in cerebral blood flow, impairs functional hyperaemia, causes vascular oxidative stress and other symptoms of cerebrovascular dysfunction that often occur before plaque formation (Niwa et al., 2000, Iadecola, 2004). Having shown that intravenous injection of claudin-5 and occludin siRNAs allowed for simultaneous TJ protein suppression, blood samples were extracted from siRNA-treated APP-Swe (4 months of age) mice 72-120 hours post-siRNA injection for measurement of plasma Aβ1-40 levels. Detection of human Aβ1-40 in APP-Swe mouse plasma was carried out by ELISA (Fig. 6.2). This analysis revealed significantly increased levels of plasma Aβ1-40 72 hours after injection in claudin-5 and occludin siRNA-treated animals compared to NT siRNA-treated animals, coinciding with the timepoint of maximal claudin-5 and occludin co-suppression (Fig. 6.3). No significant difference in plasma Aβ1-40 levels was observed between the two groups 120 hours after siRNA administration. Based on this finding, APP-Swe mice were chronically administered every three weeks (for a period of 9 months) with siRNAs targeting claudin-5 and occludin and blood samples collected 72-96 hours post-injection. Over this period, plasma Aβ1-40 concentrations were significantly and consistently higher in claudin-5 and occludin siRNA-treated animals compared to NT siRNA-treated animals suggesting that increased Aβ1-40 movement across the BBB into the blood occurs following claudin-5 and occludin co-suppression (Fig. 6.4).

6.4.2. Assessment of cognitive function and brain/plasma Aβ1-40 levels in APP-Swe mice following siRNA-mediated co-suppression of claudin-5 and occludin at the BBB

Subsequently, cognitive ability was assessed in siRNA-treated APP-Swe animals at 6 months of age by performing hippocampal-linked T-maze tests. Previous studies have shown that spatial memory is impaired in 3-6 month-old APP-Swe mice as assessed by Y-
maze and Morris water maze tests (King et al., 1999, Westerman et al., 2002, Perez-Cruz et al., 2011). The spontaneous alternation T-maze task is based on the tendency of a cognitively healthy animal to alternate between one of two goal arms when the trials are conducted in quick succession and is an established behavioural test of spatial learning and memory (Fig. 6.5; Deacon and Rawlins, 2006). Cognitively healthy adult WT mice have percentage alternation rates of approximately 80% (Deacon and Rawlins, 2006).

After conducting 18-21 T-maze trials per animal over 7 days, APP-Swe mice receiving the combined claudin-5 and occludin siRNAs displayed significantly increased alternating behavior (mean: 68.51%) compared to NT siRNA-treated animals (mean: 62.54%) (Fig. 6.6). At the end of this experiment when animals were 12 months old and following perfusion of mice to clear systemic Aβ levels of Aβ1-40 in the brains of siRNA-treated mice were analyzed by ELISA and compared to plasma Aβ1-40 levels for each animal. A significant decrease in the brain/plasma ratio of Aβ1-40 was observed in mice receiving the combined claudin-5 and occludin siRNA injections compared to NT siRNA controls (Fig. 6.7). Decreased levels of the more insoluble Aβ isoform, Aβ1-42, were also recorded in claudin-5 and occludin siRNA-treated APP-Swe mice though this finding was not statistically significant (Appendix Fig. A9).

While it would have been interesting to test the effect of increased Aβ movement across the BBB on amyloid plaque number/density, there were not enough Congo-positive plaques visible in the brains of these 12 month old APP-Swe mice for robust statistical comparison between treatment groups. This finding is in line with a previous characterization of APP-Swe mouse brains that reported numerous plaques at 15-23 months of age but minimal plaque number at 10-12 months (Kawarabayashi et al., 2001).

To assess decreased brain Aβ1-40 and increased plasma Aβ1-40 levels on the neuroinflammatory profile of claudin-5 and occludin siRNA-treated APP-Swe mice, the transcript levels of various cytokines and growth factors was assessed by Taqman RT-PCR. This analysis revealed decreases levels of NGF, TGFβ-1, GDNF and ICAM-1 in APP-Swe brains compared to a WT control but no significant differences in transcript levels of these targets between NT siRNA- or claudin-5 and occludin siRNA-treated APP-Swe mice (Fig. 6.8 – top panel). On the other hand, transcript levels of TNFα, CCL-5, IL-1β, BDNF, VCAM-1, CXCL-10 and CCL-11 were all increased in APP-Swe mice compared to a WT control (Fig. 6.8 – bottom panel). Apart from a significant decrease in levels of the pro-inflammatory cytokine TNFα in claudin-5 and occludin siRNA-treated
mice compared to NT siRNA-treated mice, no marked differences in target levels were observed between the APP-Swe treatment groups.

6.4.3. Status of Aβ transporters/receptors and peripheral organ pathology in siRNA-treated APP-Swe mice

Transcellular Aβ transport across the BBB via the LRP1 (Aβ efflux from the brain) and RAGE (Aβ influx into the brain) endothelial receptors is a known route of Aβ movement between the neural and blood compartments. As a result, it was necessary to assess whether suppression of claudin-5 and occludin at the BBB indirectly affects LRP1 or RAGE levels and thus whether increased Aβ1-40 plasma levels in claudin-5 and occludin siRNA-treated APP-Swe mice could be due to indirect modulation of transcellular Aβ transport. No significant differences in the expression pattern of LRP1 or RAGE were observed between treatment groups in protein fractions isolated from the brain vasculature, brain parenchyma or the liver (the site of LRP1-mediated systemic clearance of Aβ) (Fig. 6.9). This suggests that the increased plasma levels and decreased brain levels of Aβ1-40 in claudin-5 and occludin siRNA-treated APP-Swe mice were as a direct result of paracellular Aβ movement following claudin-5 and occludin co-suppression and not the result of changes in transcellular Aβ receptors following siRNA treatment. Furthermore, levels of the major Aβ transporter/binding partner ApoE were unchanged between treatment groups suggesting that claudin-5 and occludin co-suppression does not indirectly affect Aβ metabolism by altering ApoE levels (Fig. 6.9). In addition, there were no marked differences in brain or liver APP levels between both treatment groups. Thus decreased brain Aβ1-40 levels observed in claudin-5 and occludin siRNA-treated APP-Swe mice are not due to upstream changes in APP transgene expression following siRNA treatment (Fig. 6.9). In order to assess whether changes in Aβ transport could be due to toxic effects of chronic siRNA injections on peripheral organs, liver, lung, heart, spleen and kidney tissues from siRNA-treated APP-Swe mice were examined using H&E staining by a qualified veterinary pathologist (Prof. Sean Callanan, School of Veterinary Medicine, UCD, Ireland). These chronic injections of siRNA in APP-Swe mice did not induce any gross histopathological changes in peripheral organs (Fig. 6.10).
6.4.4. Levels of plasma Aβ1-40 in aged APP-Swe mice following siRNA-mediated co-suppression of claudin-5 and occludin at the BBB

Results presented in Chapter 3 showed that claudin-5 and occludin are decreased in the brains of aged APP-Swe mice but only in CAA-affected cerebral blood vessels. Having observed enhanced clearance of Aβ1-40 following down-regulation of claudin-5 and occludin at the BBB in young APP-Swe mice, the effect of claudin-5 and occludin co-suppression was next examined throughout the neural vasculature of aged (18 months) APP-Swe mice. Acute administration of claudin-5 and occludin siRNAs resulted in increased concentrations of plasma Aβ1-40 in aged APP-Swe mice 72-96 hours post injection of siRNAs (Fig. 6.11). Interestingly, overall plasma levels of Aβ1-40 in aged APP-Swe animals (200-600 pg/ml) were considerably lower than levels in younger APP-Swe animals (300-1200 pg/ml) in this study, suggesting that increased brain Aβ deposition corresponds to reduced plasma Aβ levels, a finding previously reported in APP-Swe mice (Kawarabayashi et al., 2001).

6.5. Discussion

Recent evidence suggests that Aβ begins to accumulate in the brain approximately 15-25 years (in familial AD) and 10 years (in sporadic AD) before onset of disease symptoms (Bateman et al., 2012, Mawuenyega et al., 2010). Importantly, this data also strongly suggests that aberrant clearance of Aβ, for example across the BBB, is likely to play a significant role in late-onset AD (Mawuenyega et al., 2010). Findings presented in previous chapters showed increased flux of Aβ along a concentration gradient following down-regulation of the TJ components claudin-5 and occludin in brain endothelial cells in vitro and in WT mice. In this chapter plasma levels of Aβ1-40 were found to be increased following systemic administration of claudin-5 and occludin siRNAs in APP-Swe mice, a transgenic mouse model of AD in which Aβ progressively accumulates in the brain. Repeated administration of claudin-5 and occludin siRNAs every 3 weeks for a period of 9 months led to a sustained increase in Aβ1-40 plasma levels over time and corresponded with better cognitive function at 6 months of age compared to NT siRNA-treated animals as assessed via T-maze tests of spatial memory. Moreover, increased plasma Aβ1-40 levels following claudin-5 and occludin co-suppression coincided with decreased levels of Aβ1-40 in the brains of claudin-5 and occludin siRNA-treated APP-Swe mice as measured by Aβ1-40 brain/plasma ratios.
Since levels of the transcellular Aβ receptors LRPI and RAGE were unaffected following down-regulation of claudin-5 and occludin in APP-Swe animals, changes in Aβ1-40 flux between the brain and blood following administration of claudin-5 and occludin siRNAs most likely reflect paracellular movement of Aβ1-40 across the BBB. While brain Aβ1-42 levels were also lower in claudin-5 and occludin siRNA-treated APP-Swe mice, this result was not significant and may reflect the inability of this more insoluble Aβ isoform to passively diffuse across the BBB. Altered Aβ movement across the BBB also raises the issue of what conformational forms of Aβ can move paracellularly following TJ protein down-regulation. Due to the size-selective nature of BBB permeability following siRNA-mediated claudin-5 and occludin co-suppression (around 3-4 kDa and less than 10 kDa), monomers of Aβ1-40 (4.4 kDa) likely represent the primary Aβ species being drained paracellularly across the BBB. While dimers of Aβ1-40 (8.8 kDa) cannot be excluded, minimal rates of Aβ1-40 dimer flux were previously observed across brain endothelial cells \textit{in vitro} following TJ protein down-regulation (as presented in Chapter 4). To verify the precise Aβ species moving out of the brain in claudin-5 and occludin siRNA-treated animals, BBB permeability assays involving the modified Aβ monomers and dimers used in Chapters 3 and 4 could be conducted in the future, though the milligram amounts of Aβ peptide isolate needed would make such an experiment time-consuming and expensive. While western blot analysis of different Aβ species in APP-Swe plasma may also be an option, the very low levels of Aβ1-40 (pg/ml) in these plasma samples would make detection difficult.

Enhanced clearance of Aβ from the brain and improved cognitive function following siRNA-mediated claudin-5 and occludin co-suppression in young APP-Swe mice suggests that controlled modulation of TJ proteins at the BBB could represent a new approach in AD treatment. However since AD is an age-related condition and the majority of AD patients are diagnosed over 60-65 years of age, a final aspect of this study sought to examine Aβ1-40 movement in aged APP-Swe animals. Interestingly, increased levels of plasma Aβ1-40 were recorded in aged APP-Swe mice treated with claudin-5 and occludin siRNAs despite these animals already having reduced claudin-5 and occludin in CAA-affected blood vessels. This suggests that controlled and timed co-suppression of claudin-5 and occludin in non-CAA-affected vessels of aged APP-Swe mouse brains may also allow efflux of Aβ monomers. However, this creates a somewhat paradoxical observation: increased paracellular movement of Aβ1-40 following siRNA-mediated TJ...
protein suppression occurs in brains where a portion of blood vessels are already deficient in claudin-5 and occludin. As discussed in the next chapter, this raises questions about the interaction of Aβ with TJ components at the BBB and the nature of TJ ‘dysfunction’ in AD and CAA pathogenesis.
Figure 6.1: siRNA-mediated co-suppression of claudin-5 and occludin in APP-Swe mice (12 months of age). Top - Immunohistochemical analysis of claudin-5 and occludin levels post injection of siRNAs in APP-Swe mice. Decreased claudin-5 and occludin vascular staining (arrows) was observed in claudin-5 and occludin (C/O) siRNA-treated animals compared to NT siRNA-treated control animals. (Scale bar = 100 μm). Bottom - Quantification of claudin-5- and occludin-positive vessels in siRNA-treated APP-Swe mice. (*P ≤ 0.05, **P ≤ 0.01, Student’s t-test, n = 4-6 animals per experimental group. Data are means ± SEM).
Figure 6.2: Representative human Aβ1-40 ELISA standard curve using synthetic human Aβ1-40 and reagents supplied in the Invitrogen Human Aβ1-40 ELISA Kit. Optical density (OD) values at 450nm were obtained over a range of 0-500 pg/ml human Aβ1-40.
Figure 6.3: Plasma Aβ1-40 levels in APP-Swe mice following systemic treatment with a single dose of NT siRNA or claudin-5 and occludin siRNAs. ELISA analysis of Aβ1-40 levels showed that Aβ1-40 levels were higher in claudin-5 and occludin siRNA-treated APP-Swe mice compared to NT siRNA-treated animals 72-96 hours after treatment with no significant differences in levels between experimental groups 120 hours after administration. This correlation between TJ protein down-regulation and increased levels of plasma Aβ1-40 levels suggests a window of BBB opening which may allow for enhanced paracellular movement of Aβ1-40 from the brain to the blood in APP-Swe mice. (*P < 0.05, Student’s t-test, n = 6 animals per experimental group. Data are means ± SEM).
Figure 6.4: Plasma Aβ1-40 levels in APP-Swe mice following intravenous administration of NT siRNA or claudin-5 and occludin siRNAs. ELISA analysis of plasma Aβ1-40 levels was performed on blood samples extracted 72-96 hours post-siRNA administration. Aβ1-40 plasma levels were significantly and consistently increased in APP-Swe mice receiving systemic claudin-5 and occludin siRNAs approximately every 3 weeks for a period of 9 months. This suggests that periodic and transient down-regulation of claudin-5 and occludin allows for enhanced movement of Aβ1-40 out of the brain in APP-Swe mice. (*P < 0.05, **P < 0.01, ***P < 0.001. Student's t-test, n = 4-6 animals per experimental group. Data are means ± SEM).
Figure 6.5: Image of T-maze apparatus used for hippocampal-linked spatial memory tests in siRNA-treated APP-Swe mice. Using the protocol described by Deacons and Rawlins (2006), the animal is first placed in the ‘Start’ position at the bottom of the maze. The door of the ‘Start’ position is then released to allow the animal to choose one of the goal arms (‘A’ or ‘B’). Once inside one of the goal arms, the door of that arm is closed and the animal is confined there for 30 seconds. The animal is then placed back at the start position facing away from the goal arms for 15 seconds, the ‘Start’ door re-opened and the animal given 2 mins to choose one of the goal arms again. If the second choice of arm is different from the first, this is scored as a positive result for ‘alternation’. If the animal chooses the same arm as the first time, this is scored as a negative result.
Figure 6.6: T-maze assessments of hippocampal-linked spatial memory as measured by percentage alternation rates in NT siRNA- or claudin-5 and occludin (C/O) siRNA-treated APP-Swe mice at 6 months of age. C/O siRNA-treated APP-Swe mice (Average % alternation = 68.51 ± 1.823) performed significantly better in the spontaneous alternation T maze tests compared to NT siRNA-treated animals (62.54 ± 1.931). (*P ≤ 0.05. Student’s t-test, n = 8-9 animals per experimental group).
Figure 6.7: ELISA analysis of brain/plasma Aβ1-40 ratios (pg/g soluble brain Aβ1-40 per pg/ml plasma Aβ1-40) in NT siRNA- or claudin-5 and occludin (C/O) siRNA-treated APP-Swe mice following periodic siRNA injections every 3 weeks for a period of 9 months. Levels of soluble brain Aβ1-40 for each animal were compared to that animal’s plasma Aβ1-40 levels over the timecourse of siRNA administrations. Decreased brain/plasma Aβ1-40 ratios in C/O siRNA-treated APP-Swe mice indicates lower levels of soluble brain Aβ1-40 and increased plasma Aβ1-40 following claudin-5 and occludin down-regulation at the BBB. (*P < 0.05, Student’s t-test, n = 9 animals per group).
Figure 6.8: Transcriptional profile of cytokines and growth factors in brain cortex of siRNA-treated APP-Swe mice and an age-matched WT control. Top – RT-PCR analysis showed that transcript levels of NGF, TGFβ-1, GDNF and ICAM-1 were decreased in APP-Swe brains compared to a WT control. No significant differences in transcript levels of these targets were observed between NT siRNA- or claudin-5 and occludin siRNA-treated APP-Swe mice. Bottom – Transcript levels of TNFα, CCL-5, IL-1β, BDNF, VCAM-1, CXCL-10 and CCL-11 were all increased in APP-Swe mice compared to a WT control. Apart from a significant decrease in TNFα in claudin-5 and occludin siRNA-treated mice compared to NT siRNA-treated mice, no marked differences in target levels were observed between the APP-Swe treatment groups. (*P < 0.05, non-parametric Mann Whitney test, n = 4-6 animals in siRNA-treated animal groups).
**Figure 6.9:** Western blot analysis of brain vascular (A), total brain (B) and liver (C) protein levels of amyloid precursor protein (APP), low-density lipoprotein receptor-related protein 1 (LRP1), receptor for advanced glycation end products (RAGE) and apolipoprotein E (ApoE) in NT siRNA- and claudin-5 and occludin (C/O) siRNA-treated APP-Swe mice. Levels of the major Aβ transporters and receptors in the brain vasculature and brain parenchyma (LRP1, RAGE, ApoE) and liver (LRP1, ApoE) were unchanged between both treatment groups. This suggests that the increased plasma levels of Aβ1-40 in C/O siRNA-treated APP-Swe mice are not the result of changes in these transporters following siRNA treatment. Similarly, there were no marked differences in brain and liver APP levels between both treatment groups. Thus decreased brain Aβ1-40 levels observed in C/O siRNA-treated APP-Swe mice are not due to upstream changes in APP transgene expression following siRNA treatment.
Figure 6.10: Hematoxylin and eosin (H&E) staining of liver, lung, heart, spleen and kidney paraffin-embedded sections from NT siRNA- and claudin-5 and occludin (C/O) siRNA-treated APP-Swe mice at 12 months of age. No gross histopathological changes were observed in peripheral organs of siRNA-treated animals and no differences between treatment groups was found. A qualified veterinary pathologist who was blind to the treatment groups also assessed these organs. (n = 4-6 animals per experimental group, scale bar for all images = 200 μm).
Figure 6.11: Plasma Aβ1-40 levels in aged (18 months) APP-Swe 24 hours to 1 week post-injection of NT siRNA or claudin-5 and occludin (C/O) siRNAs. ELISA analysis of Aβ1-40 levels in plasma of aged APP-Swe mice showed that following treatment with C/O siRNAs, there is an increase in Aβ1-40 plasma levels 72-96 hours after siRNA administration compared to NT siRNA animals but not at 24, 48 or 168 hours. This window of increased Aβ1-40 plasma levels in C/O siRNA-treated APP-Swe mice corresponds to the timepoints at which claudin-5 and occludin are co-suppressed. (*P ≤ 0.05, Student’s t-test, n = 4 animals per experimental group. Data are means ± SEM).
Chapter 7:
General Discussion
General Discussion

As the lifespans of people in the developed world reach 80 years and over, the number of new dementia and AD cases is set to dramatically rise in the near future. With nearly 40 million people living with dementia worldwide and this number set to double by 2030, this group of noncommunicable chronic diseases affects not only patients but has an enormous impact on their family members (Saxena and Wortmann, 2012). The associated costs of treating, managing and supporting people with dementia and their families will also increase with costs in the US alone projected to be over $1 trillion by 2050 (Alzheimer’s Association, 2014). Indeed, the World Health Organization has labeled dementia as a ‘public health priority’ for the 21st century because of its health, social and economic impact (Saxena and Wortmann, 2012). However, AD is the only cause of death among the top 10 causes in the US for which there is currently no effective means of prevention, no cure and no means to halt or even slow disease symptoms (Alzheimer’s Association, 2011).

The role of Aβ in AD pathogenesis was first established following the discovery that AD could be inherited in an autosomal dominant fashion as a result of a mutation in the gene coding for APP (Golde et al., 1991). Since then, the involvement of Aβ in synaptic, neuronal and vascular dysfunction has dominated AD research and over time, focus has shifted from amyloid plaques to soluble Aβ assemblies (dimers, oligomers) as the predominant neurotoxic species in AD pathogenesis (Shankar et al., 2008, Karran et al., 2011, Huang and Mucke, 2012). However following the recent setbacks associated with clinical trials of Aβ-targeting monoclonal antibodies (Doody et al., 2014, Salloway et al., 2014), it has become clear that a greater understanding of the early disease process will be key to therapeutic strategies aimed at preventing or at least ameliorating AD. There is now a consensus that AD starts years before memory problems become apparent, and that treatment when patients already have dementia may be too late (Bateman et al., 2012, Fagan et al., 2014, Gandy, 2012). Alterations in Aβ transport across the BBB interrupt the homeostatic intake and clearance of Aβ and such impaired Aβ clearance across the BBB (in addition to failure of other clearance mechanisms such as Aβ-degrading enzymes and microglial uptake) is thought to contribute to AD pathogenesis in up to 95% of sporadic cases (Mawuenyega et al., 2010, Iadecola, 2004). While RAGE and LRP1 are well-established endothelial receptors for transcellular influx and efflux of Aβ at the BBB
respectively (Bell et al., 2009), little is known with regard to Aβ movement along paracellular pathways in cognitively normal, presymptomatic or AD-affected individuals. As TJ dysfunction had previously been associated with the molecular pathology of AD (Hartz et al., 2011, Carrano et al., 2011), the main objective of this doctoral work was to fully characterize TJ components at the BBB in AD and in response to Aβ, and investigate the consequences of such TJ alterations on BBB integrity in WT mice and in the APP-Swe transgenic mouse model of AD. Levels of the major TJ proteins claudin-5 and occludin, both of which are enriched in the brain microvascular endothelium, were reduced in CAA-affected blood vessels of AD patient brains compared to brain samples obtained from patients with other neurodegenerative conditions. This phenomenon was also recorded in aged APP-Swe mouse brains compared to age-matched WT controls. However, intact claudin-5 and occludin immunostaining was observed in surrounding vessels devoid of Aβ in brains of AD patients and APP-Swe mice. Together with the finding that recombinant soluble Aβ1-40 monomers and dimers specifically down-regulate claudin-5 and occludin while up-regulating the intracellular TJ protein ZO-1 in brain endothelial cells in vitro, this led to a hypothesis that Aβ itself could regulate TJ protein levels and possibly vice versa. To assess the effects of this TJ protein down-regulation on BBB permeability and Aβ movement, in vitro transwell permeability assays and RNAi were used to suppress claudin-5 and occludin and monitor Aβ flux across brain endothelial cells. These experiments revealed that claudin-5 and occludin down-regulation confer a size-selective TJ that facilitates the passive paracellular diffusion of soluble Aβ1-40 monomers (4.4 kDa) while simultaneously excluding Aβ1-40 dimers (8.8 kDa). While in vitro models of the BBB are important systems for elucidating mechanistic functions of blood/neural transport, it is difficult to re-capitulate many essential aspects of the BBB (eg. high TEER, presence of other NVU cell types) that exists in a living system (Abbott et al., 2010). In this regard, using in vivo RNAi, co-suppression of claudin-5 and occludin pheno-copied the in vitro analyses and established a size-selective TJ to molecules 3-4 kDa in size and less than 10 kDa. Furthermore, this size-selective TJ permitted the enhanced extravasation of human soluble Aβ, isolated directly from the hippocampus of an AD patient, from blood-to-brain in WT mice. This suggested that decreased levels of claudin-5 and occludin at the BBB could facilitate movement of Aβ along its own concentration gradient via the paracellular pathway of the BBB. In a final set of experiments, the APP-Swe transgenic mouse model of AD was used to probe soluble Aβ levels on either side of the BBB when claudin-5 and occludin levels
are low. Plasma Aβ1-40 levels were significantly increased, brain Aβ1-40 levels decreased and cognitive function enhanced in APP-Swe mice when both claudin-5 and occludin were suppressed by RNAi.

These combined findings suggest a potentially beneficial correlation between the observed down-regulation of claudin-5 and occludin in post-mortem AD brains and increased Aβ movement from the brain to the blood. However, while endothelial cells and other NVU components may respond to initial extracellular Aβ accumulation by enhancing clearance mechanisms, drastic cerebrovascular and TJ dysregulation in response to chronic Aβ exposure (as in the end-stages of AD) likely compromises the BBB. This may lead to uncontrolled BBB permeability and leakage of serum proteins and peripheral immune cells into the brain parenchyma, exacerbating neuroinflammation, neural damage and cognitive impairment in AD (Erickson and Banks, 2013). Thus, changes in TJ integrity may be beneficial in early stages of AD but harmful later in the disease course. Since relatively high concentrations (micromolar amounts) of Aβ were needed to affect TJ protein levels in brain endothelial cells in vitro, this suggests that TJ complexes may only be altered in Aβ-rich settings such as AD. However rather than being a constant impermeable seal, TJ complexes are increasingly recognized as dynamic and plastic structures that can respond quickly to changes in the local micro-environment such as cytokine signals (Weber, 2012, Lampron et al., 2013, Winger et al., 2014). Thus transient changes in TJ complexes may allow for macromolecule transport in a homeostatic environment. Interaction of Aβ with brain endothelial cells for example may enable Aβ to move across both the transcellular (through LRP1/RAGE) and paracellular pathways in healthy brains. This raises the intriguing possibility of potential cross-talk between transcellular and paracellular pathways of the BBB. Interestingly, vascular LRP1 levels are reduced early in AD pathogenesis (Bell et al., 2009, Deane et al., 2004) and as reported here, both LRP1 and claudin-5/occludin levels are decreased in CAA-affected vessels of APP-Swe mice. Investigating the status of TJ protein components following changes in LRP1 and RAGE levels in brain endothelial cells is a future area of interest.

As presented in the schematic below (Fig. 7.1), the experimental results in this body of work suggest a mechanism whereby Aβ-mediated decreases in claudin-5 and occludin allows for modulation of the paracellular cleft and size-selective paracellular movement of Aβ monomers along a concentration gradient. This could occur in a homeostatic neural environment and/or in the Aβ-rich environment of AD brains. However, possibly due to
failure of other Aβ clearance mechanisms, high molecular weight oligomers form after self-association of Aβ in AD brains. These Aβ oligomers are too large to traverse the BBB and accumulate in the brain parenchyma and around cerebral blood vessels (CAA) (Fig. 7.1). This model may also help explain why acute modulation of claudin-5 and occludin levels in aged APP-Swe mice using RNAi enhances movement of Aβ monomers out of the brain despite these animals already lacking claudin-5 and occludin in CAA-affected vessels.

RNAi-mediated TJ protein suppression has been used previously to deliver small molecule drugs across the BBB to neural tissue of the brain/retina (Campbell et al., 2008; 2009; 2010) and conversely, to enhance drainage of water from brain-to-blood to alleviate brain swelling around the site of a TBI (Campbell et al., 2012). While clearance of Aβ1-40 monomers would likely have downstream effects on Aβ oligomer and amyloid plaque levels, it would also be interesting to investigate whether BBB modulation could facilitate increased brain entry of Aβ-targeting small molecule drugs in AD mouse models. In light of Aβ movement across the BBB following claudin-5 and occludin co-suppression, RNAi-mediated TJ protein suppression could also be applied to other diseases where Aβ is involved in pathogenesis. Glaucoma is an age-related ocular condition in which raised intraocular pressure (IOP) leads to degeneration of retinal ganglion cells (RGC) and optic nerve damage and is the second leading cause of blindness worldwide (Quigley and Broman, 2006). While the factors linking raised IOP to RGC death are unknown, abnormal APP processing and increased Aβ deposition in the RGC layer have been reported in experimental glaucoma models (Guo et al., 2007, Goldblum et al., 2007). Furthermore, glaucoma patients have decreased vitreous levels of Aβ (indicating enhanced retinal Aβ deposition) and altered Aβ levels in the CSF (Yoneda et al., 2011, Nucci et al., 2005). Targeting claudin-5 and occludin levels in the vasculature of the inner blood-retina barrier may therefore offer a means of reducing Aβ load in glaucomatous retinas and possibly enhance RGC function and survival. Separately, modulating levels of other TJ components of the BBB could have implications for studying disease pathology and developing therapies. For example, the TJ protein tricellulin is thought to be involved in limiting macromolecule (4-10 kDa) transport where three endothelial cells meet at the BBB (Ikenouchi et al., 2005; Krug et al., 2009). Down-regulation of tricellulin via RNAi could therefore serve as a potential avenue for delivery of large compounds across the BBB or as a second route of paracellular Aβ movement.
Over a century after Alois Alzheimer first described a case of 'presenile dementia', no effective form of therapy yet exists for this increasingly prevalent neurodegenerative condition. Despite initial failures in targeting Aβ in AD clinical trials, recent advances in diagnosing AD earlier using Aβ biomarkers and Aβ imaging (Bateman et al., 2014, Fagan et al., 2014) as well as its central involvement in cerebrovascular, synaptic and neuronal dysfunction means Aβ will likely remain the prime target of future therapeutic strategies in AD. Realistically however, lessons from other complex diseases like cancers suggest that combination drug therapy will offer the greatest chance of improving both acute symptoms and chronic disease progression in AD. Targeting Aβ and tau while also designing novel therapeutics to modulate neuroinflammation, stimulate neurogenesis and repair and enhance memory will likely be necessary to halt or reverse symptoms of dementia. The results presented here have implications for both understanding the molecular pathophysiology of AD and the development of therapies to relieve Aβ load and improve neurovascular and neuronal function in AD/CAA. Firstly, since co-suppression of claudin-5 and occludin in brain endothelial cells in vitro and in vivo enhances paracellular movement of Aβ, this suggests that reduced TJ protein levels along Aβ-laden CAA vessels in AD brains may act as a cellular response to clear accumulating Aβ. Secondly, it is possible that soluble Aβ1-40 monomer may facilitate its own movement across the TJ of the BBB in AD and thereby help explain the ‘amyloid sink hypothesis’ in which intravenously-delivered antibodies targeting Aβ can draw the molecule across the BBB by sequestering plasma Aβ (Karran et al., 2011, Toyn and Ahlijanian, 2014). In conclusion, this work has examined various aspects of BBB biology and AD/CAA pathology and proposes that controlled and targeted modulation of TJ complexes at the BBB could represent a novel means of targeting Aβ in treating AD and CAA.
**Figure 7.1:** Schematic model of paracellular Aβ movement across brain endothelial cells of the BBB. (1) In a homeostatic (healthy brain) and/or Aβ-rich environment (Alzheimer’s brain), Aβ induces degradation of claudin-5 (CLDN5) and occludin (Occ) tight junction proteins (possibly via signalling through its transcellular receptors like LRP1). (2) Subsequent opening of the paracellular cleft allows for Aβ monomer clearance from brain-to-blood along its own concentration gradient. However during AD progression, failure of other clearance mechanisms leads to the accumulation and aggregation of Aβ and the resulting high molecular weight oligomers are too large to be cleared along the paracellular cleft.
Appendix

Supplementary Result Figures

Figure A1: Scoring system for TJ protein immunoreactivity in cerebral microvessels. (1) Punctate, discontinuous and faint claudin-5 (red) staining with < 25% vessel immunoreactivity. (2) Punctate and faint claudin-5 staining with 25 – 50% vessel immunoreactivity. (3) Stronger claudin-5 signal but with short areas of punctate or interrupted staining, 50 – 75% vessel immunoreactivity. (4) Clear and intense claudin-5 signal along the length of vessel, > 75% vessel immunoreactivity. Scale bar = 20 μm.
Figure A2: Characterization of claudin-5 and occludin TJ proteins in brain microvessels of other non-AD neurodegenerative conditions. Representative images are shown from paraffin-embedded brain sections from the following conditions: Lewy Body dementia (LBD), progressive supranuclear palsy (PSP), frontotemporal dementia (FTD) and multiple systems atrophy (MSA). No amyloid plaque pathology or Aβ accumulation was observed in the brain parenchyma of these non-AD neurodegenerative donor cases as assessed by Congo Red stain and anti-Aβ immunohistochemistry. However positive Congo red staining indicating the presence of protein aggregates was observed in most of these conditions: in cases of LBD and MSA, this positive staining likely represents Lewy bodies (intraneuronal cytoplasmic inclusions containing α-synuclein); in cases of PSP and FTD, the ‘teardrop’-shaped structures are a hallmark of intraneuronal neurofibrillary tangles composed of hyperphosphorylated tau protein. In the four non-AD neurodegenerative cases presented here, intact claudin-5 and occludin vessel immunoreactivity was observed throughout the brain regions analyzed. Quantification of the number of claudin-5- and occludin-positive vessels in these conditions is presented in Fig. 3.2. Scale bar = 50 μm.
Figure A3: Immunohistochemical analysis of LRP1 expression in brain microvessels of aged APP-Swe (20 months) mice. Top – Immunostaining of vascular LRP1 (red) revealed decreased LRP1 levels in CAA-affected vessels (thioflavin S – green) of aged APP-Swe animals compared to age-matched WT controls. Despite the higher levels of vascular LRP1 in old WT animals, discontinuous LRP1 vessel staining was still observed in vessels of old WT animals. Scale bar = 50 μm. Bottom – Quantification of the number of LRP1-positive vessels in brain cryosections from WT and APP animals. (*P ≤ 0.05, Student’s t-test; n = 3-5 animals per group. Data are means ± SD).
Figure A4: Treatment of bEnd.3 cells with recombinant unmodified Aβ1-40 monomer peptide (0.1-1 μM). Western blot analysis of TJ protein levels 12 hours after treatment with increasing concentrations of unmodified Aβ1-40 monomer (without F19P) showed a dose-dependent decrease in claudin-5 and occludin but not ZO-1. These TJ protein changes mirrored the pattern observed with the modified Aβ1-40 F19P monomer. (n = 3 separate cell transfections per concentration).
Figure A5: Half-maximal inhibitory concentration (IC₅₀) measurements of claudin-5 and occludin siRNAs. RT-PCR analysis of claudin-5 and occludin mRNA levels shows that the effect of claudin-5 or occludin siRNA on target suppression is dose-dependent with target suppression retained using nanomolar (nM) quantities of siRNA. Furthermore, IC₅₀ measurements of claudin-5 and occludin siRNA when the siRNAs are added simultaneously show that RNAi pathway engagement and target suppression is maintained during co-treatment, with IC₅₀ measurements similar to those for single siRNA treatment.
**Figure A6**: Paracellular movement of FITC-Aβ1-42 across bEnd.3 monolayers. *Left* - Application of FITC-Aβ1-42 to the apical chamber of the transwell. Transfection of bEnd.3 cells with TJ-directed siRNAs increases paracellular FITC-Aβ1-42 movement across the cell monolayer to the basolateral chamber compared to NT siRNA-transfected cells and untransfected cells. The apparent permeability coefficient ($P_{app}$ - *right*) was highest for bEnd.3 cells treated with occludin siRNA alone or both claudin-5 and occludin siRNAs.
Figure A7: Animals treated with non-targeting (NT) siRNA, claudin-5 (CLDN5) siRNA, occludin (Occ) siRNA or claudin-5 and occludin (C/O) siRNAs were given a transcardial perfusion of 3 kDa (a) or 10 kDa (b) biotin-dextran and liver, lung and heart sections stained with streptavidin-Cy3 (left panels; scale bars = 100 μm). Right panel indicates streptavidin-Cy3 fluorescence measurements relative to NT siRNA control. No major differences in peripheral organ permeability to 3 kDa or 10 kDa biotin-dextran were observed between treatment groups. (n = 3-4 animals per treatment. Data are means ± SEM).
Figure A8: Immunostaining of amyloid-β (Aβ) and microvessels (via isolectin IB4-Alexa Fluor 488) in brain cortical regions of untreated, non-targeting (NT) siRNA-treated or claudin-5 and occludin (C/O) siRNA-treated animals following tail vein injection of tris buffered saline-soluble (AD-TBS) extracts from hippocampal tissue of an Alzheimer disease patient. (Images representative of 4-5 animals per experimental group, scale bar = 50μm).
Figure A9: ELISA analysis of brain Aβ1-42 levels in NT siRNA- or claudin-5 and occludin siRNA-treated APP-Swe mice following periodic siRNA injections every 3 weeks for a period of 9 months. Levels of brain Aβ1-42 were lower in animals treated with claudin-5 and occludin siRNAs compared to NT siRNA-treated animals but this result was not statistically significant. (*P = 0.0717, Student’s t-test, n = 4-6 animals per group).
siRNA sequences

Control non-targeting siRNA No.1© was obtained from Ambion. All targeting siRNAs were obtained from Dharmacon. Upon arrival, lyophilized siRNA was resuspended in 1X siRNA buffer to a stock concentration of 1mg/ml (Thermo Scientific) based on the calculation that 1 nmol siRNA = approx. 13.4 μg siRNA. The siRNA was then aliquoted and stored at -20°C for future use.

Table A1: Sequences for siRNAs used during the study.

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<th>Name</th>
<th>Strand</th>
<th>Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>siNT</td>
<td>Sense</td>
<td>UUCUCCGAACGUGUCACGUGA</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ACGUGACACGUUCGAGAA</td>
</tr>
<tr>
<td>siCLDN5</td>
<td>Sense</td>
<td>CGUUGGAAAUUCUGGGUCUUU</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AGACCAGAAUUUUCCACAGU</td>
</tr>
<tr>
<td>siOcludin</td>
<td>Sense</td>
<td>GAUAUUACUUGAUCGUGAUU</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AUCACGAUCAAAGUUAAACUU</td>
</tr>
<tr>
<td>siLRP1</td>
<td>Sense</td>
<td>GCAAAACUGUGACCGAUGAU</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>UCAUCGGUCACGCUUUGCUU</td>
</tr>
</tbody>
</table>

RT-PCR primer sequences

All primers were designed using Primer3 and stock oligos obtained from Sigma. Primer efficiency in amplifying target sequences was verified using standard curve and melt curve analysis on the 7300 Real Time PCR System (Applied Biosystems).

Table A2: RT-PCR primer sequences for tight junction mRNAs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-5</td>
<td></td>
<td></td>
<td>TTTCTTCTATGCGCAGTTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GCAGTTTGTTGCTACTTCA</td>
</tr>
<tr>
<td>Ocludin</td>
<td></td>
<td></td>
<td>ACAGTCCAATGGCCTACTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ACTTCAGGCAACCAGGTTG</td>
</tr>
<tr>
<td>ZO-1</td>
<td></td>
<td></td>
<td>CCACCTCTGTCCAGCTCTTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CACCCTGATGCTTTTCT</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td>TCACCCACACTGTGCCCATCTACGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAGCGGAACCGCTCATTGCACATGG</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Sequence (5' – 3')</td>
<td>Reverse Sequence (5' – 3')</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>NeuN/Fox-3</td>
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<td>GACGTGGACTTGGACTTGGGT</td>
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</tr>
<tr>
<td>Synaptotagmin</td>
<td>CGATGCTGAATCTGGAAGCTGA</td>
<td>GGCAGCAAGAAGACTTGGAT</td>
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<tr>
<td>GFAP</td>
<td>AGCCCTGCCAGCCTCTCCCTTAG</td>
<td>AAGGTGTGGCTGAAATGCGGCG</td>
<td></td>
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<tr>
<td>S100β</td>
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<td>TGTCACCCCTCTCGCCCAGAG</td>
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<tr>
<td>PDGFRβ</td>
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<td>TAATCCCGTCAGCATCTTCC</td>
<td></td>
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<tr>
<td>α-smooth muscle(SM)-actin</td>
<td>TCCAATGTCGCCAGGATGTA</td>
<td>GAAGGAATAAGCCAGCTTCAG</td>
<td></td>
</tr>
<tr>
<td>Claudin-5 (CLDN5)</td>
<td>TTTCTTCTATGCGCAGTTGG</td>
<td>GCAGTTGTGGCTCAGCTCA</td>
<td></td>
</tr>
<tr>
<td>Tie2</td>
<td>TCAGGGCAAAAAATGAAGACC</td>
<td>TCTAGGCCCCTTGGAGCTGTA</td>
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</tr>
<tr>
<td>Myelin basic protein (MBP)</td>
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<td>CCTGTCACCCGCTAAAGAGC</td>
<td></td>
</tr>
<tr>
<td>Myelin-associated oligodendrocytic</td>
<td>CATTGGCTATCCATTCACCT</td>
<td>AGGATCGCCTCATTTTCCCT</td>
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</tr>
<tr>
<td>basic protein (MOBP)</td>
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<td></td>
<td></td>
</tr>
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<td>AGAGGCGTGGTGATGTA</td>
<td></td>
</tr>
<tr>
<td>Protein tyrosine phosphatase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>receptor type C (PTPRC)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequences of human and mouse Aβ1-40

Differences between the two sequences are highlighted in red:

**Human**  
DAEFRHDSGY_{10}EVHHQKLVF_{20}AEVGGSNKGA_{30}IIGLMVGVV_{40}

**Mouse**  
DAEFGHDSGF_{10}EVRHQKLVF_{20}AEVGGSNKGA_{30}IIGLMVGVV_{40}

Scrambled human Aβ1-40 (Anaspec):
AEGDSHVLKEGAYMEIFDVQGHFGKIFRQVVDLGSHNVA

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Buffer solutions and preparation

10X Running Buffer (pH 8.6)
- 30.3 g Tris
- 144.2 g Glycine
- 19 g SDS (Lauryl Sulphate)
- 500 ml dH₂O
- Adjust pH to 8.6 using HCl with the aid of a pH meter.
- Transfer solution to a 1L volumetric flask and bring to the mark with dH₂O.

1.5M Tris (pH 8.8)
- 18.165 g Tris
- 80 ml dH₂O
- Adjust pH to 8.8 using HCl with the aid of a pH meter.
- Transfer solution to a 100 ml volumetric flask and bring to the mark with dH₂O.
- Store at room temperature.

0.5M Tris (pH 6.8)
- 6 g Tris
- 80 ml dH₂O
- Adjust pH to 6.8 using HCl with the aid of a pH meter.
- Transfer solution to a 100 ml volumetric flask and bring to the mark with dH₂O.
- Store at room temperature.

10% SDS (Lauryl Sulphate) (pH 7.2)
- 10 g SDS
- 80 ml dH₂O
- Adjust pH to 7.2 using HCl with the aid of a pH meter.
- Transfer solution to a 100 ml volumetric flask and bring to the mark with dH₂O.

10% Ammonium Persulfate (APS)
Prepare fresh on the day of use and mix well.
- 10% w/v APS
- 90% v/v dH₂O
Transfer Buffer (pH 8.3)
- 3.03 g Tris
- 14.42 g Glycine
- 200 ml Methanol
- 600 ml dH₂O
- Adjust pH to 8.3 using HCl with the aid of a pH meter
- Transfer solution to a 1L volumetric flask and bring to the mark with dH₂O

Tris Buffered Saline (TBS) (pH 7.4)
- 6.05 g Tris
- 8.766 g NaCl
- 800 ml dH₂O
- Adjust pH to 7.4 using HCl with the aid of a pH meter

4% Paraformaldehyde (PFA) (pH 7.4)
- 8g PFA
- 20 ml 10X PBS
- 100 ml dH₂O
- Adjust pH to 7.4 using NaOH and bring to 200 ml using dH₂O

Protein Lysis Buffer (PLB) (pH 8.8)
- 1.51 g Tris
- 4 g SDS
- 0.31 g Dithiothreitol (DTT)
- 100 ml dH₂O
- Adjust pH to 8.8 and bring to 200 ml using dH₂O
- Aliquot and store at -20°C
- Add 1 protease inhibitor tablet (Roche) per 10ml of PLB before use

Working Buffer (for murine primary brain endothelial cell isolation)
- 100 ml Ca²⁺/Mg²⁺-free Hanks Balanced Salt Solution (HBSS)
- 1 ml TC-HEPES buffer (1M)
- 1 ml Pen/Strep (final concentration 100 i.u./ml 100 μg/ml)
- 2.75 ml 22% (w/v) BSA (final conc. 0.5%)

Complete Digest Medium (for murine primary brain endothelial cell isolation)
- Ca^{2+}/Mg^{2+}-free HBSS containing:
  - 1 mg/ml Collagenase/Dispase
  - 10 mM HEPES
  - 100 i.u. penicillin/100 μg/ml streptomycin
  - 20 units/ml DNAse I
  - 0.147 μg/ml TLCK

Growth Medium (for murine primary brain endothelial cell isolation)
- EGM2-MV (with all additives) from Lonza
- 3 μg/ml puromycin (for endothelial selection)
References


Alzheimer’s Society of Ireland (2014) Understanding Alzheimer’s Disease and Other Dementias, https://www.alzheimer.ie


variants at ABCA7, MS4A6A/MS4A4E, EPRA1, CD33 and CD2AP are associated with Alzheimer’s disease, *Nature Genetics* **43**: 429-435.


Keaney, J., Campbell, M., and Humphries, P. (2011) From RNAi technology to effective therapy: how far have we come and how far to go?, Therapeutic Delivery 2(11): 1373-1378.


