TRINITY COLLEGE DUBLIN
School of Genetics and Microbiology
Smurfit Institute of Genetics

Colony Stimulating Factor 1 Receptor and Blood-Brain Barrier Maintenance

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

Conor Delaney
2021

Under the supervision of Dr Matthew Campbell
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, except where noted below.

Dr Michael Farrell, Beaumont Hospital, performed the autopsy and chromogenic staining in Figure 3.1.

Dr Eoin O’ Keeffe, Campbell Lab, Trinity College Dublin, contributed to the analysis of BBB permeability in Figure 3.8.

Dr Kieva Brennan, Doyle Lab, Trinity College Dublin, contributed to the analysis of the FACS data in Figure 3.20 and Figure 3.21.

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Conor Delaney
Summary

The colony stimulating factor 1 receptor (CSF-1R) is a receptor tyrosine kinase involved in the differentiation, proliferation and function of myeloid lineage cells. Activated by its two ligands, interleukin-34 (IL-34) and colony stimulating factor 1 (CSF-1) this receptor is additionally essential for the viability of microglia, the macrophages of the central nervous system (CNS). Less is known about the role of CSF-1R in non-myeloid cells of the CNS, with reported functions in neuroprotection as well as in regulating endothelial cell tight junction expression under pro-inflammatory conditions. Dominant mutations in CSF1R have been found to be causative of a neurological disease called adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP). Individuals carrying a heterozygous loss of function mutation in CSF1R generally begin to display symptoms in their fifth decade. Early to mid-stages of the disease involve white matter degeneration, personality changes, motor dysfunction and dementia with the disease rapidly progressing to a lethal stage after a median of 6 years. There is currently no standard treatment for ALSP, and the molecular aetiology of the disease is unknown.

In this study, a novel involvement of the blood-brain barrier (BBB) was observed in post mortem cortical sections of individuals with genetically diagnosed ALSP. The BBB is a feature of the specialised CNS vasculature, induced through the coordinated actions of pericytes, astrocytes and endothelial cells. Its primary function is to limit the passage of molecules from the blood into the brain through the expression of endothelial tight junction (TJ) proteins which seal the paracellular cleft. Early work in this study identified increased BBB permeability in ALSP by examining post mortem cortical tissue, as well as through the use of in vivo magnetic resonance imaging (MRI) assessment of BBB integrity. Furthermore, an accumulation of amyloid beta (Aβ) protein was detected at the cerebrovasculature, a pathology known as cerebral amyloid angiopathy (CAA) found in over 80% of Alzheimer’s disease patients. These vascular phenotypes in ALSP were indicative of a role for CSF-1R in BBB function and maintenance. Molecular analyses of the variant CSF-1R isoforms expressed by these ALSP patients confirmed a loss of signalling function, indicating these phenotypes to be a result of CSF-1R haploinsufficiency. Indeed, pharmacological inhibition of CSF-1R in endothelial cells in vitro resulted in decreased expression of TJ components, with an accompanying increase in endothelial monolayer permeability. As CSF-1R is known to be critical for microglial function, the effects of paracrine microglial signalling on endothelial cells in the context of CSF-1R inhibition were next examined. Conditioned media from microglia was found to be capable of regulating endothelial TJ expression, in addition to
upregulating endothelial CSF-1R, in a manner that was dependent on microglial CSF-1R expression. Using Csf1r heterozygous primary microglial and endothelial cells, unique responses of endothelial TJ gene expression to microglia conditioned media were identified when both cell types were heterozygous for Csf1r. Decreased gene expression of Cld5, Ocln and Marveld2 was observed only in this context, with wild type endothelial cells unaffected, and wild type microglia unable to induce the same response. This aberrant outcome of microglial-endothelial crosstalk indicated CSF-1R to be a mediator of intercellular communication in both of these cell types within the brain. Administration of recombinant CSF-1 and IL-34 to mice with cell-specific Csf1r heterozygosity further expanded upon this function of CSF-1R. Heterozygous loss of endothelial Csf1r resulted in lower levels of Cld5 expression within the brain microvessels, in addition to dysregulated expression of genes involved in Aβ transport across the brain endothelium. Endothelial Csf1r heterozygosity also reduced the clearance of stereotaxically injected Aβ in the mouse hippocampus, resulting in an accelerated onset of behavioural deficits. In the context of macrophage Csf1r heterozygosity, a similar dysregulation of TJ and Aβ transport gene expression was observed, with additional changes in macrophage activity at the cerebrovasculature. While Aβ levels following stereotaxic injection were unaffected by loss of macrophage Csf1r, macrophage localisation to regions of Aβ deposition was severely attenuated. This combination of reduced vascular clearance and macrophage-mediated phagocytosis of Aβ would suggest a disruption of similar mechanisms in the ALSP brain. Moreover, an examination of ALSP donor peripheral mononuclear cell populations revealed an increase in undifferentiated cells. Macrophages produced from ALSP donor monocytes also displayed a reduced phagocytic capacity, in addition to being dysmorphic. This reduced rate of phagocytosis was also seen in macrophages derived from Csf1r heterozygous mouse bone marrow.

The results presented here indicate that loss of CSF-1R results in dysfunctional endothelial cells in addition to macrophages. The endothelium is the first point of contact for peripheral macrophages localising to the brain, and as such the BBB can serve as a signalling hub for leukocyte transmigration. Poor macrophage recruitment to Aβ-laden cerebral vessels, in combination with dysfunctional endothelial cross-talk with parenchymal microglia, might be drivers of ALSP BBB pathology. Recently, hematopoietic stem cell transplantation has proven beneficial at stalling ALSP progression and reversing some of the pathologies, supporting this role for the peripheral innate immune system in ALSP. The introduction of macrophages with homozygous CSF1R into the ALSP system may disrupt this aberrant cross-talk, and restore macrophage-driven clearance of Aβ.
Acknowledgements

I have been fortunate to receive help and support over the course of my PhD, helping me work through a project which had appeared deceivingly straightforward at first. Firstly, I would like to express my gratitude to my supervisor Dr Matthew Campbell for providing me with the opportunity to work on such a novel project in his lab. His guidance over the last 4 years has been invaluable, providing advice for approaching the many different aspects of this project as well as for making progress in academia. My time in the Campbell Lab has granted me experiences and opportunities which I believe are not always part of the standard PhD, and Matt’s enthusiasm for science has inspired me to pursue a further career in research. Thank you also to Professor Michael Farrell in Beaumont Hospital for kick-starting my project and coordinating the early work with the Dublin Brain Bank. Professor Savvas Savvides, thank you for hosting me at your lab at the VIB in Ghent, and for your collaboration on the molecular work on CSF-1R. To the patients, their family members and the clinicians involved; without your engagement and eager contributions, this project would not have been possible.

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<th>Description</th>
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<tbody>
<tr>
<td>3-MA</td>
<td>3-Methy-Adenosine</td>
</tr>
<tr>
<td>AARS2</td>
<td>Alanyl-tRNA Synthetase 2</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>ADES</td>
<td>Amyloid Degrading Enzymes</td>
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<td>ADGRE1</td>
<td>Adhesion G Protein-Coupled Receptor E1</td>
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<td>AJ</td>
<td>Adherens Junction</td>
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<td>Apolipoprotein E</td>
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<td>Apolipoprotein J</td>
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<td>Amyloid-Beta</td>
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<td>BBB</td>
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<tr>
<td>BCA</td>
<td>Bicinchonic Acid</td>
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<td>BCSFB</td>
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<td>BMM</td>
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<td>CAA</td>
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<td>C-CBL</td>
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<td>CCL2</td>
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<td>CCL3</td>
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<td>cDNA</td>
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<td>CMP</td>
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<td>CNS</td>
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</tr>
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<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<td>CSF-1</td>
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<td>CSF-1R</td>
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<td>DAMPS</td>
<td>Damage-Associated Molecular Patterns</td>
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<td>DAPI</td>
<td>4′,6-diamidino-2-Phenylindole</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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</table>
DMSO  Dimethyl Sulfoxide
DNA  Deoxyribonucleic Acid
dNTP  Deoxynucleoside Triphosphates
DPBS  Dulbecco’s Phosphate Buffered Saline
DWI  Diffusion-Weighted Imaging
EAAT1  Excitatory Amino Acid Transporter 1
EAAT2  Excitatory Amino Acid Transporter 2
EAE  Experimental Autoimmune Encephalomyelitis
EC  Endothelial Cell
ECL  Enhanced Chemiluminescence
ECL1  Extracellular Loop 1
ECL2  Extracellular Loop 2
EDTA  Ethylenediaminetetraacetic Acid
EGM2  Endothelial Growth Media 2
EGM2-MV2  Endothelial Growth Media 2 Microvessel 2 Supplement
FACS  Fluorescence-Activated Cell Sorting
FBS  Fetal Bovine Serum
FDC  Follicular Dendritic Cells
FIRE  fms-Intronic Regulatory Element
FITC  Fluorescein Isothiocyanate
FLAIR  Fluid-Attenuated Inversion Recovery
GAPDH  Glyceraldehyde 3-Phosphate Dehydrogenase
GDNF  Glial Derived Neurotrophic Factor
GFAP  Glial Fibrillary Acidic Protein
GFP  Green Fluorescent Protein
GLUT1  Glucose Transporter 1
GM-CSF  Granulocyte-Macrophage Colony-Stimulating Factor
GRB2  Growth Factor Receptor-Bound Protein 2
GRP78  78 kDa Glucose-Regulated Protein
HBSS  Hanks Balanced Salt Solution
HDLS  Hereditary Diffuse Leukoencephalopathy With Axonal Spheroids
HEPES  4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
hIgG  Human Immunoglobulin G
HLA  Human Leukocyte Antigen
HO-1  Heme Oxygenase 1
HRP  Horse Radish Peroxidase
HSC  Hematopoietic Stem Cell
HSCT  Hematopoietic Stem Cell Transplantation
<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>HSP40</td>
<td>Heat Shock Protein 40 kDa</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat Shock Protein 90 kDa</td>
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<tr>
<td>hVEGF</td>
<td>Recombinant Human Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>iBRB</td>
<td>Inner Blood Retinal Barrier</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
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<td>Insulin-Like Growth Factor</td>
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<td>Junctional Adhesion Molecules</td>
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<td>Kainic Acid</td>
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<td>KLD</td>
<td>Kinase-Ligase-Dpni</td>
</tr>
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<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRP-1</td>
<td>Low-Density Lipoprotein Receptor-Related Protein 1</td>
</tr>
<tr>
<td>MARVEL</td>
<td>MAL And Related Proteins For Vesicle Trafficking And Membrane Link</td>
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<td>MBEC</td>
<td>Mouse Brain Endothelial Cells</td>
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<td>MCM</td>
<td>Microglia Conditioned Medium</td>
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<td>MCSF-1</td>
<td>Membrane-Bound Colony Stimulated Factor 1</td>
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<td>MHC-II</td>
<td>Major Histocompatibility Complex 2</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug Resistance Proteins</td>
</tr>
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<td>MS</td>
<td>Multiple Sclerosis</td>
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<td>MΦ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
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<td>NALS</td>
<td>Neuroaxonal Leukoencephalopathy With Spheroids</td>
</tr>
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<td>NGS</td>
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<td>Neuromedin U</td>
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<td>Neuromedin U Receptor 2</td>
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<td>NPC</td>
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<td>Neural Stem Cell</td>
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<td>OCLN</td>
<td>Occludin</td>
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<td>OPC</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PDGFRβ</td>
<td>Platelet-Derived Growth Factor Receptor Beta</td>
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<td>PDGFβ</td>
<td>Platelet-Derived Growth Factor Beta</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PECAM1</td>
<td>Platelet And Endothelial Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinases</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>POLD</td>
<td>Familial Pigmentary Orthochromatic Leukodystrophy</td>
</tr>
<tr>
<td>PTP-ζ</td>
<td>Protein Tyrosine Phosphatase Zeta</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
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<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
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<tr>
<td>RAGE</td>
<td>Receptor For Advanced Glycation End Products</td>
</tr>
<tr>
<td>rcf</td>
<td>Relative Centrifugal Field</td>
</tr>
<tr>
<td>rhbFGF</td>
<td>Recombinant Human Basic Fibroblast Growth Factor</td>
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<tr>
<td>rhEGF</td>
<td>Recombinant Human Epidermal Growth Factor</td>
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<td>Reactive Oxygen Species</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
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<tr>
<td>sCSF-1</td>
<td>Soluble Colony Stimulating Factor 1</td>
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<td>SDS</td>
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<td>Small Interfering Ribonucleic Acid</td>
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<td>Tris Buffered Saline With Tween-20</td>
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<tr>
<td>TEER</td>
<td>Trans-Endothelial Electrical Resistance</td>
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<tr>
<td>TJ</td>
<td>Tight Junction</td>
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<tr>
<td>TLCK</td>
<td>Tosyl-L-Lysyl-Chloromethane Hydrochloride</td>
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<td>TLE</td>
<td>Temporal Lobe Epilepsy</td>
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<td>TNF-α</td>
<td>Tumour Necrosis Factor-Alpha</td>
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<td>ULK1</td>
<td>Unc-51 Like Autophagy Activating Kinase 1</td>
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<tr>
<td>UPS</td>
<td>Ubiquitin/Proteasome System</td>
</tr>
<tr>
<td>FiMS</td>
<td>Fms-interacting protein</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
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<td>Vascular Endothelial Cadherin</td>
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Chapter 1:

General Introduction
1.1 The Blood-Brain Barrier

1.1.1 An Overview Of Specialised Central Nervous System Barriers

The human brain is an energy-demanding organ, requiring 20% of the human resting metabolism and blood flow despite it accounting for only 2% of the body’s weight\textsuperscript{1-3}. The brain holds a prioritised position within the body, even under conditions such as exhaustion or malnourishment in anorexia it can retain its mass to the detriment of other organs\textsuperscript{4,5}. Neurons, the primary cell type within the brain, are responsible for the majority of this large metabolic demand. In particular, excitatory glutamatergic neurons consume 84% of the energy supplied to the grey matter to fire action potentials, initiate postsynaptic effects and perform the recycling processes of glutamate signalling\textsuperscript{6,7}. The brain is also sensitive to damage, both from physical insult as well as from the entry of harmful blood-borne macromolecules. This is largely due to the complex connectivity of neurons and the low or absent levels of adult neurogenesis to facilitate the replacement of damaged tissue\textsuperscript{8,9}. This sensitivity extends to the inflammatory effects of activated immune cells, although the brain has specialised resident macrophages the uncontrolled infiltration of activated peripheral leukocytes can have devastating effects.

To provide for these needs, the central nervous system (CNS) has three specialised cellular barriers which function to maintain cerebral homeostasis and perform the unique functions required by the brain (Figure 1.1). The blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB) of the choroid plexus and the arachnoid barrier of the meninges are each complex multicellular barriers with critical roles in maintaining CNS homeostasis.
Figure 1.1: Barriers of the CNS. A simplified overview of the specialised barriers of the central nervous system. (a) The arachnoid barrier of the meninges formed by cells of the arachnoid membrane. (b) The endothelial blood-brain barrier of the CNS vasculature, induced and maintained by cells of the neurovascular unit. (c) The epithelial blood-CSF barrier of the ventricle. From McCully et al. (2018).
The cerebrospinal fluid (CSF)-secreting choroid plexus is found within the ventricles of the brain and is a highly vascularised tissue consisting of a monolayer of cuboidal ependymal epithelial cells and connective tissue. The fenestrated vasculature of the choroid plexus does not display the same reduced level of paracellular transport as that of the brain parenchyma\textsuperscript{10}. This allows for the extravasation of molecules usually absent in the CNS into the surrounding connective tissue. The ependymal epithelial cells of the choroid plexus express tight junction (TJ) proteins to form the BCSFB, preventing the entry of harmful substances into the CSF\textsuperscript{11}. The BCSFB endothelium is polarised, expressing adherens junctions (AJs) basolaterally and TJs apically such that molecules passing from the choroid plexus vasculature must sequentially pass through AJ and TJ complexes before entry into the CSF\textsuperscript{12}. Catenin complexes anchor AJ E-cadherin proteins to the cell F-actin cytoskeleton\textsuperscript{13}, while zonula occludens 1 (ZO-1) performs a similar role for TJ proteins occludin and claudins-1, -2, -3 and -11\textsuperscript{14,15}. CSF produced by the choroid plexus acts as a means of physical protection for both the spinal cord and brain against physical impacts, as well as contributing to the regulation of blood flow, CNS microenvironment and brain solute levels. The CSF circulating the brain makes contact with the second CNS barrier within the subarachnoid space. The arachnoid barrier is a blood-CSF barrier, comprised of specialised leptomeningeal cells which compact to form the arachnoid barrier. The barrier separates the meningeal dura mater, situated below the cranium, from the pia mater, which lies above the glia limitans\textsuperscript{16}. Similarly to the vasculature of the choroid plexus, the dura mater vasculature does not have BBB properties and the entry of blood components into the brain is hindered by the arachnoid barrier cells which express gap junction proteins such as connexin-43 as well as AJ protein E-cadherin and scaffolding protein ZO-1\textsuperscript{17}. The precise molecular makeup of the arachnoid tight junction is still under examination, however, electron micrographs of the arachnoid cell layer have captured highly dark electron-dense regions indicative of tight junctions and the TJ protein claudin-11 has been reported in the rat and human brain\textsuperscript{18,19}. Both the BCSFB and the arachnoid barrier function as secondary barriers to prevent molecules already escaping fenestrated vasculature from entering the brain, however, within the brain parenchyma, the vasculature has evolved to have even more specialised barrier properties. The blood-brain (and inner blood-retina) barrier is located at the endothelium of CNS microvessels and is maintained by the co-operative interactions of endothelial cells, astrocytes and pericytes, members of the so-called neurovascular unit (NVU, Figure 1.2). The discovery of the BBB (reviewed in Saunders et al.\textsuperscript{20}) is often attributed to initial dye-injection experiments by Paul Ehrlich and Edwin Goldmann which revealed the capacity of the CNS vasculature to both retain and exclude tracer molecules. Initial injections of water-soluble dyes into the vasculature resulted in all organs except the CNS being stained\textsuperscript{21}, providing
the first insight into the existence of a barrier between the CNS and circulatory system. Although Erlich himself disputed the existence of a specialised CNS vasculature, despite his earlier findings\textsuperscript{22}, subsequent studies by Goldmann\textsuperscript{23,24} are credited with establishing the existence of a specialised CNS vasculature. Goldmann noted that the CNS could exclude peripherally injected trypan blue while retaining it when injected into the subarachnoid space thus building on earlier similar work by Franke\textsuperscript{25} and Bouffard\textsuperscript{26} using methylene blue and trypan red respectively.

The specialised BBB of the CNS is a dynamic tissue barrier that acts to protect and regulate the neural environment through restricted permeability and selective molecule transport from blood to the brain and vice versa. This regulatory capacity of the CNS vasculature is required to such an extent that the CNS vascular system comprises up to 5% of total brain volume\textsuperscript{27}, having a surface area of 15-25m\textsuperscript{2} and a median capillary-neuron distance of 50 μm\textsuperscript{28,29}. The properties of the BBB are achieved through the co-operative actions of cells within the NVU which is comprised of pericytes, astrocytes, neurons and endothelial cells\textsuperscript{30}. While gases and small lipophilic compounds can freely cross the BBB, a highly specialised CNS endothelium establishes a size-selective barrier limiting paracellular traffic and preventing the passive diffusion of molecules as small as 400-800 Dalton (Da)\textsuperscript{31,32}. Creating an increased dependence on active transport allows the BBB to physically regulate molecular traffic via the remodelling of intercellular endothelial TJs or through luminal or abluminal transport protein localisation\textsuperscript{33,34}. This regulation of transport and junctional proteins permits the high nutrient influx required for cognitive function, such as increased luminal expression of glucose transporter GLUT1\textsuperscript{35}. Simultaneously, endogenous and exogenous toxins are excluded from the brain parenchyma via enriched abluminal efflux proteins\textsuperscript{36}.

BBB dysregulation or deterioration with age is now believed to be a compounding factor in neurodegenerative and age-associated diseases such as multiple sclerosis (MS)\textsuperscript{37}, Alzheimer’s disease (AD)\textsuperscript{38}, schizophrenia\textsuperscript{39} and type 2 diabetes mellitus\textsuperscript{40}. Changes in TJ expression and normal NVU functionality have been identified at pre-clinical stages in animal models of neurodegenerative diseases\textsuperscript{41} pointing towards the BBB as an early target in the disease pathogenesis. The changes observed in early stages of neurodegenerative diseases may indeed be part of the pathology, however, they equally may be physiological age-related changes which instead render the brain susceptible toward subsequent disease-inducing insults. Autoimmunity and neuroinflammation are often central to the pathologies seen in age-associated disorders. The “immune privileged” status of the brain is often misinterpreted as meaning the organ is
resistant to all leukocyte invasion, however, this view is incorrect and leukocyte transmigration across the BBB does occur, but remains tightly regulated.
Figure 1.2: The Neurovascular Unit. Cells of the assembled neurovascular unit. In capillaries, the basal lamina and glia limitans fuse to close the Virchow-Robins space.
1.1.2 Endothelial Cells

BBB development in the mouse embryo begins at approximately embryonic day 12 (E12) with invading mesoderm-derived angioblasts forming a vascular plexus of immature blood vessels which progressively branches to produce a vascularised brain. Endothelial cells form the BBB through downregulated transcytosis, polarised transport protein expression and reduced passage of molecules through the paracellular space due to the formation of continuous intercellular TJs. Despite these specialised endothelial features, the development of BBB properties is not an intrinsic feature of cerebral endothelial cells themselves. Early experiments performed by Stewart and Wiley revealed that the properties observed in CNS endothelial cells are a product of the CNS microenvironment itself. In their seminal studies, avascular quail brain tissue was engrafted into the chick mesodermal coelom cavity and quail mesodermal somites engrafted into chick brain. This allowed an important determination to be made; would vessels sprouting from the host tissue maintain their properties, or would they conform to the properties of the engrafted tissue. Upon injection of trypan blue, transplanted quail mesodermal tissue, but not the surrounding chick neural tissue, readily took up the dye. The opposite was observed in the chick coelom, where quail neural tissue grafts showed no dye extravasation, while the surrounding chick mesodermal tissue did. Endothelial cells growing within the transplanted brain grafts also developed tight junctions, had reduced pinocytic activity and expressed alkaline phosphatase, all of which are markers of CNS endothelial cells. This revealed that endothelial cells themselves are naïve regarding CNS identity; the neural environment instead supplies extracellular trophic factors to induce “tight” endothelial characteristics.

CNS endothelial cells have a characteristically high transendothelial electrical resistance (TEER) which is a product of the TJs formed between cells. While peripheral capillaries demonstrate TEER values of 2-20 Ω·cm², pure cultures of CNS endothelial cells can have TEER values as high as 1800 Ω·cm² which is further increased when co-cultured with astrocytes and/or pericytes. These TJs act as a physical barrier, forming compact structures at cell-cell contacts and restricting the paracellular flux of molecules. TJs are scaffolded to the F-actin cell cytoskeleton by ZO family members, which bind to and organise claudins through their C-terminal PDZ binding motifs and occludin via its GUK domains. These junctional proteins include three main families, the claudins, occludin, MARVELs and Ig-like junctional adhesion molecules (JAMs) as well as novel members such as lipolysis-stimulated lipoprotein receptor (LSR). The tight junction forms luminally within the endothelium, with PECAM-1 and VE-cadherin binding to cytoskeletal catenin complexes to form abluminal adherens junctions. Discussed in further
detail later, these junctional complexes are a major feature of CNS endothelial cells but only facilitate closure of the paracellular transport pathway. CNS endothelial cells lack fenestrae and have low rates of transcytosis, driving transport across the BBB via polarised expression of transport proteins such as the efflux protein p-glycoprotein (P-gp) and glucose transporter GLUT1. To cater for increased active transport across the cell, endothelial cells of the BBB also have an extraordinarily higher number of mitochondria per cell than endothelial cells in muscle.

Mature CNS endothelial cells are surrounded by a basement membrane, are directly in contact with mural pericyte cells and receive instruction from neighbouring astrocyte end-feet. These co-ordinated actions facilitate the maintenance of the BBB, however during the development of the BBB a stepwise induction occurs to create a functional embryonic barrier which does not appear to require the fully assembled NVU.

1.1.3 Pericytes

Pericytes are mural cells present in every vascularised tissue of the body, associating with the abluminal face of the endothelium and embedding themselves within the basement membrane which they help to produce. Connexin-43 is a critical protein which facilitates pericyte-endothelial cell contact, forming hemichannels at peg-and-socket junctions where the cell types interact. Pericytes have varied morphology, appearing as a mesh of interconnected cells in the precapillary arteriole and postcapillary venules, while pericytes interacting with capillaries themselves appear as long thin strands or helices. The arrival of pericytes at the developing mouse endothelium at E18 correlates with increased TJ expression and reduced extravasation of tracer molecules, implicating this NVU component as one of the primary CNS inducers of the barrier. Blood vessels within the CNS have the highest pericyte to endothelial cell ratio in the body and this ratio has been shown to correlate with cerebral blood flow and barrier integrity. Pericytes preferentially make contact with cerebral capillaries at endothelial cell junctions while also positioning themselves at luminal faces of astrocytic end feet; this advantageous localisation allows for the regulation of multiple aspects of the NVU such as capillary diameter, blood flow and end-feet guidance during barrier development.

Endothelial platelet-derived growth factor β (PDGFβ) has been shown to be induced by canonical Wnt/β-catenin signalling to drive pericyte recruitment through its receptor (PDGFRB). Similarly, pericyte-derived glial-derived neurotrophic factor (GDNF) was found to be a potent stimulator of claudin-5 expression in both peripheral and cerebral endothelial cells. With astrocytes arriving post-barriergenesis, the pericyte plays a critical role in establishing the early
BBB. Mice lacking either PDGFRβ or its ligand die shortly after birth and through the use of hypomorphic alleles research into the endothelial-pericyte dynamic has produced new insights into the role of pericytes within the NVU. Armulik et al. utilised the viable Pdgfβret/ret mouse expressing a variant of PDGFβ lacking its heparin sulphate proteoglycan binding ability and compared neurovascular changes against wild-type (WT) controls or Pdgfβ−/− mice with an endothelial-specific Pdgfβ knock-in construct (R26P). Initial findings showed pericyte coverage was reduced to 26 % in the Pdgfβret/ret model, while the R26P+/− and R26P+/+ mice retained 40 % and 72 % respectively. Although TJ protein levels were unchanged, junction strand morphology in the Pdgfβret/ret and R26P+/− mice deviated from WT controls. The astrocyte-enriched water channel protein aquaporin 4 (AQP4) was also re-distributed, potentially due to a loss in astrocyte end-feet guidance and polarisation. Immunohistochemical staining also revealed the transcellular movement of albumin, Immunoglobulin-G (IgG) and injected 70 kDa dextran indicating an increased transcellular permeability in the absence of TJ expression differences. These injected tracers were observed to accumulate within the endothelium of Pdgfβret/ret and R26P+/− mice only, potentially hinting at a role for pericytes in downregulating luminal transcytosis and abluminal vesicle release.

Pericytes have also been shown to display multipotency following ischemia, exhibiting a capacity to differentiate from a nestin + intermediary cell type into vascular angioblasts, neural stem cell organoids and even microglia-like cells. The capacity of pericytes to re-program themselves into microglia-like cells has also been demonstrated in the absence of ischemia. Mice were fed a chow diet containing the colony stimulating factor 1 receptor (CSF-1R) inhibitor PLX3397, which is known to reduce 99 % of brain microglia after 3 weeks. On the removal of the drug from the chow, repopulating microglia were observed to have originated from pericytes which first de-differentiated into a nestin + precursor before developing microglia properties. Pericytes may not only function as regulatory cells within the NVU but may also act as reservoirs in the case of cerebral insult, gaining expression of pluripotency transcription factors in order to re-populate the injured region with NVU cell types.

1.1.4 Astrocytes

Astrocytes are glial cells within the CNS and are the most abundant cell type within the mammalian brain. Astrocytes project polarized end-feet to ensheath more than 90 % of cerebral capillary surface area; these end-feet act to recycle neurotransmitters, provide nutrients and regulate local immune responses. Astrocytes are absent for nearly the duration of embryonic BBB development, appearing at E19 in the mouse and postnatally in the rat.
however, glial-like precursors may drive barrierogenesis through Wnt and Sonic hedgehog (Shh) ligand secretion. The CNS specific mosaic of Wnt ligand expression may be a factor also in the unique endothelial and pericyte features seen within the NVU, driving elevated PDGFβ expression relative to peripheral endothelial cells and creating a GDNF-enriched environment for BBB induction and maintenance.

It is widely believed that the role of astrocytes within the NVU is the maintenance rather than the establishment of the BBB; in adulthood, astrocytes secrete many of the ligands found within the developing CNS to preserve BBB characteristics. The Hedgehog (Hh) pathway involves the sonic hedgehog ligand (Shh) binding to cell surface receptors such as Patched, de-repressing the G-coupled protein receptor smoothened (Smo) and activating downstream transcriptional changes through the Gli family of transcription factors. Within the CNS, Smo activity in endothelial cells represses pro-inflammatory chemokine C-X-C motif ligand 8 (CXCL8) and chemokine C-C motif ligand 2 (CCL2) production and upregulates TJ and AJ components. Treatment of endothelial cell monolayers with recombinant Shh or astrocyte-conditioned media-induced downregulation of CXCL8 and CCL2; congruently an acute increase in BBB permeability and leukocyte transmigration is observed upon injection of mice with the Smo inhibitor cyclopamine. Developing mice with a conditional endothelial cell-restricted Smo deletion have reduced TJ expression, increased plasma protein leakage into the brain parenchyma and reduced astrocyte recruitment at later stages. Shh is exclusively sourced from astrocytes in the adult NVU, indicating their role in maintaining the signalling pathways established in the developing CNS. As well as their actions through the Shh pathway, astrocyte-secreted factors such as GDNF, apolipoprotein E and J (ApoE, ApoJ) and end-feet enriched transport proteins further regulate CNS endothelial cells. These include AQP4, downregulated in paediatric CNS neoplasms with dysfunctional BBB and implicated in the clearance of soluble amyloid-β (Aβ); and excitatory amino acid transporters EAAT1 and EAAT2 which take up and recycle glutamate to prevent glutamate-induced excitotoxic neuron death during stroke.

1.1.5 The Basal Lamina And Glia Limitans

The CNS vasculature has a dual set of extracellular basement membranes which are produced by the NVU, providing a structural scaffold and barrier against infiltrating cells. Interactions between integrins and laminins within the NVU also drive cellular development and maintain BBB homeostasis. β1-integrin, a cell surface molecule which binds to α-integrin subunits of the extracellular basement membranes, has been revealed to be a central protein in endothelial cell development. Loss of endothelial β1-integrin resulted in cuboidal endothelial cell morphology,
and critically a mislocalisation of claudin-5, CD31 and VE-cadherin\textsuperscript{77}. Expression of proteins involved in establishing endothelial cell polarity, Par3 in particular, was also reduced. The BBB requires stringent polarisation to maintain concentration gradients across the blood and brain, and integrin-mediated endothelial-basal lamina interactions appear to be critical for this. The basal laminal also directs and polarises astrocytic endfeet as they are recruited to the NVU. Loss of agrin, a heparan sulphate proteoglycan found in the basal lamina, disrupts the localisation of aquaporin-4 in astrocyte endfeet. The water channel protein is localised into orthogonal arrays of intramembranous particles (OAPs), however, in the absence of agrin, it is randomly distributed across endfeet membranes\textsuperscript{78}. Astrocytes isolated from agrin-null mice were found to display no alterations in levels of AQP4 expression. Instead, the polarisation of astrocyte endfeet and localisation of AQP4 into OAPs was disrupted in the assembled NVU, highlighting the role of the basal lamina in the specialisation of the individual cellular components of the BBB\textsuperscript{79}. Astrocytic expression of laminin in the glia limitans basement membrane has also been shown to be involved in BBB maintenance and AQP4 expression. Loss of astrocytic, but not endothelial, laminin resulted in increased BBB permeability and loss in either cell type decreased AQP4 expression and loss of astrocyte endfoot polarity. There was also downregulation of endothelial claudin-5 and occludin, as well a shift in pericyte morphology to a contractile state\textsuperscript{80}. Co-operative interactions between the extracellular membranes and the cells of the NVU not only help to maintain the BBB but also provide physical acellular barriers against immune cell entry into the CNS.

As leukocytes enter the brain, they first must cross both the endothelial basal lamina and glia limitans membrane. The basal lamina consists of endothelial and pericyte-produced collagen IV, fibronectin, perlecan and laminins α4 and α5 which interact with integrins and dystroglycans of infiltrating immune cells in the initial steps of leukocyte transmigration\textsuperscript{76,81,82}. Expression of laminin-α4 and laminin-α5 within the basal lamina has been shown to be sensitive to the inflammatory state of the CNS, with inflamed vasculature expressing a higher level of laminin-α4\textsuperscript{76}. Laminin-α4 expression also correlated with higher T-cell infiltration, while laminin-α5 expression appeared to restrict leukocyte crossing of the endothelium. Leukocytes which successfully cross the basal lamina must then breach the glia limitans. The glia limitans membrane, which fuses with the basal lamina in cerebral capillaries\textsuperscript{83}, has a differing laminin profile and is enzymatically degraded via matrix-metalloproteases (MMPs) produced during leukocyte entry\textsuperscript{84}. Both clodronate treatment depletion of MMP-expressing perivascular macrophages, as well as selective deletion of MMP-2 and MMP-9, was able to induce a stalling of T-cells between the basal lamina and glia limitans membrane\textsuperscript{84}. This double checkpoint nature
of the BBB extracellular membranes facilitates the maintenance of the brains immune-privileged state.

1.1.6 Junctional Proteins Of The BBB

In both the BBB and inner blood-retinal barrier (iBRB), TJs function to seal the paracellular space between neighbouring endothelial cells, or between membrane regions of the same cell (Figure 1.3). Through limiting the paracellular passage of macromolecules and solutes, TJs generate a dependency on the highly regulated transcellular transport mechanisms of the BBB endothelium.

Claudins are a large family of transmembrane proteins, 20-27 kDa in size and expressed in various tissues and cell types throughout the body. They contain four transmembrane domains, generating two extracellular loops (ECLs) which can form dimers between claudins of an identical or a different type. These two loops are believed to have discrete functions, ECL1 acts to regulate paracellular transport via pore formation while ECL2 drives the physical closure of the paracellular cleft\(^8^5\). Within the mouse brain, claudins 1, 3, 5, 11 and 12 were thought to be expressed at the endothelial tight junction, although recently the high cross-reactivity of claudin-recognising antibodies has cast doubt on the presence of claudin-12 and claudin-3 and their importance within the NVU. Claudin-12 knockout mice have no BBB dysfunction, and immunohistochemistry of brain sections using commercial claudin-12 antibodies produces false-positive immunoreactivity\(^8^6\). The use of RNA sequencing and LacZ reporter mice have now shown claudin-12 to have very low expression within the BBB endothelium, being primarily expressed in astrocytes and neurons. Similarly, the generation of a claudin-3 knockout mouse has prompted its inclusion in the list of BBB claudins considered to be an artefact of antibody cross-reactivity\(^1^5\).

In light of these revelations, claudin-1 and claudin-11 have also recently been subjected to investigation. Using mass spectrometry and validation with siRNA knockdown, claudin-11 has been shown to be present in the BBB endothelium, forming cis- and trans-heterophilic dimers with claudin-5. Reduced claudin-11 was detected in the capillaries of frontal lobe and spinal cord sections of human MS tissue, and in the brain and spinal cord of mouse experimental autoimmune encephalomyelitis (EAE) tissue. Downregulation of claudin-11 via siRNA targeting was also shown to increase endothelial monolayer permeability to a FITC-conjugated 70 kDa dextran in endothelial cell monocultures, further confirming its function in BBB maintenance\(^8^7\).

Investigations into claudin-1 have shown it to be expressed not in the resting CNS vasculature, but during chronic stroke in both mice and humans. Its expression during postischemic barrier formation may be detrimental for disease outcome, as it displaces claudin-5 from the TJ complex.
and is associated with a proinflammatory endothelial phenotype. Targeting claudin-1 with an inhibitory peptide improved barrier integrity as well as functional recovery in chronic stroke, identifying it as a potential therapeutic target to improve BBB recovery rather than a critical member of the TJ complex\textsuperscript{68}. Despite the recent changes in what is believed to be the molecular composition of BBB TJs, claudin-5 has robustly been shown to be critical for BBB formation. Early experiments using claudin-5 knockout mice revealed its loss resulted in postnatal lethality\textsuperscript{31}, and it is the most abundant claudin family member found in the CNS vasculature. Transcriptomics data has shown it to be enriched at least 500-fold respective to other family members in endothelial cells, and recent advances in single-cell RNA sequencing has allowed it to be estimated that there are over 10,000 Cld5 mRNA transcripts per cell in brain capillaries, near ten-fold the number of Actb transcripts\textsuperscript{89–92}. Claudin-5 has been implicated in neurodegenerative and neuropsychiatric disorders, and restoration of homeostatic claudin-5 levels in these diseases has become a promising therapeutic angle\textsuperscript{93}.

Occludin, a member of the MARVEL family (MAL and related proteins for vesicle trafficking and membrane link), was the first TJ protein identified\textsuperscript{48}. Unlike claudin-5 knockout mice, occludin knockouts have been shown to be viable\textsuperscript{94}. Enriched in epithelial and CNS endothelial TJs, occludin undergoes extensive post-translational modifications allowing it to dynamically regulate the TJ. Phosphorylation of the C-terminal cytoplasmic tail of occludin has been shown to drive its localisation to the luminal tight junction while unphosphorylated forms were found to be distributed across the abluminal membrane\textsuperscript{95,96}. Modification of occludin can have specific effects on the TJ depending on the amino acid residue that undergoes phosphorylation. Phosphorylation of serine 490 (S490) can result in upregulation of occludin endocytosis and dissociation of occludin-ZO-1 complexes\textsuperscript{97,98}. Treatment of bovine retinal endothelial cells with vascular endothelial growth factor (VEGF) was demonstrated to induce protein kinase C-mediated phosphorylation of occludin\textsuperscript{99}. Subsequent investigations revealed that specifically S490 phosphorylation was induced, and accompanied by ubiquitination of occludin and its degradation via the ubiquitin-proteasome pathway. Confirmation of occludin being central to the reported VEGF-induced endothelial TJ breakdown was performed by replacement of S490 with an alanine residue, which was sufficient to prevent VEGF-induced junctional breakdown. Additionally, expression of an occludin-ubiquitin chimaera in place of WT occludin could drive TJ disruption even in the absence of VEGF. Occludin’s role as a target for TJ remodelling by extracellular signals was also shown in the endothelial response to IL-17a, which has been found to facilitate T-cell entry into the brain in EAE. IL-17a treatment of mouse endothelial cells reduced TEER and expression of occludin through inducing the production of reactive oxygen species.
(ROS), shifting the endothelium to a proinflammatory state\textsuperscript{100}. The MARVEL and claudin family members can also regulate each other within the TJ, while claudin-5 appears to have a key structural role in the BBB TJ, organisational and regulatory roles appear to be fulfilled by MARVEL proteins in endothelium\textsuperscript{101}.

The endothelial TJ complex is anchored to the cell cytoskeleton through the binding of protein C-terminal domains to ZO proteins, tethering the TJ complex to F-actin\textsuperscript{46}. ZO proteins are key structural members of the BBB endothelium, with heterodimers of ZOs 1, 2 and 3 anchoring claudin and occludin to the cell cytoskeleton. As ZO-1 knockout mice display embryonic lethality, ZOs do not compensate for each other, but rather have unique expression patterns and functions in development\textsuperscript{102}. ZO-1 and ZO-2 can regulate the TJ complex itself, binding modifiers of Rho GTPases such as shroom2 and cortactin to remodel cytoskeletalanchoring\textsuperscript{103,104}. In a rat model of ischemia-reperfusion, ZO-1 was identified as a potential therapeutic target for BBB breakdown. Cell culture experiments showed a cytoplasmic re-distribution of ZO-1 after oxygen-glucose deprivation and reperfusion, accompanied by increased endothelial autophagy and MMP secretion. In subsequent \textit{in vivo} modelling, loss of observed BBB integrity was exacerbated by administration of the autophagy inducer rapamycin, which enhanced caveolin-1 mediated ZO-1 redistribution to the lysosome, and increased BBB permeability\textsuperscript{105}.

The core TJ complex also contains tricellulin, a member of the MARVEL family, and LSR which are both present at tricellular junctions\textsuperscript{106}. LSR is a member of the angulin family, and functions to recruit tricellulin to the TJ via a Pyk2-dependent phosphorylation\textsuperscript{107}. Although less research into these TJ components has been carried out with respect to the BBB endothelium, tricellulin has been found to have roles in epithelial junctional integrity\textsuperscript{108–110} and mutations in tricellulin have been found to cause deafness\textsuperscript{49}. Tricellular junctions have a central tube, an extracellular space between the three adjoining cells, which has increased paracellular flux of ions but not macromolecules\textsuperscript{111}. Targeting these, rather than the bicellular tight junction, has become a promising therapeutic angle for delivery of macromolecules across the brain without full BBB disruption.

While the former TJ proteins are central to TJ modulation and formation, the JAM protein family has a central role in regulating immune cell traffic through the BBB. JAM proteins have been implicated in establishing the apical-basal endothelial cell axis through recruiting cell polarity complexes, and in facilitating leukocyte infiltration\textsuperscript{112,113}. The actions of JAM proteins are in concert with basolaterally located adherens junctions (AJs) comprised of cadherins such as
vascular endothelial-cadherin, and nectins from the cell adhesion molecules (CAMs) family which further regulate leukocyte passage across the BBB$^{52}$. 
Figure 1.3: The Endothelial Tight Junction. The molecular composition of the endothelial tight junction. Luminally localised tight junction proteins claudin, occludin and LSR seal the paracellular cleft through anchoring to the cytoskeleton via ZO proteins. Abluminal gap and adherens junctions are comprised of JAMs, cell adhesion molecules (CAMs), CD31 and connexins. Adapted from Greene et al. (2019).
1.1.7 Transport Across The Blood-Brain Barrier

Transport across the BBB occurs via paracellular and transcellular pathways, with polarised expression of transport proteins coordinating with the sealing of the paracellular space through TJ complex formation to tightly regulate macromolecule entry into the brain. Lipid mediated free transmembrane diffusion across the BBB requires low molecular weight compounds with a high lipid solubility, meaning fewer than 8 hydrogen bonds\textsuperscript{114,115}. Should small molecules be too lipid-soluble however, they will remain sequestered within the capillary bed without diffusing into the aqueous compartment of the brain. Many reviews and articles quote a 400 Da cutoff for free diffusion of molecules into the brain\textsuperscript{116}, however, this is not a finite limit and further consideration is warranted on the topic. The compounds screened in this early study were later identified to be substrates for the p-glycoprotein efflux protein, indeed proteins measuring over 400 Da have been detected to freely pass into the brain from the blood, without evidence of a transport system, such as the 573 Da enkephalin\textsuperscript{117} and the 849 Da delta sleep-inducing peptide\textsuperscript{118,119}. An extreme scenario has been the reporting of the diffusion of the 7,800 Da chemokine cytokine-induced neutrophil chemoattractant-1 (CINC1) from the blood into the brain\textsuperscript{120}. There may be unknown mechanisms of active transport which facilitate entry of these larger compounds into the brain, or a property of these molecules that has yet to be attributed to promoting diffusion across the BBB. In contrast, evidence from drug design and delivery approaches has maintained the 400 Da cutoff as the current standard, and tracer injections in mice routinely show 443 Da biotin unable to escape the healthy CNS vasculature. Factors such as charge, molecular weight and hydrophobicity interact to form a non-linear relationship between capacity to cross the BBB and size\textsuperscript{121}, as such the BBB can be only generally considered impenetrable to molecules over 400 Da.

Regardless of the capacity of lipophilic molecules to enter the brain by passive diffusion, movement of hydrophilic molecules across the CNS endothelium requires distinct BBB transcellular transport systems (Figure 1.4). Transcellular transport systems include adsorptive and receptor-mediated transcytosis, active influx/efflux transporters and protein transporters. The classical example of a BBB transport protein is the glucose transporter GLUT1 which is expressed on the luminal and abluminal faces of the endothelium and is enriched in blood-tissue barriers, particularly the BBB\textsuperscript{122}. Morpholino-based downregulation of the GLUT1 encoding gene Glut1 in zebrafish resulted in a compromised BBB and vasogenic brain edema\textsuperscript{123}. Occludin, claudin-5, VE-cadherin and the VEGF receptor 2 were all downregulated in the vasculature of zebrafish with reduced Glut1 expression. In the A\textsuperscript{Pp}w/\textsuperscript{0} mouse model of Alzheimer’s disease, loss
of one copy of the mouse GLUT1 gene Slc2a1 exacerbated the Alzheimer’s disease pathology and induced early BBB breakdown. BBB leakage and reduced expression of claudin-5, ZO-1 and occludin were observed in Slc2a1+/− mice even in the absence of an Alzheimer’s disease background, highlighting the critical importance of GLUT1 in the BBB endothelium. Ion concentrations are also maintained by protein transporters, with abluminally dominant expression of Na+/K+ ATPase and Na+-K+-2Cl⁻ co-transporter 1 maintaining ionic concentrations in both the brain and the endothelium to both maintain the CNS microenvironment and provide ions for active transporters124. Amino acid influx into the brain is maintained by four systems (L1, y¹, n and xG⁻) which have differential but overlapping, amino acid substrates125,126. Glutamate transporters at the BBB depend on sodium gradients generated by ion transporters, with EAAT 1, 2 and 3 expressed on the abluminal face of the endothelial cell to maintain low extracellular levels of glutamate within the CNS127.

The sensitivity of the brain to toxins demands it have a means of efficient efflux of harmful compounds that arrive at the brain via the blood. Luminal expression of ATP-binding cassette (ABC) superfamily, including P-gp and multidrug resistance proteins (MRP) 1, 3 and 5128, act to efflux blood-borne xenobiotics, pharmacological compounds and toxins from the endothelium back into the blood. P-gp is a highly expressed efflux transporter at the human BBB, discovered originally due to its ability to efflux chemotherapeutics from mammalian cancer cell lines129. While humans express a single form of P-gp, rodents having two isoforms, Mdr1a and Mdr1b, which are similarly expressed in CNS endothelial cells130. P-gp has a range of substrates, and while classifying the broad range into defined classes has been proven difficult, they are most often hydrophobic or amphipathic and positively charged131. Delivery of drugs into the CNS is enhanced in Mdr1a knockout mice132,133, in particular known P-gp substrates such as ivermectin. This P-gp substrate was present in Mdr1a−/− brains at 90-fold higher concentrations than in controls, while peripheral organs displayed only 3-4 fold increases. P-gp has also displayed the capacity to function as an immunoregulatory protein, though whether it functions as a direct transporter of cytokines134,135, or simply secretion of immunomodulatory substances136, is still uncertain. Furthermore, these roles of P-gp may be limited to its activity in leukocytes rather than its expression within the BBB endothelium137. ABC efflux proteins are a boon to and bane of the CNS, protecting it from harmful peripheral compounds while simultaneously hindering the uptake of potential therapeutics.

Larger macromolecules enter the brain via transcytosis, requiring intracellular vesicles to shuttle both the transported macromolecule and bound receptor from the luminal to the
abluminal face of the endothelium. Transcytosis can be receptor-mediated, with cargo binding to cell surface proteins, or adsorption-mediated where the negatively charged cell membrane encapsulates a positively charged cargo. In both cases, vesicles can form from caveolae, small plasma membrane invaginations, or clathrin-coated pits which utilise the AP2 adaptor complex and dynamin GTPase for vesicle formation and release\textsuperscript{138,139}. Clathrin-mediated transcytosis is utilised by the transferrin receptor\textsuperscript{140} and low-density lipoprotein receptor-related protein-1 (LRP-1) for clearance of Aβ from the brain\textsuperscript{141}. CNS endothelial cells have inherently low levels of transcytosis, due to its suppression by Mfsd2a, which when knocked out of mice or zebrafish results in a leaky BBB due to increased rates of vesicular transcytosis\textsuperscript{142,143}. Mfsd2a is a fatty acid transporter and localises unsaturated phospholipids to the luminal membrane, which inhibits caveolae formation in adsorptive transcytosis\textsuperscript{144}. Overexpression of Msfd2a has also been found to be beneficial in rat models of surgical brain injury, as well as subarachnoid haemorrhage by further downregulating caveolae-mediated transcytosis\textsuperscript{145,146}. As mentioned in 1.1.3, pericytes also maintain the BBB through inhibition of endothelial transcytosis, with reduced pericyte coverage in \textit{Pdgfβ}^{ret/ret} and \textit{R26P}^{+/-} mice leading to increased transcytosis-mediated BBB permeability.
Figure 1.4: Transport Pathways Across the BBB. Limited paracellular transport due to tight junction formation generates a dependency on transcellular transport mechanisms. From Greene et al. (2019)
1.2 Adult-Onset Leukoencephalopathy With Axonal Spheroids And Pigmented Glia

Adult-Onset Leukoencephalopathy with Axonal Spheroids and Pigmented Glia (ALSP) is an autosomal dominant Mendelian disorder caused primarily by mutations in colony stimulating factor-1 receptor gene (CSF1R), although other genes have been identified in cases with no CSF1R mutation. Adult-onset leukoencephalopathies accompanied by axonal spheroids and pigmented glia were initially categorised as two main conditions, Hereditary Diffuse Leukoencephalopathy with axonal Spheroids (HDLS) and Familial Pigmentary Orthochromatic Leukodystrophy (POLD). While these clinical conditions have been known for nearly 40 years as distinct entities, the genetic cause of the two was reported within the past 10 years as being associated with dominant acting mutations in CSF1R. Due to the shared genetic aetiology of the conditions, they are now considered to be part of the spectrum of the same disease, which has been termed ALSP.

1.2.1 Clinical Symptoms And Neuropathology Of ALSP

Leukoencephalopathies are a large class of progressive heritable diseases which affect the white matter and cells of the axon-glia unit, including microglia, neurons, oligodendrocytes and astrocytes. Differential diagnosis of leukoencephalopathy is challenging; a broad range of symptoms during onset and broad symptom heterogeneity, even within families with the same mutation, can lead to an incomplete diagnosis. ALSP itself also shares overlapping symptoms with AD and MS. The early-stage symptoms have such a strong similarity to MS that the disease is often misdiagnosed as such, it is often only following the rapid deterioration of the patient that an accurate diagnosis is made. Indeed, in a large study of a Mayo Clinic ALSP cohort, all 20 examined cases, confirmed to have been ALSP by post-mortem analyses, had been misdiagnosed. Although there is currently no standardised treatment for ALSP, misdiagnosed patients do often receive symptom-based treatment, although none of these to date have been effective.

Clinical onset of ALSP is generally within the fourth or fifth decade of life, although it has been documented to present as early as 8 years of age, and the median survival post-diagnosis is 6.2-6.8 years. Early symptoms are often mild, starting with gait, mild personality changes and neuropsychiatric features such as depression, substance abuse and increased aggression. Behavioural changes often go unnoticed by the individual and neurological changes can be accompanied by a loss of insight and empathy. Rarely, optic nerve thinning or atrophy is present, generally identified when patients present with declining visual function. The disease
rapidly progresses; patient motor function reduces and is accompanied by cognitive decline, seizures, spasticity, dementia, incontinence and loss of ambulatory ability\textsuperscript{156}. In the final stage of the disease, most individuals are bedridden, often in a vegetative state in the months prior to death. The period between receiving a diagnosis and becoming bedridden can vary from months to years, making it difficult to provide a prognosis. Reported regular blood and CSF analyses of ALSP patients have shown little change in circulating profiles, making monitoring the progression of the disease reliant on neuroimaging and neurocognitive assessment\textsuperscript{152}. The reason for this age-related onset of ALSP has yet to be elucidated. It may be that the brain reaches a threshold of deposited intercellular debris or damage from previous injuries or infections. A shift in the epigenetic regulation of macrophage and neuronal function may also be responsible, revealing new sensitivities in CSF-1R mediated processes. While not addressed in this thesis, an investigation into the changes in chromatin modifications and microRNA expression that coincide with ALSP onset would potentially yield insight into the triggering event in the aetiology of the disease.

Neuroimaging studies have allowed for the observation of the general decline of the brain during ALSP progression. The general trend from onset to death is a shift from patchy, asymmetrical white matter atrophy to confluent symmetrical degeneration, accompanied by ventricular enlargement, thinning of the corpus callosum and small callosal calcifications\textsuperscript{157,158}. Although often misdiagnosed, ALSP patients will often are assessed by magnetic resonance imaging (MRI), which can be used retroactively to stage the disease or correlate with symptom presentation following post-mortem diagnosis with ALSP. One such analysis utilised scans during the presymptomatic and symptomatic stages of the disease, using T2-weighted and fluid-attenuated inversion recovery (FLAIR) imaging performed on individuals with confirmed \textit{CSF1R} variants. T2-weighted imaging can detect water-rich regions, such as CSF-filled ventricles, areas of inflammation, white matter atrophy and hypomyelination, displaying them as bright fields. The FLAIR approach also detects water-rich regions but has longer and altered imaging parameters to allow for normal high-intensity regions such as CSF-filled ventricle to be attenuated and appear dark. FLAIR can facilitate the more sensitive detection of abnormalities as the distinction between the CSF and local dysfunction is easier. Van Gerpen \textit{et al.} were able to use these techniques to identify hyperintense bifrontal periventricular foci in a presymptomatic patient MRI\textsuperscript{159}. Two subsequent imaging sessions, performed 7 and 8 years later after onset of symptoms, revealed that these foci had expanded to become symmetrical swathes of diffuse periventricular atrophy. Atrophy was also no longer limited to frontal regions of the brain, with the parietal periventricular matter, and cortical white matter displaying diffuse hyperintensity\textsuperscript{159}. A larger study looking at 16
symptomatic cases found that brain atrophy was present in 94% of cases examined, corpus callosum thinning was also present in nearly all individuals (88%). White matter lesions, present in every individual examined, were asymmetric and patchy in 37% of cases, and strikingly the spinal cord and cerebellum were found to always be spared in the disease. ALSP has hallmark neuropathology of demyelination, diffuse axonal swellings and axonal spheroids which stain positively for phosphorylated neurofilament, ubiquitin and amyloid precursor protein. Microglia laden with pigmented lipofuscin, and swollen macrophages are also common; these and activated glia are often localised to regions of the pathology rather than being pervasive throughout the brain.

1.2.2 The Genetics Of ALSP

Although known to be familial, the underlying genetics of ALSP has only been identified in the last decade. Heterozygous mutations in CSF1R are the primary cause of ALSP, with loss of function variants leading to haploinsufficiency of CSF-1R. Mutations are most often located in the kinase domain of CSF-1R (Figure 1.5), disrupting kinase activity while potentially maintaining its capacity to undergo ligand-binding and internalisation mechanisms. Since the identification of CSF1R being commonly mutated in POLD and HDLS, a multitude of case reports and retrospective sequencing studies have been published documenting over 60 novel variants in the gene. Molecular analyses of these variants are not always performed, with a lack of kinase activity assumed but ligand binding, cell surface expression and signalling capacity not confirmed. Penetrance of CSF1R mutations and genotype-phenotype correlation can vary amongst pedigrees, with rare examples of individuals presenting with classical ALSP pathology while a carrier parent is unaffected. The reasons for the incomplete penetrance of identical, dominantly acting CSF1R mutations are still unknown, with likely environmental or epistatic aspects to the disease still to be elucidated.

Bi-allelic loss of CSF-1R is extremely rare, but it has devastating effects. The earliest investigation into homozygous loss of CSF1R was in 1991. In a study of myelodysplasia syndromes driven by large deletions in the 5q region, bone marrow progenitor cell populations were identified to have a secondary loss of CSF1R on the unaffected chromosome. Patient leukocytes were screened for the presence of CSF1R on the homologous chromosome and individuals were identified as having lost both copies in their granulocyte fractions. As GM-CSF can drive the differentiation of these cells, it is not surprising that they could be produced, and the authors could only loosely tie it to leukaemia pathogenesis. Homozygous germ-line mutations in CSF1R have more recently been reported in two back to back papers, providing parallels between
observed human and mouse phenotypes in the context of homozygous CSF1R mutation. Seven individuals were identified with mutations in both copies of CSF1R, displaying skeletal malformities not observed in ALSP and neurological deficits from birth rather than in later years. Intriguingly, one of the individuals had had a child, indicating a conserved reproductive system. Brain calcifications, enlarged ventricles, corpus callosum agenesis and white matter atrophy were documented in early MRI and CT scans. Molecular analyses of the CSF1R variants revealed conserved production of the protein, although drastically decreased signalling capacity. The conclusion that these individuals had two hypomorphic alleles, rather than homozygous loss of function alleles, supported the individuals’ survival to third and fourth decades. Heterozygous kindred members also displayed no or very mild cognitive symptoms, although one heterozygous sibling has been diagnosed with MS in the absence of an ALSP pathology. No biopsy for neuropathology was performed, so it is currently not possible to determine the biochemical glial and neuronal pathologies in these individuals. A second study identified two further cases of individuals carrying homozygous CSF1R variants, and again heterozygous carriers were unaffected although the authors note that examined carriers were younger than 40, so may have been presymptomatic. Although no molecular analysis of these CSF1R variants was performed, immunohistochemical analyses of one deceased individual were performed. Calcifications, axonal spheroids and swellings and cerebellar disorganisation were observed. Iba1 and CD68 staining revealed an absence of parenchymal microglia, with Iba1 and CD68 immunopositivity restricted to enlarged perivascular cells. As CSF-1R is conserved across many evolutionary clades, non-mammalian models of CSF-1R have also been employed to examine loss of function effects. In zebrafish, there are two paralogs of CSF1R, csf1ra and csf1rb, the products of an ancient genome-wide duplication event that occurred during zebrafish evolution. Using a zebrafish model with both CSF1R homologs mutated to produce loss of function csf1ra and csf1rb, the neuronal CUX1 transcription factor was identified to be reduced in both the zebrafish model and the human cortical tissue. CUX1+ neurons project callosal axons, and the reduction of CUX1+ neurons in both cases of CSF1R loss lends support to CSF-1R signalling activating CUX1 transcription. The authors suggest microglia mediate this upregulation due to their near-absence in both models, however, this loss of CUX1 expression could also be a direct effect of neuronal CSF-1R activation.

Reports of incomplete penetrance, and widely ranging ages of onset between kindred members, also suggests that there may be epistatic effects occurring between CSF1R mutations and an individual’s genetic background. Although nearly all cases of ALSP are due to mutations in CSF1R, indeed between 10 and 25% of leukodystrophies are due to CSF1R mutations, there have
been cases with interacting secondary mutations\textsuperscript{165,170}. An example of this is a synergistic effect between CSF1R and the gene for neuromedin U receptor 2 (NMUR2). NMUR2 encodes the CNS-expressed receptor for neuromedin U, a peptide highly expressed in the hypothalamus and involved in feeding behaviour and appetite regulation\textsuperscript{171}. Variants in the neuromedin 2 encoding gene NMU have been linked to obesity in humans, although non-pathogenic variants have also been documented in the gene\textsuperscript{172}. In a study of an ALSP-affected kindred carrying a CSF1R mutation, binge eating behaviour was observed in affected individuals. Hyperphagia is not usually an aspect of ALSP clinical presentation and its onset coincided with the start of ALSP symptom presentation, namely dementia, in both the current and previous two generations. A variant in NMUR2 was identified to be carried by the kindred through the maternal line, however only those with both the NMUR2 and CSF1R mutations displayed any binge eating behaviours\textsuperscript{173}. The concept that ALSP can weaken the neural circuitry to predispose individuals to underlying, and usually non-pathogenic, genetic variants is one that has not been widely expanded upon. These epistatic gene interactions, in conjunction with personal histories of inflammatory events and CNS injuries, may be reasons for the large spectrum of ALSP clinical onset, symptom presentation and the original separation of the HDLS and POLD diseases. Although the cerebellum and spinal cord are not usually affected in ALSP, a case report of an ALSP patient with spinal cord lesions spreading rostrally with time has been published\textsuperscript{174}. However, this CSF1R mutation has been previously published with no mention of spinal cord lesions, indicating a secondary factor within this individual modified the disease presentation. An investigation into these secondary effects in ALSP cohorts is warranted to truly understand the genetic architecture of ALSP.

A second causative gene for ALSP has been suggested from recent sequencing studies. In the absence of mutations in CSF1R, a mitochondrial alanyl-tRNA synthetase 2 gene AARS2 has been identified in ALSP patients\textsuperscript{175–177}. AARS2 mutations are known to be causative in fatal infantile cardiomyopathy\textsuperscript{178} and ovarioleukodystrophy\textsuperscript{179}, and an examination of CSF1R-negative ALSP patients found biallelic mutations in the same gene. Premature ovarian failure was thought to be unique to AARS2-related ALSP and the average age of onset was about two decades earlier than in CSF1R-related ALSP. A comparative study between ALSP and AARS2-mutation related leukencephalopathy (AARS2-L) revealed further subtle differences between the two, adding to ovarian failure as the sole differentiating symptom. AARS2-L appeared to be more common in women, however, this may have been biased by having an indicative symptom that is specific to females. Comparing the two leukodystrophies, seizures were found to be twice as prevalent in ALSP, while gait and cognitive dysfunction were more common in AARS2-L. Incontinence and sensory deficits were found to be specific each to ALSP and AARS2-L respectively, although there
has since been published work documenting the loss of vision from optic nerve atrophy in ALSP. Looking at radiological imaging results, a striking lack of symmetrical hyperintensities, corpus callosum thinning, diffuse white matter lesions, calcifications or ventricular abnormalities were observed in AARS2-L\textsuperscript{180}. Although neuropathological examinations of biopsy and autopsy samples produced near-identical results, these imaging differences in addition to the striking earlier onset and ovarian failure, indicate that ALSP cases attributed to AARS2 mutations may indeed be a separate but highly overlapping disease entity. Since its publication, a single case of CSF1R-mutant positive ALSP with premature ovarian failure has been reported, further complicating the understanding of how these two genes cause such a similar pathology\textsuperscript{181}. It may be a case of unknown variants in the individuals’ background heterogeneity interacting with the documented mutations, or predisposing tissues to deterioration. With therapeutic approaches to ALSP focusing on restoring the CSF-1R dependent cell populations of patients, understanding the aetiologies of these two leukodystrophies may be important to avoid unnecessary treatments.
Figure 1.5: Loci of ALSP variants in CSF1R. CSF1R variants identified in ALSP cases. Variants are located in the protein tyrosine kinase (PTK) domain, correlating with the loss of function reported in ALSP CSF1R protein variants. Exon numbers are given in rectangles, with the PTK shaded in grey and amino acid (aa) numbers below. Red squares indicate truncating variants, black circles indicate missense and in-frame insertion/deletions and blue triangles indicate splice mutations. Purple and green diamonds represent the two variants identified in this project, ΔA781_N783 and P824R respectively. Adapted from Oosterhof et al. (2019).
1.2.3 Mouse Models Of ALSP

While full knockouts can identify specific and critical roles for CSF-1R pathway components (detailed in 1.3), the disease itself is the result of lifelong haploinsufficiency for CSF-1R throughout development, maturation and ageing. To date, there are two potential mouse models for studying ALSP, the \( \text{Csf1r}^{-/+} \) mouse itself and a mouse with an essential regulatory region deleted from the \( \text{Csf1r} \) gene.

The \( \text{Csf1r}^{-/+} \) mouse displays a milder phenotype than the full knockout, and has an age-related onset of neurological phenotypes, corresponding well to what is observed in ALSP patients. Visuospatial deficits were evident in \( \text{Csf1r}^{-/+} \) mice at 6 months, which were further accompanied by sensorimotor and olfactory deficits in \( \text{Csf1r}^{-/-} \) mice 11 months of age. Interestingly, sex-specific effects were seen with female mice becoming ataxic and males displaying depression and anxiety-like behaviour. Sex-specific effects are not observed in ALSP, with the exception being premature ovarian failure in \( \text{AARS2-L} \) and a single reported case of ALSP, a caveat to this mouse model which warrants further investigation. It may be that these differences haven’t emerged in ALSP due to differences in sex chromosome gene content between humans and mice, or the mouse neural circuitry may be more sensitive to the influences of circulating hormones. Neuroimaging revealed that thinning of the corpus callosum, white matter lesions and enlargement of lateral ventricles correlated with the severity of behavioural deficits, although only male \( \text{Csf1r}^{-/+} \) mice underwent MRI. Despite no change in brain mass or volume, on a cellular level there was an increase in cortical oligodendrocyte progenitor cells (OPCs) and microglia throughout the brain. Cortical layer V neurons in the mouse have an age-dependent increase in CSF-1R expression, and these neurons are known to produce callosal extensions, similarly to the affected CUX1+ neurons detected in tissue from \( \text{CSF1R}^{-/-} \) autopsy samples. In \( \text{Csf1r}^{-/-} \) mice there is selective age-associated neuron loss in this region, and this is also one of the regions with increased OPCs. The increase in microglial numbers, which appeared to display a proinflammatory profile of gene expression but not an ameboid morphology, is an interesting observation. As there was no compensatory increase in either CSF-1R, CSF-1 or IL-34 expression at transcript or protein level, ruling out the presence of a feedback loop attempting to induce overactivation of CSF-1R in the brain. The mechanism by which these excess microglia are being produced and sustained in the absence of one copy of \( \text{Csf1r} \) could be an important difference between the mouse and human brain. There may be a unique reserve in the mouse brain for microglia, similar to the perivascular nestin+ cell population or pluripotent pericytes mentioned in 1.1.3. The multitude of studies utilising CSF-1R inhibition to drive microglial depletion have
only documented a minor subpopulation of microglia which survive independent of CSF-1R activity\textsuperscript{183}, and total knockouts of the CSF-1R pathway components confirms its necessity in regulating microglia. This key phenotype, in conjunction with the sex-specific differences unique to the mouse ALSP model, highlights a deficit in underlying mechanistic data that will be needed before this model can be depended upon for ALSP research.

The second potential mouse model for studying ALSP is one in which an extremely conserved enhancer of Csf1r expression is deleted. The 337 bp fms-intronic regulatory element (FIRE) sequence is located in the second intron of the gene and is considered a super-enhancer of its expression. The FIRE sequence contains binding sites for transcription factors expressed in macrophages, including members of the PU.1, KLF4, CEBP, RUNX1 and AP1 families\textsuperscript{184}. While ALSP-associated mutations in the FIRE sequence have yet to be reported, the enhancer expands transcriptional control of CSF1R and increases its expression. The FIRE sequence is more highly conserved in mammals than any exon of the Csf1r gene\textsuperscript{185} and conserved elements of FIRE are identical across mammals, reptiles and birds. Homozygous deletion of FIRE in mouse embryonic stem cells resulted in a 200-fold reduction in differentiated macrophages, with few or none being produced from embryonic stem cell clones. Csf1r\textsuperscript{ΔFIRE/ΔFIRE} mice have no reproductive or growth defects, have normal tooth and bone development and display no gross brain morphological phenotypes or changes in myelination. Peripheral blood cell populations were unchanged, although Csf1r expression was abolished and CSF-1 binding confirmed to be absent. Microglia, Langerhans cells, and the tissue-resident macrophages of the peritoneum, heart and kidney were indeed found to be absent, with only perivascular Iba1\textsuperscript{+} cells being present within the brain\textsuperscript{186}. These mice may be a model to examine the microglia-specific requirements of the ALSP pathology, with the peripheral immune system intact and available for further modification. Examining the interactions between a microglia-deficient brain and a peripheral system with sequentially removed immune components could potentially facilitate the discovery of the peripheral immune cell-neuron link observed in ALSP. It may be that in the absence of microglia, and a source of local inflammation and macrophage activation within the CNS, peripheral Csf1r loss is tolerated, allowing these mice to be strong negative controls for the microglia-mediated effects of ALSP.

1.2.4 Treatment Strategies For ALSP

CNS-targeted treatment for ALSP will require a knowledge of the cellular aetiology of the pathology of ALSP; how CSF1R heterozygosity leads to the spectrum of neurological ALSP pathologies and why it is an age-related disorder are critical questions left unanswered. The
current treatment approach was discovered entirely by accident due to a misdiagnosis of a case of ALSP. An individual in an ALSP kindred was diagnosed with adult-onset metachromatic leukodystrophy, a disorder which has been shown to benefit from haematopoietic stem cell transplantation (HSCT). The patient received HSCT with her unaffected brother acting as the donor. Disease deterioration was halted within 6 months of the procedure, with the individual retaining language function and personality even 15 years later. A follow-up study of the kindred identified a mutation in \textit{CSF1R}, carried by the original patient, her mother and 3 other siblings. The brother who was the original donor for the HSCT was not a carrier of the mutation. The affecting siblings displayed classical ALSP clinical onset in their fifth decade, and two of the three had passed away at 6 years following the onset of symptoms. The HSCT recipient showed remarkable stability and didn’t develop depression, seizure activity or parkinsonism, even with only 15 \% donor chimerism at the 15-year mark. Although the mother also tested positive for the mutation, she displayed no symptoms and was further shown to be mosaic for the mutation. This would correlate with reported carriers of hypomorphic \textit{CSF1R} alleles having a reduced or absent ALSP pathology.

This initial finding opened up a new avenue for the treatment of ALSP, focusing on the peripheral immune system rather than targeting the CNS itself. A follow-up study of two ALSP patients who underwent HSCT further supported it as a treatment option for the disease and included neuroimaging and assessment of cognitive and ambulatory function. In both cases, there was an initial worsening of the condition and corresponding increases in hyperintensities detected by FLAIR following HSCT. Acute decreases in ambulatory and cognitive function were detected in one case, although both patients had displayed increased function by the 10-month follow-up. It is worth mentioning that human leukocyte antigen matching of the HSC graft was only haploidentical for the first patient, and this may have impacted the efficacy of the treatment. Neuroimaging for both cases showed a decrease in FLAIR hyperintensities and white matter lesions detected with diffusion-weighted imaging (DWI) in early follow-up, with complete resolution of diffusion abnormalities by 28 months. This improvement, from a previously unhaltable disease progression, highlights a prominent role of the peripheral immune system in CNS homeostasis. As discussed later in Chapter 3, replacement of CNS macrophages by peripheral cells requires a disruption of the BBB, either from irradiation or other sources. While one patient in this follow-up study did receive low level total body irradiation, it does not explain the efficacy of treatment in the second individual or indeed in another report of the beneficial outcome of HSCT in ALSP. An underlying BBB dysfunction, or a loss in BBB integrity in response to other treatment compounds, may be essential for this treatment. The possibility exists that targeted
BBB disruption could enhance efficacy by enhancing macrophage repopulation; as CSF1R expressing neurons are not being replaced by this treatment, a critical unidentified leukocyte function is being restored and having dramatic rescue effects within the ALSP brain.
### Summary of Clinical Findings of ALSP

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<thead>
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<tbody>
<tr>
<td><strong>Median Age of Onset</strong></td>
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<tr>
<td><strong>Median Age of Death</strong></td>
<td>50.5 (24-75)</td>
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**Sex (%)**

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<thead>
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<tbody>
<tr>
<td>Male</td>
<td>43.8</td>
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<tr>
<td>Female</td>
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**Predominant Symptoms (%)**

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<td>Motor</td>
<td>73</td>
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<tr>
<td>Gait</td>
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<tr>
<td>Incontinence</td>
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**Neuroimaging Patterns**

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<tbody>
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<td>Symmetry of Disease</td>
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<tr>
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<tr>
<td>Frontal</td>
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</tr>
<tr>
<td>Parietal</td>
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<tr>
<td>Diffuse</td>
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<tr>
<td>Periventricular White Matter Lesions (%)</td>
<td></td>
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<td></td>
<td>97.4</td>
</tr>
<tr>
<td>Subcortical U fibres</td>
<td>30</td>
</tr>
<tr>
<td>Atrophy (%)</td>
<td>96.3 (moderate – severe scoring)</td>
</tr>
</tbody>
</table>

**Table 1.1:** Summary of Clinical Findings in ALSP, adapted from Lakshmanan *et al.* 2017.

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1.3 Colony Stimulating Factor 1 Receptor

The colony stimulating factor 1 receptor, also known as CD115 and c-fms, is a class III receptor tyrosine kinase expressed at the membranes of myeloid cells, microglia, neural progenitor cells and subsets of neurons and endothelial cells. CSF-1R is activated via common structural principles by two distinct cytokine ligands, namely colony stimulating factor-1 (CSF-1) and interleukin-34 (IL-34)\textsuperscript{190–193} which are both essential for microglial viability, development and proliferation\textsuperscript{194–196}. Interestingly, human CSF-1 can bind mouse CSF-1R but the cross-reactivity does not occur in the opposite scenario and IL-34 displays no cross-reactivity between human and mouse\textsuperscript{197}. CSF-1R is also critical for myeloid lineage cell differentiation, as well as that of peripheral monocytes into circulating and tissue-resident macrophages\textsuperscript{186,198}. While CSF-1R is expressed in some cells of the adult brain under homeostasis, namely microglia, it and its ligands have been shown to be upregulated and neuroprotective in mouse models of AD and epilepsy\textsuperscript{199,200}. In addition to CSF-1R signalling being activated in response to CNS insult, IL-34 is secreted by neuronal cells under normal conditions and can enhance neuroprotective effects of microglia in response to stimuli such as oligomeric Aβ\textsuperscript{200}. Research into CSF-1R has become prominent recently due to its role in microglial viability, and the potential treatment strategy of CSF-1R targeting for microglial depletion and resolution of CNS inflammation.

1.3.1 Structure And Expression Of CSF-1R

Class III receptor tyrosine kinases such as CSF-1R and the epidermal growth factor receptor have a conserved structural architecture, including a highly glycosylated extracellular domain (ECD) consisting of five immunoglobulin-like domains which interact directly with bound ligands. This domain is linked via a transmembrane helix to an intracellular domain (ICD), which includes the split cytosolic tyrosine kinase region. Receptor activity is suppressed by the combined actions of an autoinhibitory conformation of the ECD and the intracellular juxtamembrane domain\textsuperscript{201}. CSF-1 and IL-34 bind their receptor as dimers with both homo- and heterodimerisation reported, although the alternative effects of the CSF-1:IL-34 heterodimer are still yet to be defined\textsuperscript{202}. Ligand binding occurs at the second and third extracellular IgG domain, with both CSF-1 and IL-34 having common binding sites in the second domain, despite their sequence dissimilarity\textsuperscript{190}. Binding at the third IgG domain is slightly stronger in the case of IL-34, which correlates with the reported higher binding affinity of IL-34 for CSF-1R\textsuperscript{203}. CSF-1R itself homodimerises via residues in the fourth extracellular IgG domain, and mutations in these residues can drive the transition of CSF-1R into its oncogenic isoform\textsuperscript{192}.
Due to difficulties with immunohistochemical staining of CSF-1R in tissue\textsuperscript{199}, the expression patterns reported thus far have mainly originated from FACS and reporter mouse data. In the hematopoietic cell population, CSF-1R expression is limited to the myeloid lineage, with hematopoietic stem cells (HSCs) having quite low CSF-1R expression due to inhibition by the MafB transcription factor\textsuperscript{204,205}. CSF-1R is transcriptionally activated in common myeloid precursor (CMP) cells and its expression stabilises as these CMPs further differentiate into monocytes, macrophages, myeloid dendritic cells, Langerhans cells and osteoclasts. Outside of the myeloid lineage, CSF-1R is also highly expressed in microglia and Panth cells\textsuperscript{198,206,207}, and it is actively involved in the development of each of these cell types. Although not required for their differentiation, CSF-1R’s pleiotropic activities have been documented in a range of other cell types. CSF-1R expression regulates neural stem cells (NSCs), oocytes and the developing embryo\textsuperscript{208}, as well as a variety of epithelial subtypes, including those of the renal proximal tube and colonic epithelial cells\textsuperscript{199,209,210}. Due to its role in innate immunity, tissue repair and inflammation, CSF-1R expression is not constitutive in many of these cells, but rather is upregulated in response to damage, infection or during a window in development. Within the adult brain, CSF-1R expression has been detected in the choroid plexus of the lateral ventricle, and in hippocampal and cortical neurons with expression increasing following injury with kainic acid\textsuperscript{182,199,209}.

The \textit{Csf1r} knockout mouse displays a vast range of phenotypes due to a loss of myeloid cell development. \textit{Csf1r}\textsuperscript{-/-} mice have reduced fertility and viability, with mice dying within days or 3 weeks of birth depending on the strain background. Knockouts have a reduced body size, truncated limbs, are deaf, toothless, and have impaired olfaction and severe osteopetrotic defects due to a lack of osteoclasts and osteoblasts\textsuperscript{211,212}. \textit{Csf1r}\textsuperscript{-/-} mice also have a 20-fold higher serum CSF-1 concentration than WT littermates, likely due to a compensatory upregulation of \textit{Csf1} expression. This increase of circulating CSF-1 could also be the product of attenuated \textit{CSF-1:CSF-1R} mediated governance of \textit{Csf1} expression, in combination with the loss of \textit{CSF-1R} mediated internalisation and degradation of CSF-1. Mononuclear lymphocyte and marrow HSC populations were severely reduced in \textit{Csf1r}\textsuperscript{-/-} mice, although there was an apparent compensatory increase in splenic macrophage production. When compared to \textit{Csf1}\textsuperscript{op/op} mice, which have a sporadic null mutation in both copies of \textit{Csf1}, \textit{Csf1r} knockouts have an exacerbated phenotype, and this led ultimately to the discovery of IL-34. Cerebral alterations in the \textit{Csf1r}\textsuperscript{-/-} mouse were not investigated until nearly 10 years after its discovery when the role of CSF-1R in microglial maintenance came to light. The brains of \textit{Csf1r}\textsuperscript{-/-} mice are smaller but denser than littermate controls and have enlarged lateral ventricles. As CSF-1R is expressed in NSCs, this might
suggest a role in subventricular neurogenesis, however, both pharmacological and genetic inhibition of CSF-1R has been shown to have no effect on adult sub-ventricle zone neurogenesis\textsuperscript{186,213}. Strikingly, there was an increase in cortical neural progenitor cell (NPC) proliferation, cortical cell apoptosis and the microglial population was about 6\% of that seen in controls\textsuperscript{212}.

The phenotypes observed in the brain of the $Csf1r^{-/-}$ mouse may be due to either direct dysregulation of neuronal cells, or a loss in microglial mediated CNS homeostasis. To discern which cell type was responsible, a nestin$^+$ cell-specific $Csf1r$ knockout was generated using the Cre-LoxP system\textsuperscript{207}. Loss of $Csf1r$ expression in these neuronal progenitor cells resulted in a milder phenotype then what was observed in the global $Csf1r$ knockout. Ventricle size, microglial number and brain mass were identical to WT controls, although brain size was still reduced in the nestin-specific $Csf1r$ knockouts\textsuperscript{207}. Not surprisingly, the increase in NPC proliferation and cortical apoptosis observed in the global knockout was also present in these mice, confirming that CSF-1R within the NPCs themselves regulates proliferation, rather than a secondary regulatory effect of microglia. Treatment of WT forebrain NPC cultures with IL-34 or CSF-1 confirmed that CSF-1R activation suppressed NPC proliferation and induced differentiation.

Few investigations into endothelial expression of CSF-1R have been performed, and there is a complete lack of investigations using reliable CSF-1R reporter mice in place of immunohistochemical approaches. Treatment of CNS endothelial cell cultures with IL-34 has been shown to stabilise tight junctions following co-treatment with TNF-$\alpha$ and IL-$1\beta$\textsuperscript{214}, and its expression was detected in the vessels of the endothelia of the mouse brain and spinal cord. This study also confirmed CSF-1R expression in primary mouse endothelial cell cultures using an immunohistochemical approach, albeit not using the commercial antibodies previously shown to have off-target specificity. The Human Protein Atlas reports both detection and non-detection of endothelial CSF-1R in human cortex\textsuperscript{215}, highlighting the potentially dynamic nature of endothelial CSF-1R expression, although the caveat remains that IHC detection of CSF-1R in tissue can be unreliable. Indeed, the Human Protein Atlas database itself gives this data an “Unreliable” score. As such, the current literature is still undecided as to whether CNS endothelial cells have CSF-1R expression, and whether that expression is constitutive or limited to inflammatory conditions.

1.3.2 Colony Stimulating Factor 1

Colony stimulating factor 1, also known as macrophage colony stimulating factor (M-CSF), exists in two main forms. Circulating soluble CSF-1 (sCSF-1) exists as either a glycoprotein or
proteoglycan and can carry out distal activation of cells while the transmembrane glycoprotein (mCSF-1) isoform performs local activation of CSF-1R on neighbouring cells\textsuperscript{216}. Furthermore, membrane CSF-1 isoforms, as well as membrane-bound CSF-1R, can be cleaved by TNF-\(\alpha\) converting enzyme to generate shed forms of CSF-1 and CSF-1R\textsuperscript{217,218}. Differing activities of sCSF-1 and mCSF-1 have been reported, suggesting that sensitivity to changes in CSF-1 levels may differ between cells reliant on circulating or locally produced CSF-1. Restoration of mCSF-1, but not sCSF1, was sufficient to rescue the skeletal phenotypes observed in Csf1 knockout mice\textsuperscript{219}. Another unique role for mCSF-1 was found in hematopoietic progenitor cells (HPCs), with mCSF-1 capable of supporting self-renewal and long-term proliferation, while sCSF-1 instead drove differentiation into monocyte and macrophage lineages\textsuperscript{220}. In terms of CNS macrophage regulation, there have been few reports on differential effects of CSF-1 isoforms. One observation was a striking opposing role for sCSF-1 and mCSF-1 in macrophage activation in a mouse model of Charcot-Marie-Tooth (CMT) neuropathy. By breeding a connexin32-deficient mouse model of CMT neuropathy onto a Csf1\textsuperscript{op/op} background, and then crossing the resulting strain with ones expressing specific Csf1 isoforms generated through alteration of CSF-1 cleavage sites, it was possible to selectively re-introduce either CSF-1 isoform and examine effects on peripheral nerve inflammation. Restoration of either isoform was equally capable of recruiting macrophages to the diseased nerve, likely due to the proteolytic cleavage of mCSF1 to allow it to circulate. Isoform-specific effects were observed following macrophage recruitment, while sCSF-1 induced local macrophage activation and disease progression, mCSF-1 inhibited activation and reduced the neuropathy\textsuperscript{221}. Although IL-34 is the predominant CSF-1R ligand expressed in the adult CNS, CSF-1 isoforms may have an effect in the response to macrophage recruitment to the CNS, in particular the BBB. Circulating CSF-1 has been reported to have a half-life of 10 min, with 94\% being cleared by CSF-1R receptor endocytosis and intracellular destruction in the liver\textsuperscript{222}. It may be that mCSF-1 expression acts to inhibit accidental macrophage activation, while the release of sCSF-1 serves to induce the innate immune system following infection or injury.

The Csf1\textsuperscript{op/op} mouse, homozygous for loss of function mutations in Csf1, shares many phenotypes with the CSF1R\textsuperscript{−/−} mouse. Due to retained Il34 expression, Csf1\textsuperscript{op/op} mice have a less severe postnatal lethality, and ventricle size and cortical rates of apoptosis are unaffected. Microglia also appear to tolerate the loss of Csf1, with retention of 63\% of the cortical microglial population although these microglia do display an overall reduced soma size and processes length\textsuperscript{223}. IL-34 expression has been reported to be absent from the adult mouse cerebellum, with IL-34 expression primarily in the forebrain and hippocampus, while CSF-1 expression was higher in the cerebellum\textsuperscript{224}. In fact, IL-34 and CSF-1 have been demonstrated to differ in function
in the white and grey matter of the brain, with treatments of either CSF-1 or IL-34 blocking antibodies depleting white and grey matter respectively\textsuperscript{225}. In the context of ALSP this is of note as the white matter of the brain is primarily affected, with the cerebellum spared throughout the disease progression. As with Csf1r, a nestin\textsuperscript{*} cell-specific Csf1 knockout has been examined to identify effects of neuron-specific Csf1 expression. Mice with Csf1 inactivation in nestin\textsuperscript{*} cells had regular macrophage development in the forebrain, although 60 \% of cerebellar microglia were depleted\textsuperscript{226}, mirroring the loss observed in Il34 deficient mice. Crossing Il34 deficient mice with nestin\textsuperscript{*} cell-specific Csf1 knockout mice resulted in a near-total depletion of brain microglia, with only a small population of cerebellar microglia surviving, likely due to retained Csf1 expression in non-nestin expressing cells. Temporally, microglia were depleted in the forebrains of these mice during embryonic development, however, the forebrain population had recovered by P23. This, in conjunction with the normal microglial development in embryonic Il34 deficient mice, indicates a switch from CSF-1 to IL-34 as the primary maintenance cytokine for forebrain microglia with age.

1.3.3 Interleukin-34

The discrepancies between observed phenotypes in Csf1 and Csf1r-deficient mice indicated that there was an unknown factor that could drive CSF-1R activation in the absence of CSF-1. A proteomics-wide approach was used to identify a secondary secreted cytokine that could activate CSF-1R, as well as its two other binding partners\textsuperscript{227}. IL-34 is a glycoprotein which displays a high level of amino acid sequence conservation between human, chimp (99.6 \%) and mouse (71 \%). IL-34 appears to have higher evolutionary conservation than CSF-1 outside of mammalian clades, with a readily alignable 60 \% sequence similarity between human and avian IL-34 sequences in contrast to the limited 30 \% conservation of CSF-1\textsuperscript{228}. In avian species, there also appeared to be an evolutionary constraint on IL-34 sequence deviation, with evidence of negative selection for variant IL-34 without a corresponding change in CSF-1R itself. This would mean that CSF-1R and IL-34 were co-evolutionary partners while CSF-1 appeared to have more permissive sequence changes. Surprisingly, IL-34 does not share any sequence similarity with CSF-1, or any known growth factor, but can bind CSF-1R with a higher affinity and longer duration than CSF-1 itself\textsuperscript{192}.

Apart from CSF-1R, there are two other binding partners of IL-34, receptor-type protein-tyrosine phosphatase-zeta (PTP-ζ) and the chondroitin sulphate chains of syndecan-1\textsuperscript{229,230}. Syndecan-1 can modify the bioavailability of IL-34 by sequestering extracellular IL-34 to the cell membrane and promoting interaction with CSF-1R. IL-34 was found to promote the migration of monocyte and macrophage cell lines in a syndecan-1 dependant manner. Regardless of retained
CSF-1R expression, treatment with an anti-syndecan-1 antibody could inhibit the migratory response of these cells to IL-34. PTP-ζ is expressed as either a chondroitin sulphate proteoglycan or non-proteoglycan form on multiple cancer tissues, as well as NPCs and glial cells. Binding of ligands results in its deactivation, increasing tyrosine phosphorylation of its substrates paxillin and focal adhesion kinase, potentially including CSF-1R itself in cell types co-expressing both receptors such as NPCs. Polymorphisms in the PTPRZ1 gene have also been found to be associated with schizophrenia. Whether this effect of PTPRZ1 mutation is due to interactions with the IL-34:CSF-1R signalling axis has yet to be determined as PTP-ζ is also expressed on neurons, allowing it to modulate the activity of sodium-gated ion channels and co-localise with the neuregulin receptor. As with CSF-1, IL-34 has recently been found to display membrane-bound expression through localisation dependant on the 78-kDa glucose-regulated protein (GRP78). Follicular dendritic cells (FDCs) can induce monocyte-like cell production from splenocyte cultures through IL-34 expression, and CSF-1R inhibition of splenocytes completely inhibits monocyte production. In FDCs heterozygous for GPR78, membrane expression of IL-34, as well as their ability to induce splenocyte differentiation, was significantly reduced.

Unlike Csf1op/op and Csf1r deficient mice, Il34 knockout mice lack many of the phenotypes observed to be due to disruption of the CSF-1R signalling pathway. Loss of Il34 does not affect viability, fertility, bone morphogenesis or circulating myeloid cell populations with only microglia and Langerhans cells beings depleted. IL-34 is required for the development of these Langerhans cells, and as mentioned previously, Il34 and Csf1 have non-overlapping expression apart from the hippocampus in the adult brain and appear to maintain distinct populations of microglia. During development, Il34 expression is observed at E11.5, and its expression is nearly 10-fold higher in the developing brain than that of Csf1. No compensatory changes in Il34 expression were observed in the Csf1op/op mouse, indicating distinct transcriptional regulation for the two ligands of CSF-1R. Il34 driven by the Csf1 promoter was able to rescue the Csf1op/op phenotype, suggesting that differences between the Il34 knockout and Csf1op/op mouse are the product of spatial and temporal regulatory cues rather than differing biological activities.

1.3.4 CSF-1R Activation And Processing

Inactive CSF-1R at the cell membrane undergoes a rapid transition between being in a monomeric or dimeric state, requiring ligand binding to induce a conformational change which leads to phosphorylation of eight tyrosine (Tyr) residues of the ICD (Figure 1.6). Ligand binding initiates an early wave of tyrosine phosphorylation, recruiting signalling proteins and inducing downstream activation of proliferation, migration, differentiation and cell survival pathways. The
formation of covalent disulphide bridges between ECDs of CSF-1R molecules then activates a secondary set of tyrosine and serine (Ser) phosphorylation events. This secondary event induces multiubiquitination by recruited ubiquitin ligases, inducing the internalisation of the ligand-bound receptor and targeting it for lysosomal degradation. Two main complexes form independently during CSF-1R activation, a more transient CSF-1R/Grb2/Sos complex and a CSF-1R/c-Cbl/phosphatidylinositol-3 kinase subunit 85 PI3K(p85α)/Shc/Grb2 complex which interacts with other signalling molecules. Recruitment of further signalling complexes occurs differentially at each phosphotyrosine residue, and identification of critical tyrosine residues has been carried out through the generation of CSF-1R variants with individual or multiple tyrosines replaced with structurally similar phenylalanine. As phenylalanine cannot be phosphorylated, this will result in CSF-1R variants with unaffected protein folding but a loss of tyrosine-specific signalling capacity. A similar approach is to replace all tyrosines involved in downstream signalling with phenylalanine and sequentially restore individual residues to examine which are critical and which display overlapping redundancy.

In the early stage, ligand binding induces transphosphorylation of Tyr-561 in humans (Tyr-559 in mice) which relieves the autoinhibitory control of the juxtamembrane domain and activation loop. Replacing Tyr-559 with phenylalanine (Phe) resulted in a loss of disulphide bridge formation between receptor monomers and a total loss of CSF-1R phosphorylation and ubiquitination. In the same study, the c-Cbl E3 ubiquitin ligase was demonstrated to be a critical partner for full CSF-1R phosphorylation. Tyrosine phosphorylation of c-Cbl activates its ubiquitin ligase activity, and in the context of CSF-1R, this was shown to be dependent on Tyr-599 mediated recruitment of the Src Family Kinase (SKF). The multiubiquitination of both c-Cbl and CSF-1R, inducing internalisation and targeting of CSF-1R for lysosomal degradation, appears to occur later in the signalling cascade following the dissociation of the transient CSF-1R/Grb2/Sos complex. During the early stage, tyrosine-phosphorylated c-Cbl form complexes with CSF-1R, PI3K subunit p85α, Grb2 and weakly with Sos.

CSF-1R signalling also appears to occur within the endosome as internalised receptors are shuttled to the lysosome. Taking advantage of the property that receptor internalisation is severely reduced when cells are stimulated with CSF-1 at 4 °C, it was observed that in the absence of CSF-1R internalisation ERK1/2 and Akt signalling did not occur. An elegant further experiment identified that CSF-1R internalisation was near complete approximately 15 min post-CSF-1 stimulation. Treatment of cells at this time point with GW2580, an inhibitor of CSF-1R,
induced an immediate loss of ERK1/2 signalling, reduced Akt activity and downregulated expression of CSF-1R activated genes, confirming the signalling capacity of internalised CSF-1R.
Figure 1.6: CSF-1R dimerization and conformational change. CSF-1R rapidly transitions between the monomeric (a) and dimeric (b) state. On ligand binding, a conformational change is initiated (c), relieving the autoinhibitory mechanisms of the activation loop (AL) and juxtamembrane domain (JMD). Covalent bonds between monomeric units stabilise dimerisation and initiate further tyrosine phosphorylation and ubiquitination of the intracellular domain (d). Adapted from Stanley and Chitu (2014).
1.3.5 Critical Tryosines For CSF-1R Activity: Survival And Proliferation

Extensive work by Yu et al. spanning two publications provided insights into the roles of the individual tyrosine residues of CSF-1R; Y544, Y559, Y697, Y706, Y721, Y807, Y921 and Y974\textsuperscript{249,250}. By generating an immortalised macrophage cell line from Csf1r\textsuperscript{-/-} bone marrow, it became possible to determine the effects of specific tyrosines of CSF-1R using transfection techniques. CSF-1R variants with individual tyrosines replaced with phenylalanine were retrovirally transfected into Csf1r\textsuperscript{-/-} bone marrow macrophages (BMMs), and then by treating transfected cells with CSF-1, the restored signalling capacity could be attributed to a specific tyrosine residue.

BMM’s can survive in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF, also referred to as CSF-2), so unlike microglia, these cells can grow in the absence of CSF-1R. Loss of Y807, as well as the loss of all eight tyrosines in the so-called YEF CSF-1R mutant, was the only change found to affect rates of cell death in cultures, although the conversion of Y807, Y559 and Y544 did reduce rates of cell proliferation\textsuperscript{250}. BMMs display a rounded, “fried-egg” morphology when grown in the presence of GM-CSF alone, with CSF-1 addition inducing a shift to an elongated morphology. This morphological change involves a restructuring of the cytoskeleton and was absent in cells expressing Y721F and Y974F CSF-1R. The Y706 residue was found to be involved in an inhibitory process regarding morphological changes, as its conversion produced hyper-elongated macrophages. When expression levels of the Mac-1 differentiation marker were examined, the elongated morphology of the Y702F expressing BMMs correlated with a 1.5-fold increase in Mac-1, compared with WT cells. Each of the other variant CSF-1R expressing BMMs had reduced Mac-1 expression, and this was most striking in the case of the Y559F and Y807F variants, which lowered expression to below 30 % of WT levels. Of note was that there was no inverse correlation between reduced Mac-1 and the lack of an elongated morphology of the Y721F and Y974F expressing cells, indicating that CSF-1R activation of Mac-1 expression doesn’t produce this morphological change itself.

Examination of CSF-1R tyrosine phosphorylation found that variants which resulted in reduced cell proliferation, Y544F, Y559F and Y807F, also had drastically reduced levels of tyrosine autophosphorylation. Consistent with findings that Y559 is critical for the recruitment of the c-Cbl ubiquitin ligase, the Y559F mutant had reduced CSF-1R degradation following stimulation with CSF-1. Y559 phosphorylation was also detected in the Y544F and Y807F variants, which retained CSF-1R targeting for degradation despite low levels of overall CSF-1R phosphorylation. These three residues were the focus of a follow-up add-back study, using the YEF mutant which has all eight tyrosines replaced. In this study, each of these three tyrosines were added back to
the YEF variant to determine their individual and interacting rescue capacities. Addition of Y807 resulted in a constitutively active receptor, which drove proliferation and Mac-1 expression independent of CSF-1 treatment. The addition of Y559 restored CSF-1 responsivity, with constitutive activity reduced to near-WT levels and proliferation and Mac-1 expression increased in response to CSF-1. Addition of all three tyrosine residues produced a CSF-1 responsive receptor which displayed CSF-1 induced WT levels of kinase activity, proliferation and 50% of WT Mac-1 expression.

These three tyrosine residues were identified to be critical to driving CSF-1R regulated macrophage proliferation; Y559 governing ligand recognition, Y807 driving receptor activation following CSF-1 governed release of Y559 mediated-autoinhibition, and Y544 enhancing receptor kinase activity. CSF-1R induces this proliferative response in the cell by signalling through the p85α subunit of PI3K and SFKs to activate ERK1/2, ERK5, p38 and Akt downstream signalling. The Y807-PI3K and Y807-ERK1/2 pathways are subsets of, but independent to, the Y559-SFK dependent pathways governing degradation and proliferation. Macrophage survival is also enhanced by activation of these pathways, with Y807 phosphorylation additionally activating the protein kinase C (PKC) pathway. PKC activation can deactivate Fms-interacting protein (FIMS) through its translocation to the cytosol and can work in conjunction with phospholipase C (PLC) to increase cell nutrient uptake.
Figure 1.7: Intracellular signalling of the critical tyrosine residues for macrophage proliferation and survival. CSF-1R signalling output of the essential tyrosine residues required for proliferation and survival (Y544, Y599 and Y807). Y544 phosphorylation enhances the activities of Y559 and Y807.
1.3.6 The CSF-1R Pathway In Diseases Of The CNS

Setting ALSP aside, CSF-1R has been poorly studied in the context of the CNS, rather it is utilised as a molecular tool for microglial depletion. CSF-1R inhibitors have been applied to a multitude of animal models of CNS disorders due to their ability to almost completely reduce the microglial population. Benefits of long term CSF-1R inhibition have been examined in TBI, intracerebral haemorrhage, EAE and neuronal lesion studies\textsuperscript{257-261}. These studies showed no overt focus on CSF-1R itself, but rather utilised it as a tool for macrophage depletion and observed the beneficial outcomes of the absence of inflammation. CSF-1R itself has been found to have a clear role in two main disorders of the CNS - epilepsy and Alzheimer’s disease (later discussed in 1.3.7).

CSF-1R signalling has been found to be neuroprotective in the kainic acid (KA) mouse model of excitotoxicity. This model involves a systemic or stereotaxic injection of kainic acid, an analogue of L-glutamate that can induce potent and prolonged excitatory responses in cortical neurons. KA injection can induce acute seizures within minutes of injection, followed by a latent period of spontaneous and intermittent seizures. KA injection is widely used as a model of temporal lobe epilepsy (TLE), inducing hippocampal neuronal cell death and associated microglial activation and astrogliosis\textsuperscript{262}. In the context of CSF-1R, Luo \textit{et al.} utilised the KA model to demonstrate a neuroprotective role of the CSF-1R pathway. Simultaneous administration of CSF-1 with KA was able to reduce KA-induced astrogliosis, as was CSF-1 treatment at 2 and 6 h post-KA injection. CSF-1 injected mice were protected from hippocampal cell loss and calbindin downregulation, despite undergoing seizure activity similar to controls\textsuperscript{199}. Similar outcomes were observed from IL-34 injection, indicating CSF-1R activation as a means of neuroprotection within the brain. A decrease in KA-induced microglial activation was also observed in the CSF-1 and IL-34 injected mice. To rule out a peripheral macrophage component in KA models, parabiosis was performed using GFP-expressing mice. There was no significant amount of GFP\textsuperscript{+} cells observed in the brain across all treatment groups, including untreated controls. CSF-1 was identified to be upregulated in neurons following KA administration, inversely correlating with neurodegeneration. CSF-1 was also found to be markedly absent in individual animals that did not survive the 6 h period following KA injection, and CSF-1R itself was found to be upregulated in neurons following KA injection. These neuroprotective effects were found to be mediated through CSF-1R activation of cAMP-responsive element-binding protein (CREB) phosphorylation.

CSF-1R has since been identified as a promising target in an \textit{in silico} analyses of dysregulated networks in chronic epilepsy, targeted for its role in microglia rather than neurons\textsuperscript{263}. To confirm these predictions, the pilocarpine model of TLE was used to generate mice which develop chronic
spontaneous seizures. In these mice, treatment with the CSF-1R inhibitor PLX3397 was found to reduce spontaneous seizure activity following the latent period. Mouse brains were examined and confirmed to still contain 50% of wild-type levels of microglia, as PLX3397 was administered at a reduced dose for only 7 days to examine the CSF-1R specific effects, rather than the effects of total microglial depletion. PLX3397 was shown to be ineffective at reducing acute phase seizure activity, lending support to the work of Luo et al. in highlighting the protective effects of CSF-1R in the acute phase of the KA model. Together, this indicates a dual role for CSF-1R in epilepsy. During the onset of epileptogenesis, and perhaps immediately during seizure, CSF-1R activation can have an acute neuroprotective effect, reducing hippocampal cell death through CREB phosphorylation. As the disease progresses to spontaneous seizures, microglial CSF-1R activity may exacerbate the disease through chronically activated inflammatory pathways. With mild seizure being a commonly reported symptom in early-stage ALSP, the loss of this neuroprotective function of CSF-1R may exacerbate ALSP progression.

1.3.7 CSF-1R In Alzheimer’s Disease

It can be difficult to determine the role of CSF-1R pathways in CNS disorders as many of the published studies have reported on its long-term inhibition in the context of microglial depletion. Likely, the beneficial effects observed in mice following this inhibition are not due to a loss in CSF-1R activity itself but rather the absence of CNS macrophages.

CSF-1R has been found to be involved in AD, with conflicting data suggesting it may improve Aβ uptake and clearance, but enhance the pro-inflammatory effects of the protein. In human AD samples, CSF-1R appears upregulated in microglia associating with regions of plaque264. Interestingly, Aβ deposits and plaques have been observed in 30 day-old Csf1op/op mice, mainly in the cerebral cortex but also less frequently in the amygdala and hypothalamus265. A corresponding loss in neurons in the CA1 and CA3 hippocampal regions suggested an AD-like disease progression. The pattern of Aβ deposition was also similar to that observed in CAA, although the investigation was limited and did not provide enough imaging data to confirm the occurrence of CAA in these mice. Csf1op/op mice have reduced numbers of microglia and an IL-34 dependent CSF-1R pathway, and IL-34 has since been found to have neuroprotective effects. In neuron cultures, oligomeric Aβ can induce neurotoxicity through upregulation of ROS production. Although treatment of neurons with IL-34 had no effect, when IL-34 was administered to neuron-microglia co-cultures the neurotoxic effect of oligomeric Aβ was inhibited. This neuroprotection was found to be mediated through increased phagocytic clearance of oligomeric Aβ via upregulation of the Aβ-degrading enzyme IDE and the antioxidative enzyme heme oxygenase-1.
IL-34 injection into the ventricles of APP/PS1 mice similarly improved fear-conditioned behaviour retention and upregulated microglial HO-1 expression. In support of the protective effect of IL-34 against oligomeric Aβ, peripheral administration of CSF-1 is beneficial in the APP/PS1 mouse model. Repeated CSF-1 injections increased cortical and hippocampal microglial numbers, reduced Aβ plaque burden and enhanced the Aβ phagocytic activity of microglia\(^{266}\). Even when administered after the onset of behavioural abnormalities, CSF-1 treated mice had improved behavioural outputs compared to controls. Unlike the direct CNS delivery of IL-34, the authors proposed that the protective effects of CSF-1 were due to stimulation of the peripheral macrophage population, promoting infiltration into the brain and increased phagocytic activity. This enhanced clearance could also be supported by increased resident microglial activity, as CSF-1 has been shown to upregulate microglial phagocytosis \textit{in vitro}\(^{267}\). Systemic CSF-1 injection has also been confirmed to improve cognition in the hAPP mouse model, displaying independence from Aβ burden within the brain and supporting the role of CSF-1R in maintaining cognition\(^{199}\).

As with the previously discussed KA models, this study confirmed a lack of peripheral macrophage infiltration in response to CSF-1 injection, however, this may not apply to animals repeatedly administered CSF-1.

In contrast to these beneficial effects of CSF-1R activation in mouse models of AD, long-term CSF-1R inhibition and microglia ablation has also been shown to be beneficial. In a dual approach to determining the role of CSF-1R in AD, Olmos-Alonso \textit{et al.} first examined cortical human AD tissue and found an increase in grey and white matter microglia numbers\(^{268}\). Microglia also had increased transcription of \textit{CSF1R}, \textit{CSF1} along with markers for a proliferative and pro-inflammatory microglial state such as \textit{CEBPA}, \textit{CD68}, \textit{CXCL8} and \textit{TGFB}. The \textit{CD163} marker for perivascular macrophages and \textit{CCR2} and \textit{CD34} markers of peripheral macrophages were also found to increase with disease severity. Moving to the \textit{APP/PS1} mouse model, a similar overall increase in microglial numbers, and in \textit{Csf1r} expression in plaque-associating microglia, was observed. Targeting microglia through oral administration of the GW2580 CSF-1R inhibitor for 3 months resulted in reduced hippocampal neuron loss and increased function in short term behavioural assays. The anxiety-like phenotype of the \textit{AAP/PS1} mouse was also reduced following GW2580 treatment, and microglial proliferation and activation markers were downregulated. Despite the behavioural improvements in these mice, no change in plaque burden or localisation was observed, and this would later be found to be a common trend for depletion studies. A similar study using the 3xTg-AD mouse model used the CSF-1R inhibitor PLX5526, which can deplete the brain of microglia in chow-fed mice over the course of 3 weeks. In this study, a similar improvement of memory and learning behaviours was observed in PLX5526-fed mice\(^{269}\). Using a
lower dose of PLX5526, a 30% reduction in microglia was possible to sustain and these surviving microglia had reduced association with Aβ plaques. As with the previous study using GW2580, no change in total soluble Aβ levels in the brain was detected, suggesting that the behavioural deficits caused by microglia themselves, or microglial-mediated neurodegeneration, were inhibited rather than the underlying processes leading to Aβ accumulation. This was repeated in 15-month-old 5x-FAD mice using the CSF-1R inhibitor PLX3397, again showing that the reduction in microglia could improve behaviour and reduce neuron loss without affecting Aβ levels in the brain.

Early intervention appears to be the critical factor in reducing Aβ accumulation within the brains of mouse models of AD via CSF-1R inhibition. Treatment of the 5xFAD mouse with PLX3397 at 1.5 months was able to reduce intraneural Aβ and neuritic plaque, while also improving mouse behavioural function. Using the same mouse model, Spangenberg et al. were able to show that removal of microglia prior to plaque formation could prevent further Aβ accumulation. This paper was also the one to demonstrate the plaque-to-vessel shift in Aβ deposition in the absence of microglia. Critically, a repopulation study was performed in which PLX5562 was removed from the chow and microglia allowed to recover in the brain. In brains with repopulated microglia, Aβ plaques began to develop to the same extent as in untreated mice. The vascular accumulation of Aβ was maintained even in the presence of restored microglia, supporting their role in preventing CAA occurrence, but not in clearing vessel-associated Aβ accumulations. Through seeding parenchymal Aβ plaques, microglia may effectively protect the CNS vasculature at the expense of the neuron.

Not all microglial depletion studies have found it to be beneficial, indicating a necessary consideration of the mouse models being used in each study. Using 12-month old APP/PS1 mice, Unger et al. found that not only was long-term PLX5562 treatment ineffective at changing the Aβ burden within the brain, but it also had no effect on learning in the mice. It could be that these mice had developed an AD pathology beyond the rescue capacity of the treatment. These results were highly conflicting when compared to previous microglia elimination studies using the same mouse model at 3 and 6 months of age. This study also revealed an Iba1+/TMEM119+ microglia-like population that were CSF-1R independent, resisting the ablation process. These cells stained positively for CSF-1R and displayed high CD44 expression, indicative of a peripheral origin which may explain their resistance to PLX5562. The lack of behavioural improvement in these mice is an important observation, removal of microglia alone is not enough to ablate the cognitive deficits of AD in this model.
The timing of microglia-focused therapies, as well as the genetic architecture of the AD pathology, were both revealed to have a large impact on this approach to treatment. With Aβ plaque accompanying the return of microglia to the brain, the use of CSF-1R or microglial inhibitors will likely be a long-term treatment plan should it be translated to humans. Early diagnosis and initiation of treatment will be critical, although it is unknown how long-term CSF-1R inhibition affects cerebral homeostasis and susceptibility to CNS injury. Without microglia, the brain is left vulnerable to pathogen invasion or physical injury as Alzheimer’s disease patients are unlikely to experience the controlled environment common to rodent studies. The concept of pausing AD progression through lifelong microglial depletion, halting the disorder but not treating its underlying driving factors, may prove ineffective as a feasible treatment for human AD patients. The increase in cognitive function following CSF-1 administration also indicates a requirement for CSF-1R signalling within the neurons themselves. A focus on driving beneficial CSF-1R driven microglial and peripheral macrophage function, in place of their eradication, may yield further potential immunotherapies.

1.4 Objectives of this project

The BBB is involved in CNS disorders including schizophrenia, Alzheimer’s disease, epilepsy and MS. Its regulatory role of the CNS microenvironment and immune cell trafficking into the brain makes it an ideal aetiological candidate for progressive diseases. The sensitivity of the brain to vascular dysfunction would indicate that a dysfunctional BBB could drive or exacerbate early symptoms of CNS disorders.

Given the scarcity of research published on the role of CSF-1R in maintaining the CNS vasculature, it was decided that an investigation into CSF-1R and the BBB in the context of ALSP was warranted. As the biochemical impacts of the novel CSF1R variants explored in this project were unknown, an initial characterisation of variant CSF1R expression and protein function was required. Both in vitro analyses and analyses using human donor ALSP tissue were used to attempt to determine a possible aetiology for the disease. With little published on CSF-1R in endothelial cells themselves, we further sought to determine a role for CSF-1R in endothelial cell function, namely tight junction maintenance. In vitro endothelial cell lines as well as isolated primary endothelial cells functions were examined following CSF-1R suppression and activation. To assess these endothelial-specific effects in vivo, endothelial-specific Csf1r+/− mice were developed using the Cre recombinase/LoxP system. Furthermore, as CSF-1R has a characterised role in microglial biology, potential CSF-1R dependent cross-talk between microglia and
endothelial cells was also investigated. \textit{In vitro} cell culture approaches were used, in combination with a macrophage-specific \textit{Csf1r}\textsuperscript{+/−} mouse generated in tandem with the endothelial strain.

In summary, the overall objectives of this project were to:

1. Characterise cerebrovascular pathology associated with ALSP causative mutations in \textit{CSF1R}.
2. Validate a role for CSF-1R in CNS endothelial cell homeostasis.
3. Examine the macrophage – endothelial crosstalk in the context of CSF-1R manipulation.
Chapter 2:
Materials and Methods
2.1 Cell Line Culture, Maintenance And Treatment

2.1.1 Cell Culture And Passaging

Unless otherwise stated, all cell lines used during this work were grown in Dulbecco’s modified Eagle’s medium containing 4500 mg/L glucose, GlutaMAX and 110.0 mg/mL sodium pyruvate (DMEM, Gibco) supplemented with 10 % v/v foetal bovine serum (FBS, Sigma) in a 5 % CO2 incubator at 37 °C. Passages are defined as cells subcultured from a previously grown flask and seeded in a new flask with fresh growth media. Human embryonic kidney epithelial (HEK293) cells sourced from ATCC and grown in T75 flasks to 80 % confluency before passaging. Cell culture media was removed, and cells washed twice with 5 mL Dulbecco’s phosphate-buffered saline (DPBS, Gibco) before the addition of 2 mL Trypsin-EDTA (Gibco). Flasks were returned to the incubator for 3 min to facilitate trypsinisation, tapped vigorously and 10 mL DMEM added to inactivate the trypsin. Cells were suspended in DMEM, centrifuged at 1,000 rpm for 5 min and the supernatant was removed. Following resuspension in DMEM, cells were split at a 1:10 dilution and seeded into T75 flasks in 12 mL of media. The human cervical epithelial (HeLa) cell line purchased from ATCC was cultured identically, and the mouse brain endothelial cells (bEnd.3, ATCC) were cultured similarly but seeded at a 1:5 dilution. The mouse BV2 microglial cell line kindly provided by Dr Marina Lynch (TCIN, Trinity College Dublin) was grown in T75 flasks and passaged upon reaching 80 % confluency. Growth media and suspension cells were removed, and adherent BV2 cells gently rinsed with 5 mL DPBS. Cells were gently detached using cell scrapers (VWR) and flasks rinsed with DMEM and cells centrifuged at 1,000 rpm for 5 min. The cell pellet was resuspended, and cells were split at a 1:20 dilution, seeding resuspended cells in 3 mL DMEM, 10% v/v FBS per T75 flask to promote cell attachment. Flasks were gently flooded with 20 mL DMEM, 10% v/v FBS at 3 h post-seeding.

2.1.2 Cell Counting And Cryopreservation

Cell counting was performed using the LUNA Automated Cell Counter (Logos Biosystems) as per the manufacturer’s instructions. Briefly, suspended cells were diluted 1:2 with Trypan Blue (Sigma) and 12 μL pipetted into a LUNA cell counting slide (Logos Biosystems). The focus was adjusted as appropriate to ensure cells were in view and cell seeding calculations performed using the cell concentration / mL output. For cryopreservation, cells were frozen in culture media containing 50 % v/v FBS, 10 % v/v DMSO (Sigma). Cells were diluted to approximately 2x10⁶ cells/mL in culture media, and 1 volume of FBS, 20 % DMSO was slowly added with gentle swirling to reduce cytotoxicity before freezing cells using isopropanol-filled Mr Frosty (Thermo Scientific)
cell coolers at -80 °C to slowly reduce the temperature of the cell suspension at the rate of -1 °C/min. Cells were rapidly thawed out of cryopreservation by incubating cryovials of cells in a water bath at 37 °C. Once thawed sufficiently to be decanted into 10 volumes DMEM, cells were centrifuged as previously, resuspended in DMEM and seeded into one T75 flask following the adherence promoting protocol in the case of the BV2 cell line.

2.1.3 Cell Transfection By Viromer Blue And Lipofectamine 2000 Reagents

For transfection of CSF1R expressing constructs (detailed in 2.5), HEK293 or HeLa cells were seeded in 12-well plates at 2.5x10^5 cells/well in DMEM and allowed to grow for 24 h. Transfection complexes were produced using Opti-MEM media (Gibco) and Lipofectamine 2000 (Invitrogen) as per standard protocol. Control wells were treated with Lipofectamine 2000 in the absence of plasmid. Briefly, for each well to be transfected, 500 ng of pcDNA3-EGFP plasmid containing native or variant CSF1R was incubated in 50 μL Opti-MEM and incubated at room temperature for 5 min. Separately, 1.5 μL Lipofectamine 2000 was also incubated in 50 μL Opti-MEM for 5 min at room temperature. The plasmid-Opti-MEM mixture was then added to the tube containing diluted of Lipofectamine 2000 and inverted several times before being incubated for a further 20 min at room temperature. 100 μL of Opti-MEM containing plasmid-Lipofectamine 2000 complexes was then added dropwise to each well, gently tilting the plate to encourage mixing into the culture media. Cells were returned to the incubator to grow before lysis or use in further experiments.

For transfection of Csf1r targeting small-interfering RNA (siRNA), the transfection reagent Viromer Blue (BioNTech) was used due to the difficulty of transfecting with the BV2 cell line. One day pre-transfection, 1x10^5 BV2 cells were seeded onto 1.3 cm plastic tissue culture treated coverslips (Sarstedt) in a 24-well plate. For each well to be transfected, 0.5 μL of Viromer Blue transfection reagent was pipetted directly onto the wall of a plastic 1.5 mL tube, followed by 45 μL of Viromer Blue buffer. Separately, Csf1r targeting siRNA (siGENOME Mouse Csf1r SMARTpool, Dharmacon) or scrambled non-targeting control siRNA (Silencer™ Select) was diluted to 2.75 pmol/μL and 5 μL of diluted siRNA added to the tube containing diluted Viromer Blue reagent. The tube was inverted several times and incubated at room temperature for 15 min, after which 50 μL was added dropwise to the culture media of each well.

2.1.4 CSF-1R Expression, Signalling And Turnover

To examine the expression of native and variant CSF1R HEK293 and HeLa cells were transfected as in 2.1.3 and grown for 24 and 48 h before being lysed for protein analyses.
For signalling experiments, culture media was removed from cells 24 h post-transfection, cells were rinsed once with serum-free DMEM (sfDMEM) and incubated in sfDMEM for 16 hours. Recombinant human IL-34 (R&D Systems) or CSF-1 (Kindly provided by Dr Savvas Savvides, VIB Ghent) was diluted to 10, 50 or 100 ng/mL in sfDMEM and cells were incubated in ligand for 10 or 30 min. At the indicated timepoint, media was removed, and cells were washed once with PBS on ice before lysis as described in 2.1.10.

For treatment with protein turnover regulators, cells were treated 24 h after transfection with rapamycin (0.1, 0.5, 1, 5 and 10 μM, Sigma), 3-MA (0.1, 0.2, 0.5, 1 mM, Sigma) or MG132 (0.1, 0.5, 1, 5 μM, Sigma). Cells were lysed as above at 24 h post-treatment for rapamycin and 3-MA and 7 h post-treatment for MG132. Each replicate was performed on cells of a different passage number, at least one week apart and involved separate transfection and treatments (n = 3).

For MG132 pre-treated signalling experiments, 12 hours after the media was changed to sfDMEM, cells were treated with 5 μM MG132 for 1 h before addition of 10, 50 or 100 ng/mL CSF-1 for 10 min before lysis.

### 2.1.5 Cell Stress And Toxicity Microarray

The P824R variant was selected for use in the array, and HEK293 cells were transfected as in 2.1.3 and lysed at 24 h post-transfection in TRK lysis buffer. Three wells of HEK293 cells were transfected per CSF1R variant, and RNA was isolated as detailed in 2.1.10 with an on-column DNase I digest included. RNA from each triplicate was pooled in equal nanogram amounts, and cDNA was synthesised using the RT² First-Strand Synthesis Kit (Qiagen) as per the standard product protocol, with secondary DNase I digest and genomic DNA elimination steps included. Native and P824R transfected cell cDNA were analysed using the RT² Profiler™ PCR Array Human Stress & Toxicity PathwayFinder (Qiagen) per manufacturer’s instructions, with cDNA samples run on three individual array plates each to generate n = 3 per condition. Data analysis was performed in Excel using the macros provided by Qiagen for this assay, and additional heat maps were produced in GraphPad Prism 8.0.

For later validation of heat shock protein and CXCL8 expression, both HEK293 and HeLa cells were transfected with native, ΔA781_N783 or P824R CSF-1R-expressing plasmids and both RNA and protein were isolated at 24 h post-transfection. Media was collected for later ELISA analysis (2.10) and cell lysates were analysed by western blot for markers of cell stress (Hsp90, Hsp40, GAPDH for loading control). Additionally, RNA was isolated and cDNA synthesised as in 2.9.1 for quantitative PCR (qPCR) analyses of CXCL8.
2.1.6 Generation Of BV2 Conditioned Media

For the generation of BV2 conditioned media (BV2-CM), mouse microglial BV2 cells were plated in 1 mL of DMEM at 1.25 x 10^6 cells per T25 flask. Flasks were flooded with 12 mL DMEM 2 hours later. At 24 h post-seeding, media was refreshed and BV2 cells grown for 24 h to generate conditioned media. Control media was placed in an empty T25 and incubated for the same duration. To determine the effects of CSF-1R signalling in the BV2 cells during media conditioning, PLX3397 (Plexicon) was added at 20 or 40 μM to flasks for the production of CSF-1R-inhibited BV2-CM. PLX3397 is a competitive inhibitor of CSF-1R, binding the autoinhibited confirmation at the juxtamembrane region, preventing receptor activation. Conditioned media was centrifuged at 1,000 rpm for 5 min, the supernatant passed through a 0.2 μm sterile filter and added to endothelial cultures. BV2-CM was prepared fresh for each replicate.

2.1.7 bEnd.3 Cell Treatments

Treatments were performed on confluent monolayers of bEnd.3 cells, with the exception being transfection of Csf1r siRNA as detailed in 2.1.3. Cells were seeded at 1.6x10^5 cells per well of a 12-well plate and allowed to grow to confluency over 4 days. Treatment of bEnd.3 cells with CSF-1R ligands was performed as standard, with cells treated with 1, 10, or 100 ng/mL recombinant mouse CSF-1 (R&D Systems) or IL-34 (R&D Systems). Cells were lysed for protein and RNA analyses 24 and 48 h post-treatment, with replicates using a different passage of cells and performed over a week apart (n = 3 for protein, n = 2 for RNA). Cells were similarly treated with the CSF-1R inhibitor PLX3397 (5, 10, 20 μM) for 24 and 48 h, with n = 3 for both protein and RNA.

In microglial conditioned media experiments, bEnd.3 cells were treated with control, BV2-CM or CSF-1R-inhibited BV2-CM produced as in 2.1.6 for 24 or 48 h. Cells were also treated with 20 μM PLX3397 made up in control media to control for remaining PLX3397 in CSF-1R-inhibited BV2-CM. BV2-CM was produced freshly before each replicate, and each replicate consisted of both BV2 and bEnd.3 cells of differing passages, performed at least one week apart (n = 3 for RNA and protein). For experiments involving pre-treatment of bEnd.3 cells with BV2-CM, followed by treatment with CSF-1 or IL-34, bEnd.3 cells were grown for 24 h in control media, BV2-CM or CSF-1R-inhibited BV2-CM produced with 20 μM PLX3397. At 24 h, media was removed and cells were treated with 10, 50 or 100 ng/mL CSF-1 or IL-34 made up in DMEM for a further 24 or 48 h. Replicates (n = 2) were performed at least one week apart, with freshly produced conditioned media using BV2 and bEnd.3 cells of different passage number.
2.1.8 MTS Cell Viability Assay

Cell viability assays were performed on b.End3, MBEC and BV2 cells using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) following the standard protocol with cells seeded and treated with PLX3397 in 96-well plates at 1x10⁴ cells/well. Using a darkened cell culture hood, MTS reagent (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was diluted 1:6 in culture media and 120 μL was added to each well before the plate was wrapped in aluminium foil and incubated for 30 min at 37 °C. Absorbance was measured at a wavelength 490nm using a microplate spectrophotometer (Multiskan™ FC Microplate Photometer, Thermo Scientific) and values were blank-adjusted using measurements from cell-free wells.

2.1.9 PLX3397 Endothelial Transwell Permeability Assays

Primary mouse brain endothelial cells or the b.End3 cell line (5x10⁴ cells per well) were grown to confluency on Corning HTS 24-well Transwell polyester inserts with a pore size of 0.4 μm, precoated with 1:20 fibronectin for 1 h at 37 °C and washed once with DPBS before cell seeding. Cells were treated with PLX3397 for 24 h, with PLX3397 added to both the apical and basolateral chamber. At 24 h post-treatment, 200 μL of tracer (1 mg/mL FITC–4kDa Dextran (Sigma)) in culture media, was added to the apical chamber of each well, and the cells were incubated at 37 °C. Sampling aliquots were taken from the basolateral chamber and replaced with fresh medium every 15 min for 2 hours and then transferred to 96-well plates (Nunc). FITC-tracer fluorescence was determined using a spectrofluorometer (Optima Scientific) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Relative fluorescence units were converted to values of nanograms per millilitre, using FITC-tracer standard curves, and were corrected for background fluorescence and serial dilutions throughout the experiment. The apparent permeability coefficient (Papp) for each treatment was calculated using the following equation:

\[
p\text{App}(\text{cm/s}) = \frac{dQ/dT}{A \times C_0}
\]

where \(dQ/dT\) (μg/s) is the rate of appearance of FITC-tracer on the receiver side after application, \(A\) (cm²) is the effective surface area of the insert size, and \(C_0\) (μg/mL) is the initial FITC-tracer concentration on the donor side. \(dQ/dT\) is the slope \(m(y = mx + c)\) calculated by plotting the cumulative amount \((Q)\) versus time \((s)\). Values for pApp were normalised to those of the untreated control and expressed as fold change. A graph detailing FITC accumulation over time is provided below in Figure 2.1 to demonstrate that the assay was performed during the exponential phase, prior to saturation of the basolateral chamber.
Figure 2.1: Representative graph of FITC-Dextran accumulation measured by relative fluorescence units (RFU). To demonstrate that the timecourse design was not confounded by potential saturation of the basolateral chamber with FITC-Dextran, cumulative levels of FITC-dextran were measured and graphed with time. The graph remains within the exponential phase throughout the duration of the sampling, indicating saturation and plateau phase was not reached. Coloured lines indicate samples taken from individual transwells.
2.1.10 Cell Lysis For RNA And Protein Analyses

For protein isolations, cells were washed with PBS on ice and proteins were isolated with radioimmunoprecipitation (RIPA) lysis buffer with added cOmplete™, Mini Protease Inhibitor Cocktail (Roche). RIPA buffer was added to cells and plates were shaken gently on ice to encourage lysis, followed by scraping the well with a pipette tip. Lysates were vortexed, insoluble membrane and cell debris separated by centrifugation at 12,000 rpm for 15 min, and the supernatant was retained for analysis by western blotting and stored at -20 °C.

For RNA isolations, cells were lysed similarly as above. The Total RNA Kit (TRK) lysis buffer was used in place of RIPA buffer and samples stored at -80 °C for later analysis by qPCR.

2.2 Primary Cell Isolation, Culture And Treatments

2.2.1 Primary Mouse Endothelial Cell Isolation

Mouse brain microvascular endothelial cells (MBECs) were isolated from adult mice via enzymatic digestion and bovine serum albumin (BSA) gradient separation, a protocol adapted from Assman et al. Mice were euthanised by cervical dislocation and brains removed and placed in sterile working buffer. Brains were moved to a sterile tissue culture hood where all further steps were performed. The cerebellum was removed, and brains were rolled on sterile Whatman filter paper to remove meninges and surface vessels. Featureless, smooth rolled brains were then homogenised using a 2 mL Dounce homogeniser without twisting and centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in sterile 22 % v/w BSA in DPBS by triturating with a 1 mL pipette followed by vortexing. The myelin/glial fraction of the homogenate was separated from the microvessel fraction by centrifugation at 3,000 rpm for 20 min. The upper myelin/glial layer was removed, resuspended in 22 % v/w BSA-DPBS and the spin step repeated. Microvessel pellets were resuspended and washed once in working buffer. Washed microvessel pellets were resuspended in pre-warmed complete digest media. Microvessels were digested for 1 h at 37 °C in an orbital shaker set to 200 rpm. During this digest, tissue culture plates were coated at 37 °C with human collagen IV (Sigma) and bovine plasma fibronectin (Sigma), diluted at 1:10 and 1:20 in DPBS respectively. Coated plates were washed once with DPBS before cell seeding. Digested microvessels were centrifuged at 1,000 rpm for 5 min, and complete digest media removed. Pellets were washed once with working buffer and then resuspended in Endothelial Growth Media 2 (EGM2, Lonza) supplemented with the Microvessel 2 Bullet Kit (MV2, Final
Concentration: 5% FBS, 5 ng/mL rhEGF, 10 ng/mL rhbFGF, 20 ng/mL Long R3 IGF, 0.5 ng/mL rhVEGF, 1 μg/mL ascorbic acid, 0.2 μg/mL hydrocortisone, Lonza). To select against contaminating cells such as pericytes, vessel associating microglia and astrocytes, puromycin was added at 5 μg/mL to EGM2-MV2 for the first 3 days of culture. On day 4 media was replaced with puromycin-free EGM2-MV2 and cells allowed to grow to confluency.

### 2.2.2 Primary Mouse Microglial Cell Isolation

Microglia were isolated as described in Cox et al. Whole brains were dissected from neonatal (P0) Cx3cr1-Cre<sup>+/−</sup>;Csf1r<sup>wt/Flx</sup> or Cx3cr1-Cre<sup>/−</sup>;Csf1r<sup>wt/Flx</sup> pups, chopped and added to working media (DMEM, 10% v/v FBS with added penicillin/streptomycin (100 μg/ml)). Brains were homogenised using a 5 mL pipette, passed through a 40 μm sterile mesh filter, and spun at 1,000 rpm for 5 min at 21 °C. Pelleted cells were resuspended in working media, plated in 1 mL per T25 flask and incubated in a humidified environment at 37 °C, 5% CO<sub>2</sub>. 5 mL of working media was added 3 h later following cell attachment. After 24 h, media was supplemented with 20 ng/mL CSF-1 and 10 ng/mL colony stimulating factor 2 (CSF2) (R&D Systems). Mixed glial cultures were grown for 14 days, changing media every 4 days. On day 14 flasks were wrapped with parafilm and shaken at 110 rpm at 21 °C for 1 hour to isolate nonadherent microglial cells. Suspended cells were centrifuged at 1,000 rpm for 5 min, seeded at 5x10<sup>4</sup> cells/cm<sup>2</sup> in minimal volumes of working media and grown in a 5% CO<sub>2</sub> incubator at 37 °C. After 2 h, working media containing any unattached cells was removed and replaced.

### 2.2.3 Primary Mouse BMDM Isolation

Femurs and tibiae were removed from Cx3cr1-Cre<sup>+/−</sup>;Csf1r<sup>wt/Flx</sup> or Cx3cr1-Cre<sup>/−</sup>;Csf1r<sup>wt/Flx</sup> adult mice and the fat and muscles cut away to leave clean bones. A 20 mL syringe was filled with DMEM, 10% v/v FBS supplemented with penicillin/streptomycin (100 μg/ml), a 27 G needle attached, and the bone marrow was flushed through the bones into a sterile petri dish. Aggregates were broken up by passing the cells repeatedly through a 20 mL syringe with a 19 G needle attached. The cells were transferred to a 50 mL tube and centrifuged at 1,200 rpm for 5 min. Cells were re-suspended in the medium with 25 ng/mL CSF-1 and plated into 6 dishes/mouse, with 10 mL of media per mouse. Plates were incubated at 37 °C with 5% CO<sub>2</sub>. Cells were fed on day 3 by adding 10 mL of medium with 25 ng/mL of CSF-1 per dish. On day 6, enzyme-free cell dissociation buffer (Sigma) and gentle scraping with cell scrapers was used to detach BMDMs. Cells were seeded onto 13 mm tissue culture coverslips and allowed to grow for 24 h before use.
2.2.4 Human PBMC Isolation And Macrophage Differentiation

Whole blood was collected in EDTA coated tubes and diluted 1:1 with PBS, 2 % v/v FBS. 20 mL of the blood/PBS mixture was layered onto 10 mL of Lymphoprep (StemCell Technologies) and centrifuged at 400 g for 45 min with brake and acceleration set to zero. The plasma layer was removed and PBMCs collected using a Pasteur pipet and washed twice in PBS, 2 % v/v FBS followed by a final wash in Roswell Park Memorial Institute Media (RPMI). Cells were resuspended in RPMI, 50 % v/v FBS, 10 % v/v DMSO and frozen at approximately 1x10^6 cells/vial.

To generate macrophages, PBMCs were thawed and transferred to complete RPMI (cRPMI, 100 μg/mL penicillin/streptomycin, 20 % heat-inactivated FBS, 50 ng/mL CSF-1) and grown for 48 h. After the first 48 h of growth, plates were rinsed 3 times with RPMI to remove loosely adhered cells, and allowed to grow in cRPMI until cells expanded to confluency, changing media every 2 days. Differentiated macrophages were detached using enzyme-free cell dissociation buffer and gentle scraping with cell scrapers and seeded at 2.8 x 10^4 cells/cm^2.

2.2.5 Phagocytosis Assays

For all phagocytosis assays, cells were seeded on 13 mm tissue culture coverslips at 1x10^5 cells per coverslip and allowed to grow for 24 h prior to lipopolysaccharide (LPS) treatment. Microglia, BMDM and PBMC-derived macrophages were treated with 10 ng/mL LPS for 24 h before being assayed. For siRNA knockdown experiments, BV2 cells were transfected as in 2.1.3 24 h post-seeding before continuing to LPS stimulation the following day. Latex beads, polystyrene amine-modified (yellow-green) (Sigma), were pre-opsonised in FBS (1:5) at 37 °C for 1 h. Opsonised beads were diluted 1:10,000 in media, yielding a final bead dilution of 1:50,000, and added to cells for 1 h. Cells were washed with ice-cold PBS 3 times to remove surface-bound beads, fixed in 4 % w/v paraformaldehyde (PFA) for 10 min and stained with MitoTracker Orange CMTMRos (Invitrogen) for 30 min. Coverslips were mounted onto glass microscope slides with Aqua Polymount (Polysciences) and imaged on a Zeiss LSM 710 confocal microscope.

Images were analysed by counting bead+ cells in multiple fields of view and the data expressed as % bead+ cells per genotype. For each assay, a second set of treatments was performed identically at 4 °C to control for non-specific binding of the beads to the cell surface. The number of bead+ cells counted in the control plate was used as a blank measurement during data analysis. For mouse primary microglia, n = 4 indicates 4 separate coverslips assayed in parallel. For BV-2 assays, n = 2 indicates two assays performed with four fields of view imaged. For mouse BMDM assays, n = 4 similarly indicates 4 separate coverslips assayed in parallel. For human PBMC-derived
macrophage assays, n = 4 indicates four independent assays, with 4 fields per coverslip quantified per assay. Data are presented as means of each independent assay.

2.2.6 Primary Mouse Microglia Conditioned Media Experiments

Primary MBEC cultures were isolated from adult Tie2-Cre;CsF1r$^{wt/Fix}$ and Tie2-Cre;CsF1r$^{wt/Fix}$ mice as described in 2.2.1, and primary microglia isolated from Cx3cr1-Cre$^+/−$;CsF1r$^{wt/Fix}$ or Cx3cr1-Cre$^+/−$;CsF1r$^{wt/Fix}$ as described in 2.2.2. For media conditioning, 7.3x10$^5$ microglial cells were seeded into 6-well tissue culture plates. Microglia were grown for 24 h and had media replaced and collected 24 h later. Conditioned media was centrifuged, sterile filtered and diluted at a 2:1 ratio in EGM2-MV2 before adding to confluent MBEC cultures. MBECs were lysed for RNA and protein analyses after 24 h. Microglia used for media conditioning were also lysed following conditioned media collection for RNA and protein analyses. This experiment was performed once for protein analysis, and independently in duplicate for RNA analyses, with two separate microglial and endothelial isolations, media conditioning and treatment.

2.2.7 PLX3397 Treatment Of MBECs

MBECs isolated from adult 10 week old C57BL/6 mice were allowed to grow to confluency and treated with 0, 5, 10 or 20 μM PLX3397 for 24 h and 48 h before an MTS cell viability assay was performed as in 2.1.8. The 24 h time point was chosen for further PLX3397 treatments, and cells were again treated and a FITC-4kDa Dextran permeability assay performed. Cells were lysed for RNA and protein analyses directly after the flux assay. For RNA and protein analyses, n = 2 indicates two separate primary cell isolations and treatments. For the MTS cell viability assay, n = 3 indicates 3 wells of a 96 well plate. For the FITC-4kDa Dextran flux, n = 2 indicates two separate wells of a transwell insert assayed in parallel.

2.3 Ethical Approval for Human Samples and Participation

Ethical approval was sought from the Tallaght University Hospital (TUH, formerly Adelaide and Meath Hospital, Dublin, incorporating the National Children’s Hospital (AMNCH)) / St. James’s Hospital Joint Research Ethics Committee (SJH/TUH Research Ethics Committee) for experiments involving human participants and samples. The ethical approval number issued is 2017-05 List 17(3). All experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
2.4 MRI

MRI was performed in the Centre for Advanced Imaging (CAMI) at St. James’ Hospital, and analysis was performed by Dr Eoin O’Keeffe. BBB permeability maps were created using the slope of contrast agent concentration in each voxel over time, calculated by a linear fit model as previously described. A threshold of high permeability was defined by the 95th percentile of all slopes in a previously examined control group. All imaging was performed using a 3T Philips Achieva scanner, and included a T1-weighted anatomical scan (3D gradient echo, TE/TR =3/6.7 ms, acquisition matrix 268x266, voxel size: 0.83x0.83x.9mm), T2-weighted imaging (TE/TR =80/3000 ms, voxel size: 0.45x0.45x.4mm), FLAIR (TE/TR = 125/11000 ms, voxel size: 0.45x0.45x4mm). The calculation of pre-contrast longitudinal relaxation time (T10), the variable flip angle (VFA) method was used (3D T1w-FFE, TE/TR = 2.78/5.67 ms, acquisition matrix: 240x184, voxel size: 0.68x0.68x5 mm, flip angles: 2, 10, 16 and 24°). Dynamic contrast-enhanced (DCE) sequence was then acquired (Axial, 3D T1w-FFE, TE/TR = 2.78/5.6 ms, acquisition matrix: 240x184, voxel size: 0.68x0.68x5 mm, flip angle: 6°, Δt = 6.5 Sec, temporal repetitions: 70, total scan length: 7.6 minutes). An intravenous bolus injection of the contrast agent gadobentate dimeglumine (Gd-BOPTA, Bracco Diagnostics Inc., Milan, Italy) was administered using an automatic injector after the first three DCE repetitions.

2.5 CSF-1R Sequencing And Preparation Of Expression Constructs

2.5.1 Genomic DNA Isolation

DNA was isolated from whole blood of live ALSP patients, or formalin-fixed paraffin-embedded (FFPE) tissue for post mortem samples. For whole blood, 2 mL of peripheral blood was added to 2 mL of red blood cell (RBC) lysis buffer (0.32 M sucrose, 10 mM tris, 5 mM MgCl₂, 0.75 % v/v Triton X 100, pH 7.4). The mixture was inverted, placed on ice for 3 min and spun at 3,500 rpm for 15 min at 21 °C. The supernatant was discarded, and the pellet resuspended in 2 mL RBC lysis buffer and 6 mL deionised water (dH₂O) before being centrifuged again. The washed pellet was resuspended in proteinase K digest buffer (20 mM tris, 4 mM Na₂EDTA, 100 mM NaCl, 1 % w/v SDS, 0.2 mg/mL proteinase K, pH 7.4) and incubated at 55 °C overnight. 4 mL of 5.3 M NaCl was added to the completed digest followed by 0.2 volumes of chloroform. Tubes were shaken, centrifuged, and the upper aqueous layer moved to a new tube. DNA was isolated using alcohol precipitation followed by resuspension in nuclease-free water. FFPE brain tissue was incubated with gentle shaking in xylene for 15 min at 21 °C, centrifuged at 3,500 rpm for 5 min at 21 °C, and
the supernatant was removed and replaced with fresh xylene. This was repeated twice before pelleted tissue was rehydrated by incubating in decreasing concentrations of ethanol (100 % v/v, 70 % v/v, 50 % v/v) before incubating in dH₂O. As above, the pelleted tissue was incubated overnight in proteinase K digest buffer and DNA was isolated.

100 ng of DNA was amplified by PCR in a volume of 25 μL using 1x Mango Taq reaction buffer (Bioline), 200 μM each of dNTPs, 0.2 μM of forward and reverse primers, and 1.25 units of Mango DNA Taq polymerase under the following conditions: 95 °C 5 min; (95 °C 1 min; 58 °C 1 min; 72 °C 1 min) × 34; 72 °C 5 min; 4 °C hold. This produced an amplified product of 603 bp using the following primers: forward 5′- CTCCAGCAGGGACTCCAAAG -3′ and reverse 5′- GGATGCCATAGGACCAGAC-3′. DNA from the above amplification was purified using a QIAquick PCR purification kit (Qiagen) and subjected to direct Sanger sequencing (Source Biosciences) using the forward primer (above). SnapGene Viewer was used to view DNA reads and confirm the presence of the variants.

2.5.2 Standard Bacterial Transformation And Plasmid Preparations

All steps were performed under a lit bunsen burner to ensure aseptic technique. Constructs were produced in 5-alpha competent E. coli cells (New England Biolabs). Competent cells were thawed on ice and plasmid preparations were diluted to 25 ng/μL and kept on ice. 4 μL of plasmid was added to 25 μL E. coli cells before performing heat shock at 42 °C for 30 s and placing cells on ice for 5 min. 950 μL of LB was added to transformed cells and the tube incubated at 37 °C for 1 h with gentle agitation to allow the production of ampicillin resistance factors. 150 μL was plated onto ampicillin LB-agar plates, allowed to dry and incubated overnight at 37 °C. Single colonies were selected the following day and transferred into 5 mL ampicillin LB and incubated for 16 h at 37 °C at 200 rpm. Full cultures were added to 200 mL ampicillin LB and incubated again for 16 h. 200 mL cultures were pelleted by centrifugation at 3,000 rpm for 20 min. Plasmids were isolated from bacterial pellets using the Qiagen Plasmid Maxi Kit as per the manufacturer’s instructions and stored at -20 °C.

Glycerol stocks were created by taking 700 μL of the 200 mL culture and adding 300 μL of 15 % v/v glycerol in LB. The mixture was inverted several times and frozen at -80 °C for use in subsequent preps. For plasmid production from frozen glycerol stocks, a pipette tip was firmly pushed into the frozen stock and immediately moved to 5 mL LB, incubated for 16 h at 37 °C at 200 rpm and subsequent steps followed as above.
2.5.3 Generation Of Variant CSF1R Expression Constructs

Native and ΔA781_N783 CSF1R cDNA sequences were synthesised using GeneArt (ThermoFisher Scientific) with inserts cloned into the AgeI/EcoRI site of the pcDNA3-EGFP expression plasmid (Addgene).

The P824R expression construct was generated from the native CSF1R expression plasmid using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs), following the standard protocol. Site-directed mutagenesis was performed by PCR amplification of the native CSF1R expression construct using primers containing the desired base pair change.

The Native CSF1R expression construct was amplified by PCR in a volume of 25 μL using 1 μL plasmid (25 ng/μL), 12.5 μL Q5 Hot Start High Fidelity 2X Master Mix, 9 μL nuclease-free water and 1.25 μL of each 10 μM primer stock under the following conditions: 98 °C 30 s; (98 °C 10 s; 70 °C 20 s; 72 °C 226 s) × 25; 72 °C 2 min; 4 °C hold. The following primers were used, with the mismatched base in bold: forward 5'- TGGATGGCCCCGAGAGACATCTTG -3' and reverse 5'- CTTCACAGGAGCCGGGC -3'. 1 μL of the PCR product was added to 5 μL 2X kinase, ligase and DpnI (KLD) reaction buffer, 1 μL KLD enzyme mix, 3 μL nuclease-free water and incubated at room temperature for 5 min. 5 μL of the KLD digest product was then used for bacterial transformation and growth on ampicillin LB-agar as in 2.5.2. Colonies were selected the following day and moved to 5 mL cultures for 16 h. 5 mL bacterial cultures were lysed and plasmid isolated using the Monarch Plasmid Miniprep Kit (New England Biolabs). Site-directed mutagenesis was confirmed by Sanger sequencing using the primer 5'- CTGCTTCACTTCTCCAGCCA -3'. Plasmids containing the single base pair substitution were selected for larger-scale production as in 2.5.2 and glycerol stocks made for future isolations.

2.6 Animal Husbandry And Genotyping

2.6.1 Mouse Husbandry And Ethical Approval

All experiments involving animals were ethically approved by Trinity College Dublin’s internal Animal Research Ethics Committee, as well as being covered by the Project Authorisation AE19136/P080 issued by the Health Products Regulatory Agency (HPRA) specifically for this research project.
Mice purchased from The Jackson Laboratory were housed in the Ocular Genetics specific pathogen-free mouse facility at Trinity per the HPRA regulations for housing. Mouse strains used in this project were maintained as separate Cre-only and Csf1r\textsuperscript{Flx/Flx} lines. Experimental mice were produced by crossing homozygous B6.Cg-Csf1rtm1.2Jwp/J (Csf1r\textsuperscript{Flx/Flx}) mice with heterozygous B6J.B6N(Cg)-Cx3cr1\textsuperscript{tm1.1(cre)Jung}/J (Cx3cr1-\textsuperscript{Cre}+/−) or hemizygous B6.Cg-Tg(Tek-cre)1Ywa/J (Tie2-Cre\textsuperscript{−/−}) mice, generating littermate Cre-negative controls with each pairing. Colony stocks were maintained as Cx3cr1-Cre\textsuperscript{+/−}, Csf1r\textsuperscript{Flx/Flx} and Tie2-Cre\textsuperscript{−/−}.

2.6.2 Genotyping

Mice were genotyped 6 days post-birth (P6) using DNA isolated from toe clippings. Toe clips were performed with ethanol wiped dissection scissors, and tissue samples were incubated overnight at 55 °C in tail lysis buffer with 0.02 mg/mL proteinase K. After vortexing, 0.2 volumes of chloroform was added to each tube. Tubes were shaken, centrifuged, and the upper aqueous layer moved to a new tube. DNA was isolated using alcohol precipitation followed by resuspension in nuclease-free water.

Genotyping was performed using 6.5 μL 2X DreamTaq Polymerase Mastermix (ThermoFisher), 1 μL 50 ng/μL genomic DNA, 0.1 μL each of 100 μM primers and 4.5 μL nuclease-free water. PCR products were run on a 2 % agarose-TAE gels at 100 V for 20 min. PCR cycling conditions, primers and product sizes are given in Table 2.2, and representative genotyping is shown in Figure 2.2
<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>B6J.B6N(Cg)-Cx3cr1&lt;sup&gt;tm1.1(cre)Jung&lt;/sup&gt;/J (Cx3cr1-Cre&lt;sup&gt;+/−&lt;/sup&gt;)</th>
<th>B6.Cg-Tg(Tek-cre)1Ywa/J (Tie2-Cre&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>B6.Cg-Csf1r&lt;sup&gt;tm1.2Jwp&lt;/sup&gt;/J (Csf1r&lt;sup&gt;Flx/Flx&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Cycling Program</td>
<td>95 °C 2 min s; (95°C 30 s; 58°C 30 s; 72°C 60 s) × 35; 72°C 5 min; 4°C hold</td>
<td>95 °C 2 min s; (95°C 30 s; 51.7°C 30 s; 72°C 60 s) × 35; 72°C 5 min; 4°C hold</td>
<td>95 °C 2 min s; (95°C 30 s; 60°C 30 s; 72°C 60 s) × 35; 72°C 5 min; 4°C hold</td>
</tr>
<tr>
<td>Primers (5’ – 3’)</td>
<td>Common R: GCAGGGGAATCTGATGCAAG</td>
<td>Transgene: F: GCCGGTCTGGCAGTAAAAACTATC</td>
<td>F: CATGGGCTGTGGCCTAGAGA</td>
</tr>
<tr>
<td></td>
<td>Transgene F: GACATTGCGCTTGC TGGAC</td>
<td>R: GTGAAACAGCATTGCTGTCACTT</td>
<td>R: GGACTAGCCACCATGTCC</td>
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<tr>
<td></td>
<td>Wild Type F: CCTCAGTGACGG AGACAG</td>
<td>Internal Control: F: CTAGGCCACAGAATTGAAAGATCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTAGGTGGAAATTCTAGCATCATCC</td>
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</tr>
<tr>
<td>Expected PCR Product</td>
<td>Mutant: 380 bp</td>
<td>Transgene: 100 bp</td>
<td>Mutant: 273 bp</td>
</tr>
<tr>
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<td>Internal Control: 324 bp</td>
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<tr>
<td></td>
<td>Wild type: 302 bp</td>
<td></td>
<td>Wild type: 193 bp</td>
</tr>
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</table>

Table 2.2: Mouse Genotyping PCR Conditions
Figure 2.2: Representative genotyping results for (a) Cx3cr1-Cre, (b) Tie2-Cre and (c) Csf1rLoxP/LoxP. Homozygous positive (+/+), heterozygous (+/-) and homozygous negative (-/-) results were possible for Cx3cr1-Cre and Csf1rLoxP/LoxP however for Tie2-Cre only hemizygous positive (+) or negative (-) results were possible. B indicates a reaction run with nuclease-free water added in place of template DNA to control for reagent contamination with DNA.
2.7 In Vivo Experimental Techniques

2.7.1 Stereotaxic Injection Of Aggresure Beta-Amyloid 1-42

*Tie2-Cre*;*Csf1r*<sup>wt/Flx</sup>, *Cx3cr1-Cre*<sup>+</sup>;*Csf1r*<sup>wt/Flx</sup> and *Cre*<sup>+</sup>;*Csf1r*<sup>wt/Flx</sup> mice (8–12 weeks old) were anaesthetized using a ketamine/medetomidine mixture administered via intraperitoneal injection and placed in a stereotaxic frame. An incision was made to expose the skull, and burr holes were made using a surgical drill either above the dorsal hippocampus or the medial prefrontal cortex (mPFC). Aggresure<sup>TM</sup> Beta-Amyloid 1-42 (Aβ<sub>42</sub>, AnaSpec) was reconstituted in 50 mM tris, 150 mM NaCl, pH 7.2 at 0.25 mg/mL per manufacturer’s instructions. A Hamilton syringe was loaded with 10 μL reconstituted Aβ, and the needle was slowly lowered into the dorsal hippocampus: (co-ordinates: A/P=−1.9 mm; M/L=±1.55 mm; D/V=1.75 mm). 5.0 μl of Aβ<sub>42</sub> was then injected at a rate of 0.5 μl per min, and once complete, the needle was left in place for 5 min before suturing the skin. Anaesthesia was reversed with an intraperitoneal injection of atipamezole and mice were placed in an incubator until recovered. Mice were sacrificed 3 days post-injection and brains taken for IHC. Behavioural assays were performed at 3 days post-injection for both strains, and additionally at 14 and 28 days post-injection for the *Tie2-Cre*<sup>+</sup>;*Csf1r*<sup>wt/Flx</sup> strain and Cre- controls. N = 5 mice per group for behavioural assessment, n = 4 injected mice per group for assessment of tight junction and n = 5 injected mice per group for the assessment of macrophage activity.

2.7.2 Tail Vein Injection Of Recombinant CSF-1R Ligands

Mice were secured in a 50 mL syringe with the plunger detached and a cork in each end preventing escape. Tails were secured by positioning into the guide hole of the cork, and veins made visible by positioning a lamp beneath the tail. A 31 G needle was attached to a 1 mL syringe, the mouse weighed, 100 μg/kg recombinant IL-34 or CSF-1, or 1 mL/kg PBS (vehicle for ligands) was injected. Light pressure was applied to the injection site before returning the mouse to its cage. 24 h post-injection, brains were taken for the same analyses of microvessel isolations. For RNA analyses, replicates (n) indicate microvessel lysates from individually treated mice. For microvessel analyses, n = 4 C57BL/6 mice per treatment group, n = 2 *Csf1r*<sup>+/−</sup> EC mice for vehicle injection and n = 3 for CSF-1 and IL-34 injection. For *Csf1r*<sup>+/−</sup> MΦ mice n = 4 for vehicle and IL-34 injection and n = 3 for CSF-1 injection.
2.7.3 Behavioural Assessment

All behavioural assays were performed in the Ocular Genetics unit in a quiet procedure room. Mice were habituated to the room for 1 h prior to assessment and were handled daily by the experimenter for 5 days before the first test to reduce anxiety. Maze and assay apparatuses were wiped down with 70% v/v ethanol between tests for each mouse unless otherwise indicated. Behavioural protocols were adapted from Greene et al.275.

2.7.3.1 T-Maze

Working spatial memory was assessed using the alternating T-Maze. Mice were individually placed into the start arm of a Perspex T-Maze (three arms 30 x 10 cm; wall height 20 cm) with a guillotine door preventing them from accessing the maze. Each mouse underwent 7 sessions with each session comprising of two trials. Sessions were interspaced over the course of one day, beginning in the morning and ending in the evening. During the first trial in each session, a Perspex divider was present to encourage the selection of one arm over the other. The trial began when the guillotine door in the start arm was removed and a stopwatch was started at the same time. The stopwatch was stopped when the mouse entered one of the choice arms. An arm entry was defined as the point when the entire mouse, excluding its tail, was in the arm and at this point a guillotine door was placed at the entrance of the start arm to prevent the mouse from going back into the start arm. The name of the arm selected (A or B) was noted by the experimenter and the mouse was then returned to the start arm for the second trial.

Before beginning the second trial, the divider between the two choice arms and the guillotine arm from the choice arm from the first trial was removed. The apparatus was not cleaned between individual trials for the same animal, as mice should use olfactory cues to make a choice on this task. The second trial again began when the guillotine door for the start arm was removed and ended when the mouse fully entered one of the two arms, again using a stopwatch to time performance in the task. The mouse was contained in the choice arm with a guillotine door before being returned to its home cage. The name of the arm selected was again noted. Each mouse was scored for each session using a binary system: 1 for alternating between the arms; and 0 for visiting the same arm twice in a session.

2.7.3.2 Plus Maze

Working spatial memory was assessed using the spontaneous alternation on a Plus-Maze. Individual mice were placed in the centre zone of a Perspex Plus-Maze (four 30 x 5 cm arms joined
by a 20 cm diameter central area; bounded by wall 15 cm high) and allowed to freely explore the apparatus for 8 minutes. Arm entries were counted by the experimenter when the mouse placed all four paws into an arm, delineated by the slot for guillotine doors associated with the apparatus (the doors themselves were not used in this task). Each arm entry was recorded in the order in which they occurred. Spontaneous alternation was assessed after the experiment was over; a successful alternation being defined as the mouse entering all four arms of the Plus-Maze over any 5-arm entry span (e.g. if the mouse entered the arms in the order 1, 2, 3, 1, 4, 3, 1 then it would count as two alternations out of a possible three). Errors were counted if the mouse exited an arm and then returned to that arm before visiting either of the other two arms.

2.7.3.3 Novel Object Recognition

Short-term recognition memory was assessed using the object recognition task. The object recognition task was performed in a plastic rectangular arena (38 x 43 x 18 cm) with a white base. Three similarly sized objects were used for all mice (built using Duplo lego blocks of differing shape and colour) with animals showing no preference for one particular object over the others. An overhead camera tracked animal movement, head position and proximity to each object using the ANY-Maze software. The task consisted of two 3-minute sessions starting with a familiarisation session where two objects were positioned at two fixed locations within the testing arena. This was followed by a test session where one of the objects from the familiarisation session was placed in the same location as before (Familiar object) and the second object was replaced with a third object (Novel object). At the beginning of each session, the test mouse was placed in the arena facing the centre of the arena and positioned equidistant from the two objects. The timer was started when the experimenter released the mouse’s tail initiated the tracking software. Between sessions, the arena and objects were cleaned with 70% ethanol and the mouse was returned to its home cage for the intersession interval (ISI) of 10 minutes. Performance in the test was measured by calculating the discrimination index during the test. This was calculated using the amount of time the animal’s head was within 1 cm of the object, measured using the ANY-Maze software. A positive value indicates a preference for the novel object (the expected behaviour) while a negative value indicates a preference for the familiar object. Values of 0 indicate no preference for one object over the other.

Discrimination Index =

\[
\frac{\text{Time Spent in Proximity to Object}_{\text{Novel}} - \text{Time Spent in Proximity to Object}_{\text{Familiar}}}{\text{Time Spent in Proximity to Object}_{\text{Novel}} + \text{Time Spent in Proximity to Object}_{\text{Familiar}}} \]
2.7.4 Microvessel Isolations For Molecular Analyses

Brain microvessel isolations were performed using an adapted protocol from Lee et al.\textsuperscript{276} with dextran replaced with 22 % BSA-PBS and additional spin steps added to aid removal of residual BSA from protein lysates. All reagents and tubes were kept on ice for the duration and stored at 4 °C overnight before use. Brains were homogenised in sDMEM using a Dounce homogeniser, and the homogenates pelleted by centrifugation at 3,000 rpm for 5 min. Pellets were resuspended in 22 % w/v BSA-PBS using a P1000 pipette, and the resuspended pellets were added to 9 mL 22 % w/v BSA-PBS in 30 mL universal tubes, and vortexed for 20 s. Homogenates were separated into the microvessel (pellet) and neuron/glial (floating) layer by centrifugation at 3,000 rpm for 20 min. To increase yield, the neuron/glial layers were resuspended in 3 mL 22 % w/v BSA-PBS, aliquoted into 1.5 mL tubes and centrifuged a second time at 10,000 rpm for 10 min, while keeping the initial microvessel pellets on ice. Pellets from the 1.5 mL and initial 30 mL universals were pooled and gently transferred to a 40 μm cell strainer (Corning). Pellets were rinsed with 5 mL PBS to dislodge loosely adhered cells, the strainer inverted and microvessel pellets collected by washing the strainer with 20 mL 0.5 % w/v BSA-PBS and centrifuged. The microvessel pellets were washed with 20 mL 0.5 % w/v BSA-PBS twice more before a final 20 mL PBS wash. Microvessel pellets were resuspended in 1 mL PBS, split into two tubes each and centrifuged at 10,000 rpm for 10 min. The pellets were then lysed for RNA analyses using a handheld tissue homogeniser as in 2.9. Unlike the microvessel isolation method in 2.2.1, brains were processed individually, without pooling samples from different mice. Additionally, the lack of an enzymatic digest step allows for the microvessels to be isolated along with adherent cells such as vessel-associating macrophages and astrocyte endfeet.

2.8 Western Blotting

2.8.1 Bicinchoninic Acid (BCA) Assay

Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). The standard protocol was followed without protein dilution, using a freshly prepared 50:1 preparation of BCA reagents A:B respectively, and BSA standards prepared as per manufacturer’s instructions (0, 65, 125, 250, 500, 750, 1000, 1500, 2000 μg/mL). 10 μL of protein lysate or BSA standard was added in duplicate to each well of a 96-well plate followed by 200 μL BCA reagent mix. The plate was covered and incubated at 37 °C for 30 min. Absorbance was measured at 595 nm using a microplate spectrophotometer (Multiskan™ FC Microplate
Photometer, Thermo Scientific). Readings were averaged and blank-corrected to the 0 μg/mL BSA standard before generating a standard curve; plotting average absorbance values of BSA standards against concentration and generating a standard curve. Protein concentrations were determined using the formula $y = mx + c$, where $y$ = absorbance at 595nm, $m$ = slope, $x$ = protein concentration (μg/mL) and $c$ = y-intercept.

2.8.2 SDS-PAGE Gel Preparation And Run

Sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels were cast using the ATTO apparatus with resolving and stacking gels prepared as described in Table 2.3, with 10 % w/v ammonium per-sulphate (APS) made up fresh each day in dH$_2$O. Gel components were added in order, and APS and TEMED were added to the gel mix just prior to pouring to prevent early polymerisation. Glass plates were assembled with sealing gaskets and clamped in place. 6 mL of separating gel mix was poured gently between the assembled plates and 2 mL of 70 % ethanol gently overlaid on top to provide air-free conditions for polymerisation and ensure a flat interface between stacking and separating gels. The gel was left at room temperature for 30 min to polymerise, the ethanol poured off and the remaining space washed carefully with dH$_2$O to remove residual ethanol. Plates were inverted to remove the remaining dH$_2$O and stacking gel poured such that it filled any remaining space. The comb was inserted into the unpolymerized stacking gel mix and the apparatus allowed to polymerise at room temperature for a further 30 min. Once polymerisation was complete, the rubber gaskets were removed, and gels placed into ATTO gel electrophoresis rigs pre-filled with running buffer. Gels were clamped in place with spacers, and the inner chamber filled with running buffer before combs were slowly removed. Wells were washed with running buffer by gentle pipetting to remove any gel debris. 20 μg of protein was diluted in dH$_2$O and 5X Pierce™ Lane Marker Reducing Sample Buffer (Thermo Fisher) to a final volume of 20 μL and denatured by boiling at 95 °C for 10 mins. Denatured samples were then loaded into the washed wells, with 2.5 μL of Prime Step pre-stained protein ladder (BioLegend) added to allow for molecular weight approximation. Gels were run at constant volts with a programmed voltage change, 80 V for 25 min and then 120 V for 2 h or until the loading dye front reached the base of the gel.
<table>
<thead>
<tr>
<th>Gel Component</th>
<th>Stacking Gel</th>
<th>Separating Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>6.791 mL</td>
<td>5.874 mL</td>
</tr>
<tr>
<td>0.5 M Tris-HCL pH 6.8</td>
<td>1.666 mL</td>
<td>-</td>
</tr>
<tr>
<td>1.5 M Tris-HCL pH 8.8</td>
<td>-</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>30 % acrylamide/bis-acrylamide (37.5:1)</td>
<td>1.333 mL</td>
<td>2.666 mL</td>
</tr>
<tr>
<td>10 % w/v SDS</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μL</td>
<td>10 μL</td>
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<tr>
<td>APS</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td>Final Volume</td>
<td>10 mL</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

Table 2.3: Gel Compositions for SDS-PAGE
2.8.3 Electrophoretic Transfer Of Separated Protein

SDS-PAGE gels were removed from the glass plates and equilibrated in ice-cold transfer buffer for 20 min to avoid shrinkage during transfer. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Merck Millipore) that had been incubated (“activated”) in methanol for 5 min reduce membrane hydrophobicity. Activated membranes were equilibrated in ice-cold transfer buffer for 5 min before being overlaid onto 3 pieces of Whatman filter paper, pre-soaked in ice-cold transfer buffer, and placed onto an Enduro Semi-Dry Blotter transfer rig. The SDS-PAGE gel was then carefully placed on top of the PVDF membrane, overlaid with 3 further pieces of pre-soaked Whatman filter paper and a 5 mL stripette was rolled over the assembly to remove bubbles that would interfere with transfer. The transfer rig lid was secured and separated proteins transferred at a constant 12 V for 2 h.

2.8.4 PVDF Membrane Immunoblotting

All washing, incubating and equilibration steps were performed on a benchtop orbital shaker. On completion of protein transfer, PVDF membranes were removed from the gel-filter paper assembly and rinsed with dH₂O to remove any gel debris. Membranes were then incubated in methanol for 5 min and allowed to air-dry on dry filter paper to enhance protein binding to the membrane and reduce noise. Dried membranes were stained with Ponceau-S solution (Sigma) to visualise protein, ensure adequate transfer and guide membrane cutting. Stained membranes were cut into sections depending on the target proteins to be immunoblotted. Cut membrane sections were washed in Tris Buffered Saline with Tween-20 (TBS-T) for 5 min twice or until Ponceau-S staining had been removed. Membranes were then incubated in methanol for 5 min, equilibrated in TBS-T and unbound membrane blocked for 40 min at room temperature in 5 % w/v non-fat dried milk in TBS-T (Marvel). Blocked membranes were washed for 5 min in TBS-T three times, and moved into individual containers containing primary antibody. Blots were incubated overnight (16 h) in primary antibody (diluted in TBS-T with 3 % w/v BSA, full list in Table 2.4) at 4 °C. The following day primary antibody solution was removed, and blots washed for 5 min in TBS-T three times before being incubated in horseradish peroxidase (HRP) conjugated secondary antibody in TBS-T for 2 h at room temperature. Blots were washed in TBS-T as before and placed in dH₂O before imaging by chemiluminescence.

2.8.5 Detection Of HRP And Densitometry

To detect bound HRP-conjugated secondary antibody, Strong Enhanced Chemiluminescence (ECL) Substrate (Advansta) and the C-Digit (LiCor) imaging system were used. A 1:1 mixture of
WesternBright ECL Luminol/enhancer solution and Peroxide Chemiluminescent solution (Advansta) was prepared and incubated for 2 min at room temperature. Incubated membranes were then placed in the mixture and left for 2 min before transferring to the C-Digit machine. Blots were placed face down, covered in the hydrogen peroxide-ECL mixture and imaged for 12 min using the high sensitivity method. The Image Studio Lite software was used for densitometric analyses, with band quantification values normalised to an Actin or GAPDH loading control before being expressed as fold change relative to untreated control. All densitometry was performed between samples run on the same SDS-PAGE gel and transferred onto the same membrane.

2.9 RNA Analyses

2.9.1 RNA Isolation And cDNA Synthesis

RNA was isolated from TRK lysis buffer using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek), first precipitating RNA with 1 volume of 70 % ethanol, binding the precipitated RNA to a spin column, and performing washes as per standard protocol before eluting RNA in 45 μL of nuclease-free water. RNA yield as quantified in ng/μL using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and diluted to 10 ng/μL. 20 μL cDNA synthesis reactions were performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using 10 μL of 10 ng/μL RNA, 2 μL 10X random primers, 2 μL 10X reaction buffer, 0.8 μL 100 mM dNTPs and 1 μL reverse transcriptase. The reaction mix was vortexed briefly before being run on a PCR cycling machine under the following conditions: 25 °C 1 min; 37 °C 120 min; 85 °C 5 min; 4 °C hold. The PCR product was diluted 1:10 with nuclease-free water before being used in qPCR analyses.

2.9.2 Quantitative PCR

Quantitative PCR was performed on a StepOnePlus (Applied Biosystems) machine using SensiFAST™ SYBR® Hi-ROX Kit (Bioline) and primers listed in Table 2.5. 10 μL reaction volumes were used, with 2.5 μL cDNA, 5 μL SYBR, 0.5 μL of pre-mixed 10 μM forward and reverse primer and 2 μL nuclease-free water in each well. The following PCR conditions were used for qPCR which includes both the amplification stage and melt-curve analyses: 95 °C 20 s; (95 °C 3 s, 60 °C 30 s ) x40; 95 °C 15 s, 60 °C for 1 min, 0.3 °C increments with data collection to 95 °C, 95 °C 15s. Data analyses were performed using the delta cycle threshold (ΔC_t) method. C_t values from each sample’s gene of interest were normalised to that of the Actb housekeeping gene to generate the delta C_t (ΔC_t) for each well. For each gene of interest, control ΔC_t values were averaged and
subtracted from all ΔCₜ values to generate delta delta Ct (ΔΔCₜ). Gene expression was calculated and presented as fold-change relative to untreated controls by calculating $2^{(-\Delta\Delta Ct)}$.

Samples were run in duplicate for each gene, and within each experiment, all samples were analysed for individual gene expression in parallel whenever possible. For samples run on separate plates, thresholding values used by the StepOnePlus software for Cₜ calculation were made uniform across each plate to account for any differences in PCR amplification.

**2.10 Enzyme-Linked Immunosorbent Assay (ELISA)**

For ELISA analyses, cell culture supernatant was removed from transfected HEK293 cells, centrifuged at 1,000 rpm for 5 min and frozen at -80 °C.

The Human IL-8/CXCL8 DueSet ELISA (R&D Systems) was used to quantify secreted CXCL8 in HEK293 cells transfected with native and variant CSF-1R. CXCL8 ELISA wash buffer (TBS-T), block buffer (TBS-T, 1 % w/v BSA) and reagent diluent (TBS-T, 0.1 % w/v BSA) were prepared and sterile filtered before use. CXCL8 capture antibody was diluted to a 20 mg/mL concentration in PBS and 100 μL was pipetted into the wells of a 96-well plate. The plate was sealed with an adhesive film incubated overnight at 4 °C with gentle shaking. The following day, capture antibody was removed and the plate washed three times with wash buffer by completely submerging in wash buffer, inverting and shaking over a sink and finally forcefully blotting against paper towels to remove remaining droplets. 300 μL of blocking buffer was added to each well and incubated with gentle shaking at room temperature for 90 min before the washing procedure was repeated. Human recombinant CXCL8 standards were prepared by diluting the provided stock solution to 2000 μg/mL in reagent diluent and performing serial 2-fold dilutions. 100 μL of each standard and undiluted tissue culture media supernatant was added in duplicate, the plate covered with another adhesive film and incubated at room temperature with gentle shaking for 2 h. Wells were emptied by aspiration and the plate washed three times as previously described before adding 100 μL of detection antibody to each well. The plate was sealed, incubated for 2 h at room temperature with gentle shaking before being aspirated and washed as before. Provided Streptavidin-HRP was diluted 1:200 with reagent diluent, and 100 μL added to each well. The plate was covered in aluminium foil to protect it from light and incubated for 20 min at room temperature. Aspiration and washing were performed as before, and 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate solution (Thermo Scientific) prepared by mixing equal parts TMB solution and hydrogen peroxide. 50 μL TMB solution mix was added to each well, and
the plate was wrapped in foil and incubated for 20 min. After 20 min, a colourless to blue colour change was observed and 50 μL TMB Stop Reagent (Sigma) added to halt the reaction, turning the solution yellow. After tapping to ensure thorough mixing, and removing air bubbles by passing the plate through a fine mist of 70 % v/v ethanol, absorbance at 450 nm was measured on a Multiskan™ FC Microplate Photometer. Values were blank corrected using the 0 μg/mL standard and pg/mL concentrations determined using a standard curve. Analysis was performed in GraphPad Prism 8.0, using 4-parameter logistic (4PL) non-linear regression for generating a standard curve for sample concentration interpolation.

2.11 Immunocytochemistry (ICC)

HEK293 cells were seeded on 1 % v/v fibronectin-coated 1.3 mm tissue culture coverslips (Sarstedt) in DMEM, 10 % v/v FBS. 24 h after plasmid transfection as in 2.1.3, cells were fixed for 10 min at room temperature with PFA, washed twice with PBS and incubated with 5 % v/v normal goat serum (NGS) before overnight incubation with polyclonal rabbit anti-CSF-1R at 4 °C. Cells were then immersion washed twice with PBS and incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody for 2 h at room temperature, washed three times with PBS and counterstained with Hoechst 33258 to visualize nuclei. Stained coverslips were inverted, and mounted onto glass microscope slides with Aqua Polymount. Macrophages were seeded in poly-L-lysine coated 8-well chamber slides (Ibidi) at 2.8 x 10^4 cells/cm^2. Macrophages were grown for 72 h, fixed in 4 % w/v PFA for 10 m at RT and stained as above with rat anti-human CD68 primary antibody and 594-conjugated goat ant-rat IgG secondary antibody. 200 μL of PBS was added to each chamber and slides were imaged without inversion.

ICC of PLX3397-treated MBECs was performed as above, with cells seeded in EGM2-MV2 growth media. After incubation in PLX3397 for 24 h, cells were fixed in 4 % w/v PFA for 10 m at RT and stained as above with polyclonal rabbit anti-claudin-5 or anti-ZO-1 at 4 °C. Cells were then immersion washed twice with PBS and incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody for 2 h at room temperature, washed three times with PBS and counterstained with Hoechst 33258 to visualize nuclei. Stained coverslips were inverted, and mounted onto glass microscope slides with Aqua Polymount.
2.12 Immunohistochemistry (IHC)

FFPE sections of autopsied ALSP cortical sulci were de-paraffinized by dipping 20 times in xylene and rehydrated stepwise through 20 dips in decreasing ethanol concentrations (100 %, 70 %, 50 % v/v) followed by 20 dips and a 2 min incubation in dH$_2$O. Sections were permeabilised by incubating in methanol for 15 min at -20 °C, and the tissue was outlined with a liquid repellent Super PAP Pen (Ted Pella, Inc.). Tissue was blocked in 5 % v/v NGS, PBS, 0.1 % v/v Triton X 100 for 45 min, and sections were incubated in primary antibody in PBS, 1 % v/v NGS, 0.1 % v/v Triton X 100 overnight at 4 °C (antibody details are given in Table 2.4). Slides were immersion washed three times in PBS and incubated with fluorophore-conjugated goat secondary antibodies for 3 h at room temperature, washed with PBS as before, and counterstained with Hoechst 33258 to visualize nuclei.

For IHC of flash-frozen mouse brains, mice were sacrificed by rising CO$_2$ and cervical dislocation, and the brains quickly removed and embedded in optimal cutting temperature compound (OCT, VWR), snap-frozen in liquid nitrogen and stored at −20 °C prior to slicing on a cryostat. Mouse brain cryosections (20 μm thick) were post-fixed in ice-cold methanol for 10 min at room temperature and washed three times in PBS. Sections were then incubated with 5 % v/v NGS in PBS, 0.5 % v/v Triton X 100 before overnight incubation with primary antibodies and isolectin B4 at 4 °C. Following three washes in PBS, sections were incubated with fluorophore-conjugated goat secondary antibody for 3 h at room temperature, washed three times with PBS and counterstained with Hoechst 33258. Sections were mounted and with Aqua Polymount and imaged with a Zeiss LSM 710 confocal laser scanning microscope.

For both ICC and IHC, isotype control staining was performed to test for potential non-specific binding of the secondary antibody. For each of human cortical sections, mouse cortex sections, fixed b.End3 cells, fixed HEK293 cells and fixed primary human macrophages isotype control staining was performed. Tissue and cells were processed as in 2.11 and 2.12, with the omission of primary antibody. Sections were incubated with secondary antibodies as indicated in Figure 2.3, testing each isotype for each tissue or cell type, and counterstained with DAPI.
Figure 2.3: Isotype control staining of tissue and cells. (a) Human cortical sections incubated with goat anti-rabbit-Cy3 and goat anti-mouse-488. (b) Human cortical sections incubated with goat-anti-rat-594. (c) Primary human macrophages incubated with goat anti-mouse-Cy3. (d) HEK293 cells incubated with goat anti-rabbit-Cy3. (e) Mouse cortical sections incubated with goat anti-rabbit-488 and goat-anti-rat-594. (f) b.End3 cells incubated with goat anti-rabbit-Cy3. Scale bars indicate 40 μm.
2.13 Image Acquisition And Analyses

Images were acquired using the ZEN Black Edition (Zeiss) software and a Zeiss LSM 710 confocal microscope. Laser intensity, gain and pinhole diameter were each kept consistent within experiments for each combination of staining carried out. Images were exported as .czi files to keep metadata intact and imported into ImageJ (National Institutes of Health, Rockville, MD, USA) using the BioFormats plugin.

For quantification of Aβ-injected mouse hippocampi, pixel intensity was thresholded uniformly across all images and integrated pixel intensity values between the injected and uninjected hippocampus were compared. Quantification was expressed as integrated pixel intensity. Replicates indicate individual mice injected with Aggresure Amyloid-β. Tight junction expression in PLX397-treated MBECs was quantified using the same method, comparing PLX3397-treated coverslips to untreated and expressing the data as integrated pixel intensity fold change.

2.14 Fluorescent Automated Cell Sorting

Fluorescent automated cell sorting (FACS) and analyses were performed by Dr Kiva Brennan. PBMCs isolated from HDLS donor blood were seeded in round-bottomed 96-well plates in RPMI media with 10 % FBS at 4x10^5 cells per well and incubated in a 5 % CO₂ incubator at 37 °C overnight. Cells were centrifuged at 300 g for 5 min and blocked for 10 min with 50 μL 10 % human AB serum, 1 % FBS in PBS. Blocked PBMCs were incubated in Live/Dead Aqua for 30 min, washed in PBS and incubated in fluorochrome labelled primary antibody diluted 1:10 in PBS, 1 % FBS for 20 min at 4 °C. Cells were washed twice with PBS 1 % FBS and analysed by flow cytometry immediately. Gating during analysis was based on fluorescence minus one controls. Flow cytometry was carried out on a BD LSFRFortessa cell analyzer and analyzed using FlowJo software (Tree Star). All antibodies and reagents used are given in table Table 2.4: Antibodies and Staining Compounds with Suppliers and Indicated Dilutions. For antibody applications, WB indicates use for western blot, ELISA indicates use in DuoSet assays, FACS indicates use for automated cell sorting and IF indicates use in immunofluorescent tissue or cell staining.
2.15 Statistical Analyses

With the exception of the Cell Stress and Toxicity Array, all statistical analyses were performed in GraphPad Prism 8.0. All data were tested for normality using the omnibus K2 (D’Agostino-Pearson) test. Two-group data were analysed by Student’s t-test, experiments with greater than two groups were analysed by one or two-way ANOVA, with post-tests for multiple comparisons as indicated in figure legends. In all cases, p < 0.05 was considered significant, and error bars are presented as the standard error of the mean.
2.16 List Of Buffers And Solutions

Unless otherwise indicated, buffers and solutions were made up in dH₂O. Solutions were pH -adjusted by dropwise addition of HCL or NaOH and measuring pH with a glass electrode and meter (Lennox).

250 mL 0.5 M Tris-HCL, pH 6.8
- 15 g Trizma base

250 mL 1.5 M Tris-HCL, pH 8.8
- 45 g Trizma base

200 mL 10 % w/v Sodium Dodecyl Sulphate (SDS), pH 7.2
- 20 g SDS

1 L Transfer Buffer, pH 8.3 (not pH adjusted)
- 0.37 g SDS
- 2.9 g Glycine
- 5.8 g Trizma base
- 200 mL methanol
- Store at 4 °C

1 L Running Buffer, pH 8.3 (not pH adjusted)
- 1 g SDS
- 14.4 g Glycine
- 3.03 g Trizma base
1 L Tris Buffered Saline (TBS), pH 7.4
- 6.05 g Trizma base
- 8.766 g NaCl

1 L Tris Buffered Saline with Tween-20 (TBS-T), pH 7.4
- 1 L TBS
- 500 μL Tween-20

1 L Phosphate Buffered Saline (PBS), pH 7.4
- 2.56 g Na₂HPO₄·7H₂O
- 8 g NaCl
- 0.2 g KCl
- 0.2 g KH₂PO₄

50 mL 22 % BSA
- 11 g BSA
- Correct to a final volume of 50 mL with DPBS
- Sterile filter and store at 4 °C

500 mL Tail lysis buffer, pH 8.0
- 6.04 g Trizma base
- 0.73 g EDTA
- 1 g SDS
- 5.84 g NaCl

250 mL Radioimmunoprecipitation Assay (RIPA) Lysis Buffer, pH 8.0
- 1.51 g Trizma base
- 2.19 g NaCl
- 1.5 g Sodium deoxycholate
- 2.5 mL Triton 100
- 0.25 g SDS

- 1 cOmplete Mini protease inhibitor tablet added to 10 mL RIPA buffer aliquot freshly prior to use
- Store protected from light at 4 °C

100 mL Complete digest medium for MBEC Isolation

- 1 mL Pen/Strep (stock of 10,000 μg/mL penicillin/streptomycin)
- 1 mL 1 M TC-HEPES buffer
- 1 mL 100 mg/mL collagenase/dispsase (Sigma)
- Correct volume to 100 mL with Ca²⁺/Mg²⁺-free Hanks Balanced Salt Solution (HBSS)
- Sterile filter, aliquot and store at -20 °C until day of dissection.

- 5 μL TLCK (stock of 0.147 mg/mL) and 50 μL DNase I (stock at 2,000 U/mL) added per 5 mL digest medium prior to microvessel pellet digest

100 mL Working Buffer For MBEC Isolation

- 2.75 mL 22 % w/v BSA
- 1 mL Pen/Strep (stock of 10,000 μg/mL penicillin/streptomycin)
- 1 mL 1 M TC-HEPES buffer
- Correct volume to 100 mL with Ca²⁺/Mg²⁺-free Hanks Balanced Salt Solution (HBSS)
- Sterile filter and store at 4 °C

500 mL Liquid Broth (LB)

- 5 g NaCl
- 5 g Tryptone
- 2.5 g Yeast extract
- Autoclave for 20 m at 121 °C, allow to cool to 40 °C before adding 2.5 mL ampicillin (Ampicillin stock solution at 0.2 g/mL)
200 mL Ampicillin LB-Agar

- 200 mL LB (antibiotic-free)
- 3 g agar
- Autoclave for 20 m at 121 °C, allow to cool to 40 °C before adding 1.25 mL ampicillin (Ampicillin stock solution at 0.2 g/mL)

2 mL 10 % APS

- 0.2 g APS
- 2 mL dH₂O
- Prepare freshly on day of SDS-PAGE

100 mL 4 % PFA, pH 7.4

- 4 g PFA
- 80 mL PBS
- Heat with a stirrer to no more than 65 °C and add NaOH dropwise until PFA is driven into solution
- Pour through a 40 μm filter before use

1000 mL 50X TAE

- 242 g Trizma base
- 57.1 mL 100 % glacial acetic acid
- 100 mL 0.5 M EDTA (pH 8.0)
### 2.17 List Of Antibodies And Staining Reagents

<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>Supplier</th>
<th>Dilution</th>
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</thead>
<tbody>
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<td>Actin</td>
<td>Rabbit Polyclonal Antibody</td>
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**Table 2.4:** Antibodies and Staining Compounds with Suppliers and Indicated Dilutions. For antibody applications, WB indicates use for western blot, ELISA indicates use in DuoSet assays, FACS indicates use for automated cell sorting and IF indicates use in immunofluorescent tissue or cell staining.
### 2.18 List Of RT-PCR Primers

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<th>Reverse Primer (5’ – 3’)</th>
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<td>ACTTCAGGACCAGGAGGTGT</td>
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<td>Mouse Tjp1</td>
<td>GCATGTTCAACGTATCCAT</td>
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<td>Mouse Marveld2</td>
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<tr>
<td>Mouse Actb</td>
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<td>CGATCCCTGAGATGACACA</td>
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<td>Mouse Rage</td>
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*Table 2.5:* RT-PCR Primer Sequences. Primer sequences were determined using the NCBI Primer-BLAST resource, with each primer product required to span exon-exon boundaries.
2.19 Full List Of Genes Examined In The Human Stress & Toxicity Pathwayfinder

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**Table 2.6:** Full List of Genes Included in the RT² Cell Stress and Toxicity Profiler
Chapter 3:
Characterisation Of Two ALSP Kindreds And Novel CSF-1R Variants
3.1 Abstract

In this chapter, work detailing molecular analyses of two familial cases of ALSP will be presented. We identified two Irish kindreds with unique familial mutations in *CSF1R* and family histories of early-onset dementia. Further examination of these cases revealed extensive BBB breakdown in the disease, leading to a hypothesis that the BBB is affected by the presence of *CSF1R* mutations. With a BBB component suggested in this disease, donor tissue was requested from the Dublin Brain Bank for IHC analyses, allowing for three ALSP brains to be assessed. Following confirmation of mutations in *CSF1R*, expression constructs were produced for *in vitro* use. Affected siblings were also contacted and peripheral blood collected. MRI of one pre-symptomatic individual was also possible, allowing for a quantification of *in vivo* BBB integrity to be obtained.

3.2 Introduction

3.2.1 Perivascular Macrophages

Microglia, the resident macrophage population of the CNS, are transiently recruited to the vasculature following vessel damage, but it is the perivascular macrophages, a population of yolk-sac derived leukocytes, that take up residence in the perivascular space of post-capillary venules. This is also the site of peripheral immune cell entry into the brain, which facilitates perivascular macrophages in safeguarding the brain from dysregulated leukocyte infiltration. The perivascular (or Virchow-Robins) space exists between the basement membranes of the glia limitans and abluminal vessel wall of post-capillary venules, with these membranes fusing in capillaries and smaller arterioles. Early bone marrow chimaera experiments investigating perivascular macrophages indicated that this population was undergoing constant turnover, with replenishment from the peripheral myeloid system. Further experiments which incorporated shielding of the brain during whole-body irradiation revealed that BBB damage during the irradiation process confounded results by allowing peripheral monocytes to enter the brain and differentiate into previously detected CNS macrophages. On shielding the brain, or using methods of macrophage depletion that did not involve radiation, this recruitment was greatly diminished. Furthermore, an approach using parabiosis, the surgical connection of the circulatory systems of two animals, revealed that perivascular macrophages of WT mice were not replaced by peripheral monocytes of the connected GFP+ mice over time. Perivascular macrophages are involved in the clearance of vessel-associated Aβ in Alzheimer’s disease, potentially through the activities of scavenger receptor class B type I which is upregulated in perivascular macrophages associating with Aβ-laden vessels. Perivascular macrophages have also been
implicated as detrimental in cerebrovascular disease. A recent study in mice demonstrated that chronic alcohol exposure drives an accumulation of this population of macrophages within the perivascular space, which then primes the brain for increased stroke lesion volume\textsuperscript{285}.

The Virchow-Robins space is a primary site of peripheral leukocyte entry into the brain\textsuperscript{286,287}, strategically positioning perivascular macrophages as gatekeepers of leukocyte transmigration across the BBB. Perivascular macrophages upregulate major histocompatibility complex class 2 (MHC-II) following CNS injury, indicating a role in antigen recognition and presentation to T-cells\textsuperscript{288}. This capacity is most clearly seen in EAE, a mouse model of MS which induces autoimmunity to myelin by concomitant injection of Freund’s adjuvant, pertussis toxin and myelin peptides\textsuperscript{289}. Early stages of the model feature an increase in perivascular macrophage numbers which precedes T-cell crossing of the BBB, and selective depletion of perivascular macrophages was found to inhibit the onset of clinical symptoms\textsuperscript{290}. A similar population of meningeal macrophages in the arachnoid barrier has been identified to perform a similar role, determining the resident perivascular macrophage population to be central in CNS infiltration of peripheral leukocytes in EAE.

\subsection{3.2.2 Cerebral Amyloid Angiopathy}

Cerebral Amyloid Angiopathy (CAA) occurs in up to 80 \% of cases of Alzheimer’s disease, as well as in the healthy elderly and over 30 \% of cases of non-Alzheimer’s dementia\textsuperscript{291}. CAA is characterized by an accumulation of Aβ in the parenchymal space, within vessel walls of arteries (namely the cerebral, leptomeningeal and parenchymal arteries) or around small to medium-sized blood vessels. The underlying mechanism that leads to Aβ accumulation in this perivascular pattern is still not fully understood, and it can occur sporadically or as part of a progressive disorder such as Alzheimer’s disease. It is accepted, however, that CAA can contribute to dementia onset and cognitive decline through increasing susceptibility for microbleeds, cerebral ischemia and chronic activation of pro-inflammatory mechanisms in the surrounding parenchyma\textsuperscript{292,293}.

Two main types of CAA exist, CAA type 1 involves capillaries and larger vessels while CAA type 2 affects larger arterioles and leptomeningeal vessels. Aβ accumulates in the space between the pericytes and glia limitans, the same compartment in which perivascular macrophages reside. Aβ itself is produced from a transmembrane amyloid precursor protein (APP) which is cleaved by the BACE β-secretase, forming the C-terminal fragment (β-CTF) and soluble N-terminal fragment (sAPP-β). The sAPP-β product undergoes further cleavage by the γ-secretase complex to produce
two main neurotoxic Aβ species, a 40-amino acid (Aβ\textsubscript{40}) and a 42-amino acid (Aβ\textsubscript{42}) isoform. While Aβ\textsubscript{42} is primarily associated with parenchymal plaques, Aβ\textsubscript{40} is the principal isoform found in CAA\textsuperscript{294,295}. Alternative \(\alpha\)-secretases ADAM-9, 10 or 17 can also cleave APP, producing non-amyloidogenic products sAPP-\(\alpha\) and \(\alpha\)-CTF. This pathway is thought to be neuroprotective as the cleavage products are incapable of forming Aβ and have even been displayed to have neurotrophic and neuroprotective properties\textsuperscript{296,297}. The \(\alpha\)-secretase pathway is thought to be the primary form of APP processing under physiologic conditions, with APP upregulation or mutations in genes encoding APP itself or secretase machinery shifting the pathway towards \(\beta\)-secretase cleavage.

Injury from CAA stems from vascular dysfunction, namely cerebral haemorrhage or ischemia due to constricted blood flow. This latter form of injury can be detected as white matter hyperintensities in T2-weighted MRI and correlates strongly with cognitive dysfunction in individuals diagnosed with CAA\textsuperscript{298}. Aβ\textsubscript{40} accumulation at the cerebrovasculature may precede NVU dysfunction, causing damage through its accumulation, or it may be the product of reduced clearance at the BBB. Of note in CAA is the primary vascular pathology; the absence of parenchymal plaque and the associated neurodegeneration indicates that specific cerebral drainage or perivascular clearance mechanisms are being affected. Despite its high comorbidity with AD, care should be taken not to consider CAA and AD as one disease entity but rather as two distinct but overlapping diseases with an Aβ component. Amelioration of cerebrovascular Aβ pathology could have clinical benefit in both CAA and AD however, as co-incidence of CAA in AD results in more severe and accelerated cognitive decline\textsuperscript{299}. NVU-targeted therapeutics may help resolve CAA and its secondary contribution to the clinical symptoms of AD. For the clinical treatment of AD however, additional treatment targeted to the mechanisms driving AD-specific pathologies such as neurodegeneration and neuroinflammation will likely also be required.

3.2.3 NVU Dysfunction In CAA

While the BBB has been extensively investigated in AD, investigation of the NVU in the specific context of CAA has been less common. Although the endothelium of the BBB displays Aβ clearance capacity\textsuperscript{300}, CAA may be the product of combined cellular dysfunction within the NVU. Aβ accumulation at the basement membrane and in the larger CNS vessels points towards NVU dysfunction as a contributing factor to CAA. In CAA, Aβ accumulation at the cerebrovasculature can result in increased vasoconstriction and dysregulation of vascular tone\textsuperscript{301}. This exacerbates the reduced cerebral blood flow in the diseased brain, which can add to cognitive impairment and Aβ accumulation within the brain\textsuperscript{302}. Aβ can induce oxidative stress in the associated...
vasculature and a critical innate immunity scavenger receptor has been found to drive Aβ-induced NADPH oxidase expression. CD36 is expressed in macrophages as well as cerebral endothelial cells, and its deletion is beneficial in ameliorating vascular oxidative stress and dysfunction in the Tg2576 mouse model of AD\textsuperscript{303,304}. Selective deletion of CD36 in aged Tg2576 mice was also beneficial, restoring LRP-1 and ZO-1 expression, reducing CAA load and improving cognition without affecting plaque burden. Pericyte and smooth muscle cells were also protected by CD36 deletion, proposing that cognitive dysfunction observed in AD may not be simply due to Aβ plaque and associated neuron loss. Specific protection of vasculature can by itself restore some cognitive function, and CAA Aβ deposition appears to have at least some unique mechanisms governing it\textsuperscript{305}.

The primary transporters of Aβ across the BBB are LRP-1 and the receptor for advanced glycosylation endpoints (RAGE) which are responsible for brain Aβ efflux and influx respectively (further discussed in \textit{5.2.1}). RAGE expression levels are mediated by the concentrations of its substrates and displays increased expression in aged and AD brains\textsuperscript{306}. In contrast, LRP-1 displays reduced expression in AD brains, and its expression is reduced during ageing\textsuperscript{307,308}. These age-related changes alone make the brain susceptible to Aβ accumulation over time. Expression levels of TJ proteins claudin-5, occludin and ZO-1 have also been reported to be downregulated in Aβ-laden capillaries\textsuperscript{309}. Areas of reduced TJ expression also had local microglial nitric oxide synthase-2 (NOX-2) upregulation, suggesting an increase in ROS to be driving changes in TJ expression. CD31 has also been reported to be decreased in the CAA vasculature\textsuperscript{310} and fibrinogen extravasation in both this and the previous study demonstrated an increase in BBB permeability. Claudin-5 and occludin have been reported to be reduced in the CAA vasculature in aged Tg2576 mice as well as in human CAA sections\textsuperscript{311}.

3.2.4 Macrophage Involvement In The Aetiology Of CAA

Stimulation of perivascular and peripheral macrophages in CAA has recently become a focus in developing therapeutics for CNS disorders, in stark contrast to the emerging investigations showing the acute benefits of microglial ablation. Stimulating the recruitment of peripheral monocytes and macrophages has been shown to reduce cognitive deficits in mouse models of both AD and tauopathy\textsuperscript{312}. Long-term treatment of mice with a PD-L1 blocking antibody stimulated the recruitment of monocyte-derived macrophages to the brain parenchyma and reduced neuron loss while slowing cognitive decline\textsuperscript{313}. In the context of CAA, this is especially relevant as perivascular macrophages, and indeed CSF-1R dependent peripheral monocytes, have been shown to have a direct role in the clearance of vessel-associated Aβ. The mouse monocyte
population can be separated into two heterogeneous groups, defined by the expression of Ly6C, CX3CR1 and CCR2. While the inflammatory population (Ly6CHiCX3CR1MidCCR2) displays CSF-1R independence, the patrolling (Ly6ClowCX3CR1highCCR2) monocyte population requires CSF-1R for their differentiation and function. Using in vivo two-photon microscopy imaging, it was shown that patrolling monocytes are actively recruited to the Aβ-laden vasculature in APP/PS1 mice, crawling against the blood flow toward vascular Aβ, phagocytosing deposits and detaching back into the bloodstream. Specific depletion of the patrolling monocyte population resulted in a reduction in vessel-associating monocytes and increased cerebral Aβ plaque. This loss in vascular Aβ clearance having a knock-on effect of increasing parenchymal plaque lends strength to the hypothesis that an equilibrium exists between parenchymal Aβ and circulating or perivascular Aβ. A loss in peripheral monocyte activity could drive early steps in CAA development and might be a compounding factor in AD where CAA development further exacerbates the mechanisms driving parenchymal plaque deposition. A similar approach in the TgCRND8 mouse model of AD focused on the resident perivascular macrophage population. Targeted depletion of this constitutively phagocytic population led to a five-fold increase in Aβ coverage of the CNS vasculature. Stimulation of perivascular macrophages turnover through the administration of chitin had the opposite outcome, reducing vascular levels of Aβ. Chitin-stimulated mice demonstrated a 60% reduction in Aβ coverage of CNS vessels, demonstrating the capacity of this cell population to drive vascular-associated Aβ clearance. Microglia and astrocytes were ruled out as contributors to this increased clearance, pointing towards a possible localisation of Aβ clearance mechanisms for vascular and parenchymal Aβ pathologies. The evidence for vasculature-specific Aβ clearance mechanisms was additionally supported by a recent microglia depletion study. Treating the 5XFAD mouse AD model with the PLX5622 CSF-1R inhibitor depleted microglia from the brain after 5 days. Loss of microglia was accompanied by a reduction of parenchymal Aβ plaque formation, supporting the function of microglia as initiators of Aβ plaque development. This loss of parenchymal Aβ was accompanied by an increase in Aβ association with the cerebrovasculature. This occurrence of CAA was absent in untreated 5XFAD mice, and claudin-5 levels were decreased in Aβ-associated vessels. It was interesting to note that PLX5622 treatment did not affect global levels of Aβ, but rather drove a plaque-to-vessel shift in Aβ deposition, the reverse of what was observed when patrolling monocytes were depleted. This indicates that microglia may perform a protective role in inducing plaque formation, shielding the cerebrovasculature from developing CAA. Microglia having a role in parenchymal Aβ clearance, versus the patrolling monocytes and resident perivascular macrophages regulating vascular clearance, could explain the separate pathologies of CAA and AD. As microglia begin to fail or
become chronically pro-inflammatory, Aβ deposition could shift towards the vasculature in later stages of AD leading to secondary CAA development.

3.3 Results

3.3.1 Characterisation Of ALSP Kindred Pedigrees And CSF1R Variants.

The first case of ALSP brought to our attention was an individual who presented in her fifth decade with dementia and motor function deficits in line with ASLP. Her brain was donated to the Dublin Brain Bank and was found to have post mortem neuropathology in line with the disease (Figure 3.1). Macroscopically, there was an enlargement of the ventricles and atrophy present in the frontal lobe (Figure 3.1 a, b). On a molecular level, there was extensive axonal demyelination and the presence of axonal spheroids which stained positive for neurofilament (Figure 3.1 c, d). Two surprising findings were the presence of perivascular deposition of Aβ and phosphorylated Tau protein (p-Tau) (Figure 3.1 e-h). These pathologies are more commonly found in Alzheimer’s disease and chronic traumatic encephalopathy, respectively, indicating that there may be a BBB component in the disease. The individual’s sister was also deceased and it was possible to obtain FFPE tissue of both brains for immunohistochemical analyses and DNA extraction. A family pedigree was constructed (Figure 3.2, a) with the original case (II-B), her sister (II-C) and her two children (III-A, III-B) affected. Of the two affected individuals in generation III, one is currently in a nursing home while the other has begun to display symptoms in his fifth decade. Sequencing of the CSF1R gene using DNA isolated from FFPE tissue confirmed the presence of heterozygous a nine base-pair (bp) in-frame deletion (c2342_2350del) in exon 19 (Figure 3.2, c). This deletion leads to the loss of A781, R782 and N783 in the kinase region of CSF-1R. These three amino acid residues, highly conserved across mammals and fish, comprise a left-handed helical domain in the structure of the protein (Figure 3.2, e). Loss of such a highly conserved region was predicted to be deleterious for the function of the protein. Inputting the single loss of A781, R782 and N783 gave predicted deleterious scores of -11.63, -12.5 and -13.33 respectively in Protein Variation Effect Analyzer (PROVEAN, J. Craig Venter Institute). The PROVEAN tool computes whether an amino acid change has a biological impact on protein function by comparing homologous protein sequence conservation. The loss of all three resides had a cumulative score of -22.494, again with a deleterious prediction.

Our collaborating neuropathologist had published in 2003 on a case of late-onset neuroaxonal leukoencephalopathy with spheroids (NALS) which displayed rare CAA. This individual had
presented in his fifth decade with dementia and personality changes, and their clinical state progressively deteriorated before death occurred 7 years post-onset. We requested tissue from this case and sequenced CSF1R, finding a heterozygous cytosine to guanine transversion in exon 18 (c.C2471G). This change would result in a proline to arginine substitution at residue 824, which is located in the activation loop of CSF-1R. This substitution has a PROVEAN score of -7.338, predicting a deleterious effect, allowing the re-classification of this diagnosis from NALS to ALSP. A pedigree for this family was produced and sequencing confirmed (Figure 3.2, b, d), with two of individual I-B’s offspring being affected. At the beginning of this project, individual II-C was hospitalised, allowing for initial blood collection and DNA isolation. During this research, II-C’s condition deteriorated and they passed away in 2019, with their brain also being donated to the Dublin Brain bank. We confirmed the presence of the mutation in individual II-B using the sequencing service Centogene (Genotgene, Germany). Clinical Laboratory Improvement Amendments (CLIA)-accredited sequencing was required as individual II-B had yet to receive a diagnosis through the healthcare system and required an official gene test result.
Figure 3.1: Neuropathology of the original ΔA781_N783 ALSP case. (a) Extensive atrophy is evident in the frontal lobe. (b) Cystic encephalomalacia is evident in the right inferior frontal lobe secondary to a historical intraparenchymal haemorrhage. (c) Secondary de-myelination observed with Luxol-Fast Blue stain. (d) Wallerian degeneration observed with spheroids apparent following phosphorylated neurofilament staining. (e, f) Profound CAA, involving meningeal and intraparenchymal blood vessels. (g, h) Extensive phosphorylated Tau staining displaying a perivascular pattern of deposition. (Autopsy performed by Prof Michael Farrell, Beaumont Hospital).
Figure 3.2: Heterozygous CSF1R mutations in two familial cases of ALSP. Genetic pedigrees and sequencing of two familial cases of ALSP. In the pedigree shown in (a), a nine base-pair in-frame deletion in individuals II-A and II-B (b) resulted in a deletion of an alanine-arginine-asparagine loop (e). A cytosine to guanine transversion in the second family (b) led to a proline to arginine replacement in individuals II-B and II-C (e). Protein changes are indicated above family pedigrees, with the genetic mutations indicated below Sanger sequencing of affected individuals. Molecular modelling displaying the locations of the P824 (red) and A781_N783 (purple) regions, and their conservation across six species is shown in panel (e), kindly provided by Dr Savvas Savvides, Ghent University.
Perivascular accumulation of p-Tau and Aβ is indicative of a vascular component in ALSP. This component could either be a direct dysfunction of cells within the NVU or dysregulation of events localised at the vasculature such as perivascular macrophage activity. We first sought to determine whether the pathologies observed in these familial ALSP cases included BBB breakdown. Figure 3.3 shows IHC of post mortem donor tissue from carriers II-B and II-C of the A781_N783 CSF-1R variant, as well as carrier I-B of the P824R variant. Staining of the cortical sulci for Aβ and claudin-5 (Figure 3.3, a) confirmed the presence of CAA around larger, non-capillary vessels in each case, although individual II-C from the A781_N783 kindred had a markedly less severe pathology. Claudin-5 staining also appeared to be more continuous in the less severe case, lacking the speckled distribution seen in Aβ-laden vessels of II-B and I-B. This less severe pathology also correlated with BBB integrity. Staining for blood macromolecules human immunoglobulin-G (hIgG) and fibrinogen (Figure 3.3, b, c) revealed BBB leakage in individuals II-B and I-B. BBB integrity appeared to be maintained in individual II-C, with no visible hIgG or fibrinogen extravasation. This increase in BBB permeability, allowing 150 kDa hIgG and 340 kDa fibrinogen passage into the parenchyma, may be preceded by CAA. The loss of BBB integrity was not specific to either CSF-1R variant, indicating that this difference is likely due to individual disease progression rather than a genotype-specific effect.

As CSF-1R has a well-established role in macrophage and microglial function, we next aimed to examine the macrophage and glial populations present within the ALSP brain. As mentioned in 3.2.4, the absence of macrophage subsets can drive the development of CAA. Staining for glial fibrillary acidic protein (GFAP), a marker for reactive astrocytes, showed the presence of perivascular astrogliosis (Figure 3.4, a). This astrogliosis was not accompanied by microglial activation, as Iba1+ microglia displayed a ramified morphology even in the presence of reactive astrocytes (Figure 3.4, b). The presence of microglia in the context of CSF1R mutations was not surprising given their presence in mouse models of ALSP. CD163+ perivascular macrophages were also present in the perivascular compartment, associating with the vasculature along with CD68+ cells (Figure 3.4, c, d). These CD68+ cells were present at the vasculature independent of CAA severity. In the case of II-B, recruitment of CD68+ cells to an Aβ-laden vessel was observed, with CD68+ cells associating directly with the deposited Aβ.

Initial staining of cortical sections for tight junction components occludin and ZO-1 (Figure 3.5) suggested a potential loss in occludin expression. While there was robust ZO-1 immunopositivity at the vasculature, occludin immunopositivity was weak. Repeating the IHC with a second
occludin antibody, co-staining for claudin-5, revealed this to be an artefact from poor antibody performance with a strong overlap in occludin and claudin-5 immunopositivity (Figure 3.6).
Figure 3.3: CAA and BBB breakdown in ALSP. Immunohistochemistry of vessels of the cortical sulci showing perivascular Aβ deposition (green) and speckled claudin-5 (red) staining (a). BBB breakdown is present in two of the three examined cases, with hlgG (green) extravasation beyond the pericytes labelled by PDGFRβ (red) in (b). Fibrinogen (green) extravasation is also present in the same cases, exuding beyond the claudin-5 (red) stained vessels in (c). CSF-1R variants in white indicate the familial mutation carried in each case and scale bars indicate 30 μm. Note that there is no claudin-5 staining the third panel of (c).
Figure 3.4: The cerebral macrophage landscape of ALSP. (Figure legend on the following page).
Figure 3.4 (previous page): The cerebral macrophage landscape of ALSP. Immunohistochemistry of cortical sulci sections showing astrocytic GFAP expression (green, a and b). Iba1+ microglia (red, b) are present in all three cases, displaying a ramified morphology even in the presence of perivascular astrogliosis indicated by GFAP signal intensity (green, b). The presence of perivascular macrophages is confirmed by CD163 staining (green, c). Vessels are marked by claudin-5 (red) staining in both (a) and (c). Phagocytically active CD68+ (red, d) perivascular cells are present in all three cases, even in the context of weak Aβ (green, d) signal intensity. CSF-1R variants in white indicate the familial mutation carried in each case and scale bars indicate 30 μm.
Figure 3.5: Immunohistochemistry of cortical sulci sections showing overlapping occludin and ZO-1 expression. Occludin (green) and ZO-1 (red) staining of cortical sulci sections. Occludin staining is minimal due to poor Invitrogen 33-1500 antibody performance, shown by superior staining in Figure 3.5. CSF-1R variants (left) indicate the familial mutation carried in each case and scale bars indicate 30 μm.
Figure 3.6: Immunohistochemistry of cortical sulci sections showing overlapping occludin and claudin-5 expression. Occludin (green, NBP1-87402) and claudin-5 (red) expression in cortical sulci sections. CSF-1R variants (left) indicate the familial mutation carried in each case and scale bars indicate 30 μm.
A potential confounding factor with the use of post mortem tissue is that the results obtained are from end stages of the disease, following hospitalisation and secondary events. To determine whether BBB dysfunction could precede or coincide with the clinical onset of ALSP, MRI was conducted on individual III-B from the A781_N783 kindred. III-B is a male in his fifth decade presenting with mild motor dysfunction. From T2-weighted and FLAIR imaging, dorsal non-fluent asymmetrical white matter hyperintensities were present in the left hemisphere. Symmetrical central and biparietal periventricular hyperintensities were present as well (Figure 3.7, a, b). The presence of an asymmetrical and largely parietal pathology correlated with the published neuroimaging studies of early-stage ALSP.

Using dynamic contrast-enhanced MRI (DCE-MRI) with injected gadobentate dimeglumine (Gd-BOPTA), it was possible to quantify the extent of Gd-BOPTA extravasation from the vasculature into the brain parenchyma. The linear dynamic model of quantifying Gd-BOPTA extravasation measures the accumulation of contrast agent in the brain over the duration of a scan. This approach measures the rate of contrast agent accumulation assuming a constant $K_{\text{trans}}$, or rate of contrast agent transfer from the blood to the parenchyma and vice versa. The extent of BBB dysfunction can then be determined by plotting increments of contrast agent accumulation and determining the slope of the plotted data. Voxels in the 95th percentile or higher were determined to be a measure of increased Gd-BOPTA extravasation. This method has recently been published by the Campbell lab and collaborators in O’Keeffe et al.\textsuperscript{318} and the collected baseline data sets from 50 control subjects were used here for normalising the data to a common baseline and generating the percentile classifications (Figure 3.8). BBB dysfunction was evident in individual III-B, particularly in the deep white matter of the anterior and posterior regions of the brain. The prevalence of BBB dysfunction this early in the disease course indicates that BBB dysfunction may be one of the early events in the progression of ALSP, rather than a secondary effect due to cerebral deterioration.
Figure 3.7: T2-weighted MRI and FLAIR of a carrier of the ΔA781_N783 CSF1R variant. MRI of a presymptomatic individual with a confirmed mutation in CSF1R. White arrows indicate white matter hyperintensities detected in T2 (a) and FLAIR (b) imaging. Hyperintensities still displayed some asymmetry in dorsal regions (left) while periventricular hyperintensities were symmetrical (centre, right).
Figure 3.8: Slope over T1 quantification (Linear Dynamic Model) of in vivo BBB integrity. T1 weighted DCE-MRI of individual III-B from the A781_N783 kindred. Values and colour mapping (right) indicate the rate of Gd-BOPTA accumulation. MRI analyses performed by Dr Eoin O’Keeffe.
3.3.4 Variant CSF-1R Has Reduced Expression

While CSF1R mutations in ALSP are largely loss-of-function, some gain of function variants have been documented in the disorder\textsuperscript{319}. As neither of the variants present in the kindreds detailed in 3.3.1 had previously been reported, expression constructs were generated to validate the predicted deleterious impact. The cDNA sequence of native and ΔA781_N783 CSF1R was cloned into the pcDNA3-EGFP, and constructs were sequenced for confirmation (Figure 3.9 a, b). An expression construct for the P824R variant was generated from the native CSF1R expression plasmid using site-directed mutagenesis (Figure 3.9 a, c). All plasmid stocks were confirmed to be homogenous for respective CSF1R genotypes by Sanger sequencing before being used in further experiments. As CSF1R cDNA sequences were inserted into the AgeI/EcoRI site, the expression is driven by the constitutive CMV promoter and as such, any expression differences between constructs were predicted to be post-transcriptional.

To determine whether variant CSF-1R is expressed and properly localised to the cell membrane, constructs were transfected into HEK293 cells. Both homozygous and heterozygous conditions were generated to determine whether there was a dominant-negative effect of variant CSF-1R (Figure 3.10, a). As CSF-1R is processed in the Golgi apparatus of the cell, it undergoes a series of glycosylations before being localised to the cell membrane. This results in two bands when analysed by SDS-PAGE, the lower molecular weight immature isoform and the fully processed higher molecular weight membrane localised isoform. As shown in Figure 3.10 (a), both CSF-1R variants fully underwent Golgi processing, with membrane isoforms present at 24 and 48 h post-transfection. A striking difference in expression levels was observed between native and variant CSF-1R, with variant CSF-1R having reduced protein expression at both 24 and 48 h, with no corresponding decrease in mRNA expression (Figure 3.10, b). Co-transfection with native CSF-1R did increase the membrane isoform fraction, however, it did not rescue the reduced duration of expression. Even following Golgi processing, the membrane isoform may not display correct localisation and cell-surface expression. To determine whether variant CSF-1R was potentially sequestered and targeted for degradation prior to membrane localisation, ICC was performed for
CSF-1R, using the ΔA781_N783 expression construct (Figure 3.10, c). No difference in CSF-1R localisation was observed, with variant CSF-1R detected at the cell membrane.
Figure 3.9: CSF-1R Expression plasmid and verified sequences. CSF1R cDNA expression sequences were inserted into the pcDNA3-EGFP plasmid (a). Sanger sequencing in (b) and (c) show confirmed deletion (region bordered between yellow and blue highlights) and sequence change required to produce the ΔA781_N783 and P824R variants respectively.

Figure 3.10: Native and variant CSF-1R expression in HEK293 cells. (Figure legend on following page).
Figure 3.10: Native and variant CSF-1R expression in HEK293 cells. (a) Western blot of whole-cell lysates from HEK293 cells for CSF-1R and Actin. The higher molecular weight band for CSF-1R represents fully-processed and membrane localised protein, indicating membrane expression is present in all scenarios. Cells were transfected with 600 ng of native or variant CSF-1R expression constructs as indicated above or 300 ng of each in the case of dual plasmid transfection. (b) Corresponding densitometry of blots presented in (a) with mRNA quantification below. (c) Immunocytochemistry confirming membrane localisation of the ΔA781_N783 variant (**** p < 0.0001, *** p < 0.0005, ** p < 0.005 one-way ANOVA with Sidak's post-test for multiple comparison, n = 3 biological replicates).
3.3.5 Variant CSF-1R Lacks Signalling Capacity And Is Targeted For Degradation

As variant CSF-1R was found to be fully processed and localised to the membrane, we next decided to determine whether the variant proteins retained any signalling capacity. As detailed in 1.3.4, ERK1/2 is phosphorylated following CSF-1R activation allowing it to be used to determine receptor signalling capacity. HEK293 cells transfected with native, variant or both native and variant CSF-1R expression constructs were serum-starved to ablate endogenous ERK1/2 activity. Following treatment with either CSF-1 or IL-34, cell lysates were examined for phosphorylated-ERK1/2 to determine whether CSF-1R signalling was intact. As shown in Figure 3.11 and Figure 3.12, 10, 50 and 100 ng/mL concentrations of CSF-1 induced robust phosphorylation of ERK1/2 at 10 min in cells expressing native CSF-1R. A similar response was also observed in native CSF-1R expressing cells treated with 50 and 100 ng/mL of IL-34, indicating that treatment conditions were sufficient for CSF-1R activation. In contrast, cells expressing either variant form of CSF-1R showed no response to ligand treatment. In cells expressing both native and variant CSF-1R, lower levels of ERK1/2 phosphorylation were observed, indicating that variant CSF-1R did not display a dominant-negative effect. As an equal total amount of plasmid was transfected for each treatment, the reduced ERK1/2 phosphorylation observed in the dually transfected cells is likely due to only half as much native CSF-1R being expressed. To confirm an absence of CSF-1R signalling, rather than a reduction in the rate of activation, the signalling time-course was extended to 30 min using CSF-1 which was shown to produce stronger ERK1/2 phosphorylation in Figure 3.13. At 30 min post-treatment with CSF-1, there was no increase in phosphorylated-ERK1/2 in cells expressing ΔA781_N783 (Figure 3.13, a) or P824R (Figure 3.13, b) CSF-1R, confirming a loss of signalling capacity rather than a reduced rate of activation.
Figure 3.11: Western blot for phosphorylated and total ERK in HEK293 cells transfected with native and ΔA781_N783 CSF-1R and treated with CSF-1 (a) or IL-34 (b) for 10 minutes, at 36 h post-transfection. Horizontal line indicates untreated cells, with increasing concentrations 10, 50 and 100 ng/ml. Corresponding densitometry displays the ratio of phospho-ERK to total ERK, normalised to the ratio of the untreated control for the native receptor (***, p < 0.0001, ***, p < 0.005, **, p < 0.01, *, p < 0.05, one-way ANOVA with Sidak’s post-test for multiple comparison, n = 3 biological replicates).
Figure 3.12: Western blot for phosphorylated and total ERK in HEK293 cells transfected with native and P824R CSF-1R and treated with CSF-1 (a) or IL-34 (b) for 10 minutes, at 36 h post-transfection. Horizontal line indicates untreated cells, with increasing concentrations 10, 50 and 100 ng/ml. Corresponding densitometry displays the ratio of phospho-ERK to total ERK, normalised to the ratio of the untreated control for the native receptor (**** p < 0.0001, *** p < 0.0005, ** p < 0.01, * p < 0.05, one-way ANOVA with Sidak’s post-test for multiple comparison, n = 3 biological replicates).
Figure 3.13: CSF-1 treatment for 30 min does not reveal a slower activation state. Western blot of whole-cell lysates from HEK293 cells transfected with native, ΔA781_N783 (a) or P824R (b) CSF-1R expression constructs. Cells were transfected with 600 ng of plasmid, or 300 ng of each in the dual transfection conditions. The horizontal line indicates cells without ligand treatment, with increasing concentrations 10, 50 and 100 ng/mL CSF-1. Cells were lysed at 30 min post-treatment.
A caveat to the signalling study reported above is that variant CSF-1R was shown to have reduced and more transient expression compared to the native isoform. A lower rate of expression or active targeting of the variant receptor for degradation would impact the receptor’s ability to dimerise and activate downstream signalling pathways. We next sought to identify the mechanism through which variant CSF-1R was being degraded, hypothesising that targeting this pathway may restore variant CSF-1R levels sufficiently to rescue signalling. Identification of CSF-1R post-translational regulatory mechanisms could also provide a means of restoring haploinsufficiency in ALSP patients. Reviewing the literature, the lysosomal inhibitor 3-methyladenosine (3-MA) has previously been shown to reduce signalling-induced CSF-1R degradation, while the proteasomal inhibitor MG132 failed to do the same.

As 3-MA is an inhibitor of autophagy, we first investigated the effects of autophagy induction and inhibition on CSF-1R expression using rapamycin and 3-MA respectively. HEK293 cells were again transfected with native, ΔA781_N783 or P824R CSF-1R and treated 24 h later with rapamycin or 3-MA. Rapamycin is an inhibitor of the mammalian target of rapamycin (mTOR) pathway and can upregulate autophagy through releasing mTORC1-mediated inhibition of Unc51 Like Autophagy Activating Kinase 1 (ULK1). Inhibition of mTOR has also more recently been shown to also increase global protein degradation pathways, including the ubiquitin-proteasome system (UPS). Treatment with rapamycin led to a significant 4- and 2.3-fold increase in native and ΔA781_N783 CSF-1R isoforms respectively (Figure 3.14). While increases in P824R levels did not reach significance, there was a nearly 2-fold increase in response to treatment. A similar but more potent increase was seen in cells treated with 3-MA, which can inhibit autophagy through PI3K-mediated formation of the autophagosome. In response to 3-MA, native, ΔA781_N783 and P824R CSF-1R levels were each significantly increased (Figure 3.15). In contrast to what was observed in rapamycin-treated cells, the P824R isoform was most responsive to 3-MA, increasing 8.3-fold while the ΔA781_N783 and native CSF-1R isoforms increased by 7.4 and 6.3-fold respectively. Although in both rapamycin and 3-MA treated cells the total levels of CSF-1R were increased, an interesting trend is the opposing sensitivities of CSF-1R isoforms to the respective inducer and inhibitor of autophagy.

As targeting of autophagy failed to reveal the means through which variant-specific CSF-1R is targeted for degradation, we next examined the effect of UPS inhibition. As previously mentioned, treatment with the proteasome inhibitor MG132 has been shown to have no effect on activation-induced CSF-1R degradation. Using PLX3397 as a positive control for CSF-1R degradation, transfected cells were treated at 24 h post-transfection with either PLX3397 or
MG132 for 7 h. A variant-specific increase in CSF-1R expression in response to MG132 was observed, with a 2.8 and 2.9-fold increase of ΔA781_N783 and P824R respectively, and no change in native CSF-1R levels (Figure 3.16). With a means to now selectively upregulate variant CSF-1R, we next repeated the signalling experiment performed in Figure 3.13, this time pre-treating native and ΔA781_N783-transfected cells with MG132 to upregulate variant CSF-1R levels. Treatment with CSF-1 again induced robust ERK1/2 phosphorylation at each timepoint in native CSF-1R expressing cells (Figure 3.17, a), and to a lesser extent in cells expressing both native and ΔA781_N783 CSF-1R (Figure 3.17, c). Despite MG132 pre-treatment, however, no signalling was restored in cells expressing ΔA781_N783 CSF-1R alone (Figure 3.17, b). This result confirmed that variant CSF-1R observed in these cases of ALSP was indeed incapable of signalling and that the previously observed signalling deficit was not simply due to a low rate of expression.
Figure 3.14: Rapamycin increases cell CSF-1R concentrations. Western blot for CSF-1R in HEK293 cells transfected with native and ΔA781_N783 (top) or P824R (bottom) CSF-1R and treated with rapamycin (a). Horizontal line indicates untreated cells, with concentrations 0.1, 0.5, 1, 5, 10μM. Corresponding densitometry (b) shows densitometry expressed as fold-changes relative to the untreated control for each genotype (black bar) (one-way ANOVA with Dunnett post-test for multiple comparisons to untreated, **p < 0.005, ***p < 0.0005, n = 3 separate cell transfections and treatments).
Figure 3.15: 3-MA increases cell CSF-1R concentrations. Western blot for CSF-1R in HEK293 cells transfected with native and ΔA781_N783 (top) or P824R (bottom) CSF-1R and treated with 3-MA (a). Horizontal line indicates untreated cells, with concentrations 0.1, 0.2, 0.5, 1mM. Corresponding densitometry (b) shows densitometry expressed as fold-changes relative to the untreated control for each genotype (black bar) (one-way ANOVA with Dunnett post-test for multiple comparisons to untreated, *p < 0.05, ***p < 0.0005, ****p < 0.0001, n = 3 separate cell transfections and treatments).
Figure 3.16: MG132 preferentially increases variant CSF-1R concentrations. Western blot for CSF-1R and Actin in HEK293 cells transfected with native and ΔA781_N783 (top) or P824R (bottom) CSF-1R and treated with MG132 or PLX3397 (a). Horizontal line indicates untreated cells, plx indicates treatment with 20μM PLX3397, with increasing MG132 concentrations 0.1, 0.5, 0.1, 5μM. Corresponding densitometry (b) shows densitometry expressed as fold-changes relative to the untreated control for each genotype (black bar) (one-way ANOVA with Dunnett post-test for multiple comparisons to untreated, *p < 0.05, **p < 0.005, n = 3 separate cell transfections and treatments).
Figure 3.17: MG132 pretreatment does not restore signalling. Western blot for phosphorylated and total ERK1/2, and CSF-1R in HEK293 cells transfected with (a) native CSF-1R, (b) ΔA781-N783 CSF-1R or (c) both native and ΔA781-N783 CSF-1R. Cells were treated with 5μM MG132 for 1 h, followed by treatment with CSF-1 for 5, 10 or 30 min. The horizontal line indicates cells without ligand treatment, with increasing concentrations 10, 50 and 100 ng/mL CSF-1.
3.3.6 The Proteasomal Targeting Of Variant CSF-1R Is Not Accompanied By Cell Stress

As variant CSF-1R had now been shown to be actively targeted by the UPS, we next determined whether this increased burden in proteasomal degradation was accompanied by a response of the unfolded protein response (UPR). Toxic accumulation of mutant or misfolded proteins within the cell can lead to endoplasmic reticulum stress and activation of the UPR. The UPR can then upregulate chaperone protein expression in an attempt to clear aggregated protein, or can ultimately induce apoptosis. We used a qPCR array approach to examine whether genes associated with cell stress and toxicity were differentially expressed in cells expressing native or P824R CSF-1R. As shown in Figure 3.18, only 2 genes out of 84 differed significantly in native and P824R CSF-1R expressing cells. CXCL8, which encodes for CXCL8, and DDIT3 which encodes for DNA damage-inducible transcript 3 (DDIT3) (also known as the C/EBP homologous protein (CHOP)) were both upregulated in native CSF-1R expressing cells relative to cells expressing P824R CSF-1R. As these were the only differentially expressed genes, out of the profile of 84 examined genes (full list in 2.19), these changes are likely a product of native CSF-1R activity rather than a cell response to CSF-1R accumulation.

An immediate response to protein accumulation may not be captured at the transcript level, as such the protein levels of chaperone heat shock protein 90 (HSP90) and heat shock protein 40 (HSP40) were also examined. No change in either chaperone protein was seen in cells expressing any of the three CSF-1R isoforms at 24 h or 48 h (Figure 3.19, a), confirming the PCR array results for P824R. Transcript levels of CXCL8 were also confirmed by qPCR for all three CSF-1R isoforms, as cells expressing the ΔA781_N783 CSF-1R isoform had not been included in the PCR array. CXCL8 expression was increased in all transfected cells, however, there was a lower expression in cells expressing variant CSF-1R (Figure 3.19, b), and this was also observed when secreted CXCL8 levels were examined by ELISA (Figure 3.19, c).
Figure 3.18: Stress and toxicity associated gene expression changes in P824R versus native CSF-1R expressing cells. Graphical representation of gene expression changes detected by the RT² Profiler™ PCR Array Human Stress & Toxicity PathwayFinder. The black line indicates a fold change ($2^{-\Delta Ct}$) of 1. The purple lines indicate a 2-fold change in gene expression. Blue dots represent genes which did not display significant changes in expression. The red and yellow dots represent CXCL8 and DDIT3 respectively which were significantly downregulated in the P824R samples. (Relative to native CSF-1R expressing cells: CXCL8, -4.3 fold change, $p = 0.000203$; DDIT3, -2.11 fold change, $p = 0.001122$; n = 3 independent transfections per genotype)
Figure 3.19: Chaperone protein levels and CXCL8 expression following CSF-1R expression construct transfection. (a) Western blot of cell lysate from untreated (Unt) and transfected HEK293 cells for CSF-1R, GAPDH (red arrow), HSP90 and HSP40. Cells were transfected with empty vehicle (EV), native, ΔA781_N783 or P824R CSF-1R. Altered CXCL8 mRNA expression (b) and secretion (c) were quantified at the 24 h timepoint by qPCR and ELISA respectively. (one-way ANOVA with Dunnett post-test for multiple comparisons to native CSF-1R transfected samples, *p < 0.05, **p < 0.005. RNA was isolated from n = 2 independent transfections for (b), and media was collected from n = 3 independent transfections for (c).)
3.3.7 ALSP Donor Macrophages Are Dysmorphic And Have A Reduced Phagocytic Capacity

While CSF-1R is essential for microglial viability, it also serves a role in peripheral macrophage differentiation and activity. Furthermore, a loss in peripheral macrophage function has been implicated in the aetiology of CAA as discussed in 3.2.4. Through contacting clinicians involved in the original cases of ALSP in the kindreds involved in this project, we were able to collect peripheral blood from individual III-A and individual II-C from kindreds carrying ΔA781_N783 and P824R CSF-1R respectively.

Initial analyses of cell populations within donor ALSP PBMCs uncovered the presence of an increased number of lineage negative cells compared to control populations (Figure 3.20). These consist of cells which do not express cell surface markers of T-cells (CD3), macrophages (CD14), natural killer cells and neutrophils (CD16, CD56), or mature (CD19) and maturing (CD20) B-cells. This increase could be attributed to reduced CSF-1R driven differentiation, although there was no consistent trend in T-cell, B-cell or macrophage counts when examined by genotype (Figure 3.21). To further define the differentiation capacity of ALSP monocytes, monocyte-derived macrophages (MDMs) were produced from donor PBMCs. There was a striking difference between the produced macrophages, with 30% of macrophages produced from variant CSF-1R expressing monocytes having a shrivelled and more rounded morphology (Figure 3.22). These dysmorphic cells were reminiscent of those reported in Yu et al. (2008); monocytes with homozygous CSF-1R tyrosine replacements displayed aberrant morphologies upon differentiation into macrophages. This strong effect of a heterozygous state of CSF-1R functionality was hypothesised to extend to the phagocytic capacity of these macrophages, as deficits in peripheral macrophage phagocytosis have already been attributed to the development of CAA. Using pre-opsonised latex beads, the phagocytic capacity of native and variant CSF-1R expressing MDMs was found to be significantly different (Figure 3.23). While bead uptake occurred in 43 % of native CSF-1R expressing MDMs, this was reduced to only 27 % of ΔA781_N783 and 25 % of P824R CSF-1R expressing MDMs. To rule out potential secondary factors such as ALSP donor infection or lifestyle, this was repeated using mouse bone marrow-derived macrophages (BMDMs). BMDMs were produced from wild-type mice (Csf1r+/−; Cre−) mice and mice heterozygous for Csf1r in macrophages (Csf1r+/−; Cx3cr1-Cre+) or endothelial cells (Csf1r+/−; Tie2-Cre+). Loss of one copy of Csf1r in macrophages was sufficient to yield a significant decrease in phagocytic activity, replicating what was observed in the case of macrophages produced from ALSP donor PBMCs.
Figure 3.20: FACS gating parameters and lineage counts of ALSP PBMCs. Gating for live cells using side and forward scatter indices and LiveDead Aqua staining (a and b respectively). (c) lineage staining using the CD3/14/16/19/20/56 cocktail. (FACS analysed by Dr Kiva Brennan)
Figure 3.21: Lymphocyte populations in ALSP PBMCs. FACS results for CD3 (T-cells), CD14 (macrophages) and CD19 (B-cells) from ALSP donor PBMCs. (FACS analysed by Dr Kiva Brennan)
Figure 3.22: Macrophages generated from ALSP donor monocytes are dysmorphic. (a) Immunocytochemistry of macrophages differentiated in vitro from control, ΔA781_N783 or P824R PBMCs. Cells were stained for DAPI (blue) and CD68 (red). White arrows show dysmorphic macrophages, quantified in (b) scale bars indicate 30 μm. (** p < 0.0005, one-way ANOVA with Dunnet’s multiple comparison test, n=4 differentiations).
Figure 3.23: Phagocytosis is reduced in ALSP and Csf1r<sup>−/−</sup> macrophages. Immunocytochemistry of macrophages differentiated in vitro from control, ΔA781_N783 or P824R PBMCs (a) and mouse bone marrow (b). Macrophages were stimulated with LPS and exposed to fluorescent opsonised latex beads for 1 hour before fixation and quantification of bead+ cells via microscopy. Cells were stained using MitoTracker (red) and DAPI (blue), genotypes of mice are given above images. (c, d) Quantification of phagocytic activity expressed as percentage bead+ cells, blank-corrected to values collected at 4 °C. (P-values were calculated using ANOVA with Tukey’s multiple comparison test, *p < 0.05, **p < 0.005, n = 4 independent assays, with means of each assay shown on each graph.)
3.3.8 Microglial Phagocytosis Is Not Similarly Inhibited By Csf1r Loss.

A deficit in peripheral macrophage phagocytosis could contribute to reduced clearance of vascular Aβ, increasing the rate of accumulation around the CNS vasculature. A corresponding decrease in microglial phagocytosis could further increase cerebral levels of Aβ. To determine the impact of Csf1r loss on microglial phagocytosis, a latex bead assay was performed using the BV2 microglial cell line. Treatment with Csf1r targeted siRNA effectively downregulated CSF-1R protein expression and phagocytosis was unaffected by this inhibition (Figure 3.24, a-c). We additionally isolated primary microglia from the mouse lines used in the BMDM assay to examine whether heterozygous genetic loss of Csf1r could impact phagocytic capacity. While these microglia did exhibit higher levels of phagocytosis than the BV2 cell line, there was no effect of heterozygous Csf1r loss on phagocytic capacity (Figure 3.24, d, e). This surprising resilience of microglia to Csf1r loss highlighted a CSF-1R dependency limited to the peripheral macrophage compartment in the context of phagocytosis.
Figure 3.24: Phagocytosis is not reduced in Csf1r deficient microglia. (Figure legend on following page)
Figure 3.24: Phagocytosis is not reduced in Csf1r deficient microglia. (a) Western blot of lysate from BV2 microglia treated with a Csf1r targeting siRNA. (b) Quantification of phagocytic activity of cells assayed in (c) expressed as percentage bead+ cells, blank-corrected to values collected at 4 °C. (c) Immunocytochemistry of BV2 cells untreated or treated with non-targeting (NT) or Csf1r targeting siRNA. BV2 cells were stimulated with LPS and exposed to fluorescent opsonised latex beads for 1 hour before fixation and quantification of bead+ cells via microscopy. Cells were stained using MitoTracker (red) and DAPI (blue). (d) Immunocytochemistry of microglia isolated from neonatal mouse brains and subjected to the same assay as (c). Genotypes are given above the images. (e) Quantification of phagocytic activity of cells assayed in (d) expressed as percentage bead+ cells, blank-corrected to values collected at 4 °C. (P-values were calculated using ANOVA with Tukey’s multiple comparison test. For BV2 cells the experiment was performed in duplicate with n = 2 coverslips quantified per replicate. For primary microglia, n = 4 independent assays were performed.)
3.4 Discussion

Here, we have provided a characterisation of ALSP donor brains with a focus on the cerebrovasculature, an aspect under-represented in the current literature on the disease. We have identified two ALSP kindreds with unreported variants in CSF1R and constructed pedigrees for each. This has allowed us to match IHC analyses of donated post-mortem tissue to \textit{in vivo} neuroimaging and peripheral leukocyte characterisation. In this chapter, we present three cases of ALSP that demonstrate CAA as an addition to the documented pathology of the disease. Severe BBB breakdown was also present in two of these cases, allowing macromolecules as large as 340 kDa entry into the brain parenchyma. Furthermore, perivascular astrogliosis was present in each case with astrocytes potentially being activated by pro-inflammatory mediators extravasating into the parenchyma. As discussed previously, there is strong evidence that an absence of peripheral and CNS macrophage populations can contribute to the generation of CAA. Despite carrying heterozygous mutations in CSF1R, the perivascular macrophage compartment was intact with CD163$^+$ and CD68$^+$ cells present at the cerebrovasculature, and microglia displayed a surveilling ramified morphology. Iba1$^+$ cells did not display a clustering around the cerebrovasculature, and together with CD163 being a specific marker for perivascular macrophages this indicates that CAA in ALSP is not simply due to an absence of peripheral macrophages. In the ALSP case with reduced perivascular Aβ deposition, the blood-brain barrier was intact to the large molecules investigated. Whether BBB dysfunction precedes or follows Aβ deposition and neurodegeneration is a critical question in CAA. If BBB dysfunction is a preceding and causative factor in the disease, it becomes a promising prophylactic therapeutic target, being easier to deliver drugs to than the parenchyma itself. Using DCE-MRI, we have demonstrated that detectable BBB dysfunction is present at the onset of clinical ALSP, with increased permeability to the ~667 Da contrast agent Gd-BOPTA. This loss of BBB integrity may contribute to progressive symptoms such as seizure in ALSP, allowing epileptogenic blood components such as the 66 kDa albumin into the parenchyma as permeability increases to the extent observed at post-mortem$^{324}$.

As the CSF1R variants presented in this project had yet to be reported and characterised, we performed a series of experiments to determine their activity and expression. Using the PROVEAN software to predict the impact of amino acid changes on protein structure, it was predicted that both variants would be deleterious to the function of CSF-1R. Through the generation of plasmid expression constructs, we have shown here that these CSF-1R variants not only are unresponsive to ligand treatment but also are actively targeted for proteasomal degradation. This active
targeting is an important finding as CSF-1R functions as a dimer, with native-native, native-variant and variant-variant dimerisations theoretically possible. Should there be an equal expression of both functional and variant CSF-1R, which we have shown to be fully processed and membrane localised, this would reduce the fraction of active CSF-1R dimers to 25% at the cell membrane. Active targeting of variant CSF-1R for degradation may serve to partially rescue this effect, increasing the ratio of native to variant CSF-1R at the cell membrane and promoting native-native dimer conformations.

A conflicting result in these experiments was the conserved response of CSF-1R to inhibitors and activators of autophagy, with CSF-1R protein levels increasing in response to both. A possible explanation of this could be the reported dual ability of 3-MA to induce and inhibit autophagy through manipulation of PI3K isoform activity. The mTOR pathway inhibited by rapamycin similarly impacts PI3K activation, and this could contribute to the signalling cascades occurring at the membrane following early CSF-1R activation. These effects of rapamycin and 3-MA may serve to inhibit non-specific recycling of CSF-1R independent of autophagy. In contrast, direct inhibition of the proteasome with MG132 yields an isoform-specific effect, restoring variant CSF-1R protein levels. As such, variant CSF-1R must be targeted by ubiquitin ligases, or perhaps through early or spontaneous activation of the recruited c-Cbl E3 ubiquitin ligase, to promote its proteasomal degradation. Indeed, we did not assess whether ligand binding and early-stage CSF-1R activity, which would involve c-Cbl recruitment, were present in the variant isoform. Should early-stage activation still occur, this would initiate recruitment of PI3K complexes which could then be subjected to the effects of 3-MA and rapamycin.

Despite the increased burden on protein turnover from variant CSF-1R transfection, there were few differential changes in gene expression indicating cell stress or toxicity in HEK293 cells. Out of a panel of 84 genes, the two differential changes in gene expression were in CXCL8 and DDIT3, of which CXCL8 was confirmed to be differentially expressed in native and variant CSF-1R expressing cells. It is of interest to note that both gene expression and secretion of the pro-inflammatory cytokine CXCL8 were increased under all plasmid transfection conditions. This may, therefore, be a response to plasmid transfection rather than a product of CSF-1R signalling itself. Repeating the experiment using a control, CSF1R-negative plasmid would help to clarify the source of these transcriptional changes. Native CSF-1R function may then enhance this upregulation or stimulate CXCL8 secretion, leading to the subsequent differences observed between CSF-1R isoforms. A limitation of this approach was the use of HEK293 cells, which may better tolerate protein turnover in comparison to neurons, endothelial cells or microglia. These cell types express endogenous CSF-1R however, which would confound potential results as a
homozygous variant CSF-1R state would be technically demanding to achieve, and endogenous CSF1R promoter expression would require silencing.

Analyses of ALSP donor PBMCs revealed an increase in lineage-negative cell counts that was consistent across both genotypes, potentially representing a pool of undifferentiated cells in the circulating blood. This population may be the one that is replaced with one responsive to differentiation factors following HSCT. ALSP donor PBMCs were dysmorphic and had a reduced phagocytic activity compared to those expressing native CSF-1R. To confirm that this decrease in phagocytosis was due to loss of CSF-1R, and not due change in ALSP circulatory cell populations or viability, macrophages were produced from mice with heterozygous genetic loss of Csf1r. Mouse macrophages with one copy of Csf1r (Cx3cr1-Cre+) displayed the same loss of phagocytic activity as those isolated from ALSP donor PBMCs. Tie2-Cre+ macrophages did not display a significant decrease in phagocytosis. This line was included in these analyses to examine whether the transient Tie2 expression reported in HPCs impacted macrophage function. The absence of a significant change in phagocytosis between the Tie2-Cre+ macrophages and either control or Cx3cr1-Cre+ indicated that LoxP excision by Cre-recombinase may be heterogeneous in Tie2-Cre+ macrophages depending on transient expression during HPC development. This crucially advised for later chapters that Tie2-Cre+ microglia and macrophages could not be considered truly homogeneously wild-type. These results from both ALSP donor and Csf1r+/− macrophages suggests that there may be a loss of peripheral macrophage phagocytosis contributing to CAA in ALSP. The presence of CD163 and CD68 positive cells at the BBB, therefore, is not fully indicative of efficient ongoing clearance processes within the ALSP brain. Additionally, Csf1r loss in microglia isolated from the same mouse lines had no impact on phagocytosis, further indicating a critical role for peripherally sourced macrophages in the clearance of vascular Aβ.

The data presented in this chapter indicates that CSF-1R variants in these two kindreds are loss of function, with the produced protein product actively degraded with no detriment to the cell. This finding confirms that haploinsufficiency for CSF1R can drive the development of CAA and affect BBB integrity as well as peripheral macrophage function. This basic finding allows for the use of a heterozygous CSF1R state as a model of events that may lead to CAA, ruling out potential gain-of-function, partial activity or secondary effects on cell viability. There has still yet to be any molecular biological study investigating the deterioration of ALSP patients. Whether the BBB changes observed in MRI precede or are the product of the neuropathological and glial pathologies is still unknown. As with most neuropathologies, early work on ALSP has been severely limited to analyses of postmortem tissue. This limitation has thus far made staging of
the disease quite difficult, and elucidating the cascade of molecular events driving symptom onset and progression has been near-impossible. Currently, progression can be monitored only through monitoring the increasing symmetry and fluency of white matter lesions, combined with an ongoing neurological assessment. These methods are secondarily limited to monitoring progression after the clinical onset of the disease, documenting rapid patient decline rather than causative biological changes. These limitations have led to a lack of a prognostic tool for those diagnosed with ALSP-associated mutations in CSF1R. A rapidly progressive disease such as ALSP would benefit immensely from a prophylactic treatment approach, targeting early events that precede clinical disease onset. Here, we suggest that BBB and peripheral macrophage function may be potential targets for early intervention and disease monitoring in ALSP.
Chapter 4:
Identifying A Role For CSF-1R In Endothelial Cell Maintenance
4.1 Abstract

In this chapter, we aim to validate a role for CSF-1R in endothelial cells and to determine the effects of CSF-1R loss in endothelial cells and associating microglia. From the work presented in Chapter 3, a loss in BBB integrity accompanies ALSP in cases with severe CAA. To determine whether this BBB dysfunction is directly due to loss of endothelial CSF-1R, we inhibited CSF-1R activity in the b.End3 cell line using PLX3397. Suppression of CSF-1R activity resulted in reduced occludin and claudin-5 expression in these cells, accompanied by an increase in endothelial monolayer permeability to a 4 kDa dextran. Furthermore, we demonstrate changes in claudin-5 and ZO-1 expression and increased permeability in primary mouse brain endothelial cell cultures following PLX3397 treatment. Intriguingly, activation of CSF-1R signalling through treatment with CSF-1 or IL-34 induced a turnover effect, with an acute reduction of tight junction protein expression followed by a later transcriptional upregulation. With CSF-1R having a well-established role in microglial homeostasis, we investigated the effects of resting and CSF-1R inhibited microglia on endothelial cells using conditioned media produced by the BV2 microglial cell line. Endothelial CSF-1R was found to be upregulated by microglia conditioned media, with inhibition of microglial CSF-1R diminishing this effect. In the case of occludin and ZO-1, changes in expression differed in response to conditioned media from resting or CSF-1R inhibited microglia. We further investigated whether this response to microglia conditioned media could prime endothelial cells for CSF-1 or IL-34 treatment through upregulation of CSF-1R. Ligand treatment of endothelial cultures pre-treated with conditioned media, however, revealed the effect of the microglia conditioned media to be the main determinant of observed tight junction expression changes. With microglia themselves having demonstrated CSF-1R dependent effects on tight junction expression, we next sought to elucidate whether microglial CSF-1R, endothelial CSF-1R or indeed a synergistic effect of both, determined this endothelial response to microglial conditioned media. Using primary endothelial and microglial cells isolated from mice lacking one copy of Csf1r, the CSF-1R signalling axis of both cell types were found to affect tight junction and CSF-1R pathway component gene expression. Endothelial cells heterozygous for Csf1r, but not wild-type endothelial cells, displayed a unique sensitivity to conditioned media produced by microglia heterozygous for Csf1r.
4.2 Introduction

4.2.1 Microglia

Although the brain is a relatively immune-privileged organ, it has active populations of specialised leukocytes which survey the CNS for infection, the build-up of toxic accumulates and entry of material from the blood. Leukocyte entry occurs both in autoimmune diseases such as MS, but also as part of CNS homeostasis where leukocytes will traffic into the brain at the postcapillary venule to sample the CNS microenvironment.

The primary resident macrophages of the brain are microglia and perivascular cuff macrophages (discussed in 3.2.1). Microglia are a distinct myeloid cell population which, unlike peripheral macrophages, are derived from the embryonic yolk sac rather than bone marrow. Microglia function to monitor the brain for pathologic changes or disturbed ionic balances, extending long processes from a compact cell body (soma) in what is referred to as a ramified morphology. Resting microglia somata are relatively immobile, although their branching processes undergo constant dynamic restructuring, extending and retracting processes to sample the cerebral microenvironment. It is estimated that the complete extracellular space of the brain is sampled by microglia once every few hours, making these cells the frontline responders to CNS insult. Upon detection of CNS injury or the presence of pathogens, microglia undergo a morphological shift from a ramified to an amoeboid morphology, contracting cell processes and enlarging the cell body. Microglia are thought to display a spectrum of activation states, with pro-inflammatory neurodegenerative M1-like and wound-healing neuroprotective M2-like polarised states occupying either end of the spectrum. It is thought that in diseases with a chronic inflammatory component, this balance between M1 and M2 polarised microglia is upset, generating an overactive M1 microglia population. Recently there have been increasing numbers of microglial ablation studies performed to examine whether the presence of microglia is a detrimental aspect of neuropathologies. Many of these studies have found that ablation of microglia can ameliorate cognitive deficits and promote brain recovery in disease models of haemorrhage, Alzheimer’s disease, epilepsy, and ischemia. An interesting finding from these is that total ablation of microglia does not affect resting behaviours of mice, indicating that the primary purpose of microglia may be to respond to CNS insult, rather than maintain CNS homeostasis. Whether the beneficial outcomes of microglial depletion are due to inhibition of a general innate immune response and pro-inflammatory effects, or an actual therapeutic resolution of the neuropathology has yet to be determined. Use of chemical inhibitors of tumour necrosis factor-alpha (TNF-α) and NF-kB in diabetes for example, and of a caspase-1 inhibitor
Microglial activation can have direct effects on the BBB and the BBB can similarly induce microglial activation, allowing for crosstalk between microglia responding to CNS insults and the BBB endothelium responding to peripheral factors. Activated microglia secrete chemokines such as IL-6, TNF-α, CCL2, CCL3 as well as ROS. These can directly affect the BBB through modifying TJ expression or increasing recruitment of peripheral leukocytes. Lipopolysaccharide (LPS) is a potent inducer of microglial activation through the binding of Toll-like receptor 4 (TLR4) and inducing NF-κB signalling. LPS treatment of rat brain endothelial-microglia co-cultures has been shown to induce disruption of tight junction localisation, whereas LPS treatment of endothelial cultures in the absence of microglia had no effect. This effect was further shown to be inhibited in the presence of an NADPH oxidase inhibitor, indicating microglia-secreted ROS can disrupt TJs. Similarly, increased permeability observed in LPS-treated endothelial-microglial co-cultures, but not in endothelial cultures alone, was prevented by the administration of TNF-α neutralising antibodies. Microglia are often activated by invading pathogens or damage-associated molecular patterns (DAMPs) released from damaged cells. When in proximity to the CNS vasculature, however, the BBB itself can drive microglial activation through secretion of pro-inflammatory molecules such as MMP-3 in response to injury or through its dysfunction by allowing macromolecules such as fibrinogen or albumin into the brain. This communication between microglia and the CNS endothelium requires functional cells of both populations, later in this chapter we provide data to suggest that loss of microglial or endothelial CSF-1R disrupts this cross-talk, resulting in a dysregulated endothelium.
4.3 Results

4.3.1 CSF-1R Inhibition Via PLX3397 Reduces Endothelial Tight Junction Component Expression

As ALSP is caused by heterozygous loss of CSF1R we sought to determine whether endothelial cells displayed sensitivity to CSF-1R manipulation. The mouse endothelial cell line b.End3 was treated with increasing concentrations of PLX3397, a small molecule inhibitor of CSF-1R, for 24 or 48h. Although there were no significant changes in ZO-1 expression, protein levels of both occludin and claudin-5 were significantly decreased at 24 h post-treatment (Figure 4.1). This downregulation of occludin was maintained at 48 h, while claudin-5 expression demonstrated a recovery. At a transcriptional level, expression of both Ocln and Cld5 was decreased at 24 and Ocln downregulation maintained at 48 h (Figure 4.2, a). These changes in protein and gene expression were accompanied by an increase in endothelial monolayer permeability. Treatment of b.End3 cells grown on Transwell inserts with PLX3397 increased the flux of a FITC-conjugated 4 kDa dextran at 24 h (Figure 4.2, b), although this data is limited by a lack of biological replicates. These results were further validated using primary mouse endothelial cells, with an increase in permeability and reduced claudin-5 and occludin protein levels observed at 24 h (Figure 4.3, a, b). Similarly to the results observed in b.End3 cells, Cld5 was again significantly downregulated at 24 h post-treatment with PLX3397 (Figure 4.4, a). Looking at the CSF-1R pathway, expression of Csf1r, its ligand Csf1 and the alternate IL-34 receptor Ptprz1 were also increased in response to the highest concentration of PLX3397, potentially pointing towards an auto-inhibitory function of CSF-1R within endothelial cells (Figure 4.4, b). To ensure that the changes observed in both expression and permeability were not due to a cytotoxic effect of PLX3397, a cell viability assay was performed. For both b.End3 and MBEC treatments, cell viability was not significantly reduced by PLX3397 treatment (Figure 4.5). In addition, ICC for claudin-5 and ZO-1 was performed on treated MBEC cultures, demonstrating intact cell-cell contacts and monolayer integrity at all concentrations (Figure 4.6). Quantification of this ICC revealed a significant decrease in both claudin-5 and ZO-1 immunopositivity in response to PLX3397 treatment. Together, these data indicate that inhibition of CSF-1R in endothelial cells has a robust impact on tight junction expression and endothelial permeability.
**Figure 4.1:** PLX3397 treatment of b.End3 cells reduces tight junction protein expression. (a) Western blot of lysates from PLX3397 treated b.End3 cells for tight junction proteins ZO-1, Occludin and Claudin-5. The horizontal line indicates untreated cells, with increasing PLX3397 concentrations (5, 10, 20 μM). Corresponding densitometry is given in (b) for both 24 (left) and 48 (right) h treatments. (One-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.0005, n = 3 independent experiments)
Figure 4.2: PLX3397 treatment of b.End3 cells reduces tight junction gene expression. (a) Gene expression changes at 24 (left) and 48 (right) h in PLX3397 treated b.End3 cells shown by qPCR for Tjp1, Oc1n and Cld5. (b) Transwell permeability flux assay using 4 kDa FITC-Dextran at 24 h post-treatment. (n = 3 independent experiments for qPCR, n = 2 technical replicates for FITC-Dextran flux, (One-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005, n = 3 independent experiments)
**Figure 4.3**: PLX3397 treatment of MBECs reduces tight junction protein expression. **(a)** Western blot of lysates from PLX3397 treated MBECs for tight junction proteins ZO-1, Occludin and Claudin-5. The horizontal line indicates untreated cells, with increasing PLX3397 concentrations (5, 10, 20 μM). **(b)** Transwell permeability flux assay using 4 kDa FITC-Dextran at 24 h post-treatment. (n = 1 for western blot, n = 3 technical replicates for flux assay, one-way ANOVA with Dunnett’s post-test for multiple comparisons)
Figure 4.4: PLX3397 treatment of MBECs alters tight junction gene expression. (a) Tight junction gene expression changes at 24 h in PLX3397 treated MBECs shown by qPCR for *Tjp1*, *Ocln* and *Cld5*. (b) CSF1R pathway gene expression changes at 24 h in PLX3397 treated MBECs shown by qPCR for *Csf1*, *Il34*, *Csf1r* and *Ptprz1*. (One-way ANOVA with Dunnett’s post-test for multiple comparisons, *p* < 0.05, **p** < 0.005, ***p*** < 0.0005, *n* = 3 independent experiments)
**Figure 4.5:** PLX3397 induced decreases in cell viability do not reach significance in b.End3 cells and MBECs. MTS cell viability assay for PLX3397 treated b.End3 cells (a) and MBECs (b). (n = 5 technical replicates for b.End3 cells, n = 3 technical replicates for MBECs, one-way ANOVA with Dunnett’s post-test for multiple comparisons)
Figure 4.6: PLX3397 decreases Claudin-5 and ZO-1 immunopositivity in MBECs. ICC for Claudin-5 (a) and ZO-1 (b) with the corresponding quantification (left). PLX3397 concentrations are labelled in white and scale bars indicate 40 μm. (One-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, n = 2 coverslips per treatment)
4.3.2 CSF-1R Activation Leads To Tight Junction Turnover In Endothelial Cells

As pharmacological inhibition of CSF-1R had induced downregulation of tight junction proteins, we next examined the effect of CSF-1R activation on endothelial cells. Confluent monolayers of b.End3 cells were treated with CSF-1 or IL-34 and tight junction components examined at 24 and 48 h post-treatment. There was no significant change in tight junction protein expression in response to IL-34 treatment, despite a CSF-1 induced downregulation of both occludin and claudin-5 at 24 h (Figure 4.7). Changes in occludin and claudin-5 expression were not maintained to 48 h, with expression restored to untreated levels.

Tight junction transcript levels were unaffected by CSF-1 at 24 h (Figure 4.8). Only Tjp1 expression responded to CSF-1 and IL-34 treatment, with a general upregulation observed in treated cells at 48 h. Of note is the apparent dosage effect in the transcriptional responses to treatment, particularly in the case of Tjp1. While 10 ng/mL CSF-1, and 10 and 50 ng/mL IL-34, could induce an increase in Tjp1 expression, expression was unchanged in cells treated with higher concentrations. These data point towards CSF-1R activation as a possible short term regulator of tight junction component turnover.
Figure 4.7: CSF-1 but not IL-34 treatment decreases Claudin-5 and Occludin in b.End3 cells. (a) Western blot of lysates from CSF-1 and IL-34 treated b.End3 cells for CSF-1R and tight junction proteins ZO-1, Occludin and Claudin-5. The horizontal line indicates untreated cells, with increasing CSF-1 or IL-34 concentrations (10, 50, 100 ng/mL). Corresponding densitometry is given in (b) for both 24 (left) and 48 (right) h treatments. (One-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005, n = 2 independent experiments)
**Figure 4.8**: Transcriptional changes in response to CSF-1 and IL-34 treatment decreases Claudin-5 and Occludin in b.End3 cells. Tight junction gene expression changes at 24 (left) and 48 (right) h in CSF-1 and IL-34 treated b.End3 cells. The horizontal line indicates untreated cells, with increasing CSF-1 or IL-34 concentrations (10, 50, 100 ng/mL). (One-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005, n = 3 independent experiments)
4.3.3 Microglia Conditioned Media Upregulates Endothelial CSF-1R

The results presented in this chapter thus far have indicated a role for CSF-1R in regulating tight junctions of endothelial cell cultures. As CSF-1R is essential for microglia in vivo, we next examined whether there was any CSF-1R dependent crosstalk between microglia and endothelial cells. To determine this, the mouse microglial cell line BV2 was used to produce microglia conditioned media (CM) under normal growth conditions (BV CM), and under CSF-1R inhibition using PLX3397 (Figure 4.9). Although PLX3397 is reported to be stable in aqueous solutions for 24 h, b.End3 cells were also treated with 20 μM PLX3397 to control for any residual drug in culture media.

As CSF-1R is required for microglial viability, the effect of PLX3397 on BV2 viability was first assessed to ensure effects observed from CM were not simply due to apoptosis or the release of cell contents due to cytotoxic effects. Treatment for 24 h resulted in a significant decrease in cell viability at 80 μM, likely due to PLX3397 cytotoxicity rather than a response to CSF-1R inhibition as the BV2 cell line can grow in the absence of CSF-1 or IL-34 (Figure 4.10, a). The 20 and 40 μM concentrations were chosen for the generation of CSF-1R inhibited BV2 CM, considered as low and high CSF-1R inhibition respectively. We have detected a robust upregulation of CSF-1R protein levels in response to BV CM, with this increase present to a lesser extent in response to 20 μM PLX3397 treated-BV2 CM (BV20 CM) (Figure 4.10, b, c). There was no increase in endothelial CSF-1R in response to either 40 μM PLX3397 treated-BV2 CM (BV40 CM) or PLX3397 itself, indicating that CSF-1R activity may regulate this ability of microglia to increase endothelial CSF-1R expression. This effect also extended to tight junction expression, with ZO-1, occludin and claudin-5 expression reducing in response to BV40 CM and PLX3397 itself (Figure 4.11, Figure 4.12, a). While ZO-1 and occludin expression were unchanged by BV or BV20 CM, claudin-5 expression was uniquely sensitive, decreasing in response to each microglia CM treatment. At a transcriptional level, Tjp1 was downregulated by BV40 CM, with Tjp1 additionally downregulated by PLX3397 alone. Intriguingly, Ocln expression was upregulated under the same conditions as CSF-1R with BV CM and, to a lesser degree, BV20 CM increasing transcript levels. This may explain the absence of any changes in occludin at the protein level under these conditions, in contrast to claudin-5. Taken together, these results point towards crosstalk between microglia and endothelial cells that is reduced or absent in CSF-1R inhibited microglia.
**Figure 4.9:** Schematic for media conditioning and treatment of b.End3 cells. Media was conditioned by BV2 microglia, either untreated or treated with PLX3397. Confluent monolayers of b.End3 cells were then treated with tissue culture media supernatant (BV = media from untreated BV2 cells, BV20 and BV40 = media from BV2 cells grown in 20 μM and 40 μM PLX3397 respectively) or 20 μM PLX3397.
Figure 4.10: BV2 conditioned media upregulates CSF-1R in b.End3 cells. (a) MTS cell viability assay for PLX3397 treated BV2 cells. (b) Western blot for CSF-1R of b.End3 cells treated with different BV2 conditioned media or PLX3397, with corresponding densitometry in (c). (n = 5 technical replicates for (a), n = 3 independent BV2 media conditionings and b.End3 treatments for (c). one-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, ***p < 0.0005, ****p < 0.0001)
**Figure 4.11**: Microglia conditioned media downregulates b.End3 tight junctions. Western blot of lysates from treated b.End3 cells for tight junction proteins ZO-1, occludin and claudin-5. Blots representative of n = 3 independent BV2 media conditionings and b.End3 cell treatments. Treatment abbreviations are as indicated in Figure 4.8.
Figure 4.12: Tight junctions are downregulated by microglia conditioned media. (a) Densitometry of tight junction protein changes following conditioned media treatments. (b) Changes in Tjp1, Ocln and Cld5 transcription following conditioned media treatments. (n = 3 independent BV2 media conditionings and b.End3 treatments, one-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.005, ****p < 0.0001)
4.3.4 Microglia Conditioned Media Has Limited Effect On Tight Junction Response To Endothelial CSF-1R Activation

As microglia CM could upregulate endothelial CSF-1R, microglia may serve to prime the endothelium for CSF-1R activation. During microglial localisation to the CNS endothelium, they may upregulate local CSF-1R expression to confer further CSF-1 or IL-34 sensitivity to the vasculature. To examine this potential role for microglia, we first pre-treated b.End3 cells with control, BV or BV20 CM for 24 h to induce endothelial CSF-1R upregulation (hereafter referred to as ‘controls’, ‘BV CM cells’ and ‘BV20 CM cells’ respectively). At 24 h, we then treated with CSF-1 or IL-34 and examined tight junction protein changes at 24 or 48 h post ligand treatment. Observed changes were classed as “ligand-specific” if there was a significant change in response to CSF-1/IL-34 that could not be attributed to the effect of the microglia CM. Overall, and as summarised later by Table 4.1, the main source of tight junction variation was determined to be from the CM pre-treatment, rather than ligand treatment. This indicates that the microglial stimulus which upregulates CSF-1R and induces the associated tight junction changes, may not serve to regulate tight junctions through a secondary activation of endothelial CSF-1R.

Treatment with CSF-1 at 24 h was observed to induce a CM-specific change in ZO-1 expression, with 100 ng/mL CSF-1 significantly upregulating ZO-1 relative to both the untreated control and untreated BV20 CM cells (Figure 4.13). While claudin-5 was increased in BV CM cells relative to the untreated control, there was no significant effect of ligand treatment. A dose-specific downward trend of claudin-5 expression, similar to that observed in Figure 4.7, was seen in both BV CM and BV20 CM cells. At 48 h post-ligand treatment, a ligand-specific effect was seen in both occludin and claudin-5 expression levels in treated controls which, albeit being at a later time point, corresponds to what was observed in the previous CSF-1 treatments (Figure 4.14). Significant ligand-specific downregulation of occludin was observed in controls treated with 50 and 100 ng/mL CSF-1. Claudin-5 similarly underwent ligand-specific downregulation in controls in response to all concentrations of CSF-1. Across all microglial CM pre-treatments, occludin protein expression was decreased relative to the untreated control. The same pattern of claudin-5 downregulation was also observed, with claudin-5 in BV CM cells reduced to the same extent as 100 ng/mL CSF-1 treated controls. A significant ligand-specific increase in occludin was, however, evident in BV CM cells, with 50 ng/mL CSF-1 upregulating occludin relative to untreated BV CM cells.
Figure 4.13: At 24 h, endothelial tight junction response is dependent on microglia CSF-1R state and not CSF-1. (a) Western blot of lysates from b.End3 cells pretreated with control or microglia conditioned media and stimulated with CSF-1 for 24 h in DMEM. Horizontal lines indicate no ligand treatment, with increasing concentrations of CSF-1 (10, 50, 100 ng/mL) (b) Densitometry of tight junction and CSF-1R protein changes following conditioned media treatments. Asterisks (*) indicate significant changes relative to the untreated unconditioned (control-media grown) cells, octothorpes (#) indicate significance relevant to the untreated cells within each conditioned-media group. (n = 2 independent BV2 media conditionings and b.End3 treatments, one-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05.)
Figure 4.14: At 48 h, endothelial tight junction response is dependent on microglia CSF-1R state and not CSF-1. (a) Western blot of lysates from b.End3 cells pretreated with control or microglia conditioned media and stimulated with CSF-1 for 48 h in DMEM. Horizontal lines indicate no ligand treatment, with increasing concentrations of CSF-1 (10, 50, 100 ng/mL). (b) Densitometry of tight junction and CSF-1R protein changes following conditioned media treatments. Asterisks (*) indicate significant changes relative to untreated unconditioned (control-media grown) cells, octothorpes (#) indicate significance relevant to the untreated cells within each conditioned-media group. (n = 2 independent BV2 media conditionings and b.End3 treatments, one-way ANOVA with Dunnett’s post-test for multiple comparisons, * or # = p < 0.05, **p < 0.005, ***p < 0.005.)
Treatment with IL-34 similarly yielded few ligand-specific upregulations. At 24 h post-treatment, only occludin displayed any significant changes. BV CM cells had reduced occludin expression in the absence of ligand, and when treated with 100 ng/mL IL-34 (Figure 4.15). At 48 h, all BV CM cells had significantly reduced ZO-1 expression regardless of IL-34 treatment. Occludin and CSF-1R were significantly decreased in untreated and 10 ng/mL IL-34 treated BV20 CM cells. CSF-1R was additionally decreased in 100 ng/mL IL-34 treated BV CM cells. Only claudin-5 displayed a ligand-specific effect in the pre-treated cells. In BV CM cells, claudin-5 was significantly upregulated by 50 and 100 ng/mL IL-34. The increase at 100 ng/mL was also significant when compared to untreated BV CM cells. Although it did not reach significance, there was a strong trend in occludin expression in BV CM cells, with a dose-dependent decrease in expression in response to IL-34. The results of these treatments suggest that microglia CM is responsible for the majority of the changes observed in tight junction expression. A two-way ANOVA, comparing ligand effects with those induced by CM alone is also supportive of this conclusion (Table 4.1). In the case of CSF-1, CM was the primary source of claudin-5 changes at 24 h, and of occludin changes at both 24 and 48 h. There was a significant interaction between the effects of CSF-1 and CM treatment at 48 h for both claudin-5 and occludin, however, particularly in the case of claudin-5 this may be due to the changes observed in CSF-1 treated control cells. With regard to IL-34, the effect of CM was a significant source of ZO-1, CSF-1R and occludin protein variation at 48 h in treated cells.

Overall, these findings indicate that the hypothesis of microglia priming the CNS vasculature for further CSF-1R activation may be incorrect. Rather, in the context of the b.End3 cell line at least, the secreted factors produced during the media conditioning have a greater effect than the ligand treatments themselves. These effects may mask the later changes brought on by ligand treatment and CSF-1R activation, reducing their effect on tight junction protein expression as seen with occludin in CSF-1 treated control versus BV CM cells (Figure 4.14). The CM may alternatively activate pathways downstream of CSF-1R itself, removing the need for ligand treatment as demonstrated by BV CM cells expressing claudin-5 equivalent to controls treated with 100 ng/mL CSF-1 (Figure 4.14). Two potentially promising results may be the significant upregulation of claudin-5 in conjunction with the concomitant downregulation of occludin in response to IL-34 at 48 h (Figure 4.16). Neither of these changes was seen in the IL-34 treated control or BV20 CM cells, suggesting them to be specific to endothelial cells treated with CM produced by microglia without CSF-1R inhibition.
Figure 4.15: At 24 h, endothelial tight junction response is dependent on microglia CSF-1R state and not IL-34. (a) Western blot of lysates from b.End3 cells pretreated with control or microglia conditioned media and stimulated with IL-34 for 24 h in DMEM. Horizontal lines indicate no ligand treatment, with increasing concentrations of IL-34 (10, 50, 100 ng/mL) (b) Densitometry of tight junction and CSF-1R protein changes following conditioned media treatments. Asterisks (*) indicate significant changes relative to the untreated unconditioned (control-media grown) cells, octothorpes (#) indicate significance relevant to the untreated cells within each conditioned-media group. (n = 2 independent BV2 media conditionings and b.End3 treatments, one-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005.)
Figure 4.16: At 48 h, endothelial tight junction response is dependent on microglia CSF-1R state and not IL-34. (a) Western blot of lysates from b.End3 cells pretreated with control or microglia conditioned media and stimulated with IL-34 for 48 h in DMEM. Horizontal lines indicate no ligand treatment, with increasing concentrations of IL-34 (10, 50, 100 ng/mL). (b) Densitometry of tight junction and CSF-1R protein changes following conditioned media treatments. Asterisks (*) indicate significant changes relative to the untreated unconditioned (control-media grown) cells, octothorpes (#) indicate significance relevant to the untreated cells within each conditioned-media group. (n = 2 independent BV2 media conditionings and b.End3 treatments, one-way ANOVA with Dunnett’s post-test for multiple comparisons, * or # = p < 0.05.)
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**Table 4.1:** p-Values from two-way ANOVA analyses of treatments in Figures 4.13 - 4.16. Significant sources of variation between groups are shown in bolded text. (n = 2 per group, using data presented in previous figures. Asterisks indicate significant p-values, *p < 0.05, **p < 0.005, ***p < 0.005, ****p < 0.0001.)
4.3.5 Both Endothelial And Microglial CSF-1R Contribute To Microglial-Endothelial Crosstalk

From the previous findings of this chapter, we have identified a role for CSF-1R in endothelial cells and tight junction expression. We have also identified that microglia secrete, in a CSF-1R dependent manner, a soluble factor which upregulates endothelial CSF-1R. Despite this upregulation, however, the response of endothelial tight junction expression to CSF-1 or IL-34 is not enhanced under these conditions. Should the secreted factors produced during the media conditioning have a greater or downstream effect than ligand treatment, perhaps the CSF-1R mediated difference lies in what secreted factors are produced, and how the endothelial cells respond to them. To investigate this hypothesis, primary microglia and endothelial cells were utilised to remove potential confounding effects of using PLX3397 and cell lines. Using the Csf1r^flx/wt mouse and the endothelial and macrophage-specific Cre recombinase lines Tie2-Cre and Cx3cr1-Cre respectively, we also could reproduce the ALSP scenario of both the microglia and endothelial cells being heterozygous for functional CSF-1R. Additionally, we also set up conditions wherein only the microglia or endothelial cells were heterozygous for Csf1r to determine whether the observed differences were due to Csf1r loss in either cell type alone. The transcriptional response to microglia CM treatment was assessed in endothelial cells as shown in the schematic below (Figure 4.17). Hereafter, microglia isolated from Csf1r^flx/wt;Cx3cr1-Cre^* or Csf1r^flx/wt;Cx3cr1-Cre^+ mice will be referred to as Csf1r^+/+ and Csf1r^-/- microglia respectively. Similarly, endothelial cells (ECs) isolated from Csf1r^flx/wt;Tie2-Cre^* or Csf1r^flx/wt;Tie2-Cre^+ mice will be referred to as Csf1r^+/+ and Csf1r^-/- ECs respectively.

Microglia isolated from these strains were initially assessed to ensure downregulated Csf1r transcription. Transcriptional analyses of CSF-1R pathway components confirmed a significant 50% reduction in Csf1r transcript, as well as no significant changes in Il34 or Csf1 expression in Csf1r^-/- microglia. The alternate IL-34 receptor PTP-ζ was also significantly upregulated in these cells. Should this also be reflected at the protein level, there may be an overabundance of PTP-ζ which would inhibit CSF-1R in the absence of IL-34. This may be a mechanism through which Csf1r^-/- microglia compensate for the increased extracellular IL-34 due to reduced CSF-1R binding, internalisation and eventual degradation.
Figure 4.17: Schematic showing the sources of microglia and endothelial cells used for experiments detailed in Figures 4.18 – 4.21. Text beneath cell culture dishes indicates the applied conditioned media coming from Csf1r homozygous microglia (+/+, green) or heterozygous microglia (+/−, yellow). Purple endothelial cells are homozygous for Csf1r while blue indicates endothelial cells heterozygous for Csf1r.
Figure 4.18: Transcription of CSF-1R pathway components in Csf1r+/+ and Csf1r−/− microglia used for media conditioning experiments. Quantification of gene expression of CSF-1R pathway components in the microglia isolated from Csf1rflx/wt; Cx3cr1-Cre− (Csf1r+/+ microglia) and Csf1rflx/wt; Cx3cr1-Cre+ (Csf1r−/− microglia) mice. (n = 2 microglial isolations, unpaired t-test with Welch’s correction, **p < 0.005.)
**Figure 4.19:** Western blot of conditioned media treated $Csf1r^{\text{flx/wt}}$ ECs and $Csf1r^{\text{flx/wt}}$ ECs. Western blot of endothelial cell lysates for tight junctions ZO-1, occludin and claudin-5. Endothelial cells were treated with control media (Unt), or conditioned media from $Csf1r^{+/+}$ microglia (+/+) and $Csf1r^{+/+}$ microglia (+/-).
Treatment of \textit{Csf1r}\textsuperscript{+/+} and \textit{Csf1r}\textsuperscript{+/-} ECs with \textit{Csf1r}\textsuperscript{+/-} and \textit{Csf1r}\textsuperscript{+/-} microglia conditioned media (MCM) revealed genotype-specific differences in the EC response to MCM. As shown in Figure 4.19, ZO-1 and occludin changes were subtle in response to treatment. In contrast, claudin-5 appeared to be increased in response to MCM treatment, with \textit{Csf1r}\textsuperscript{+/-} ECs displaying higher basal levels which were further increased by MCM treatment. On a transcriptional level, differential tight junction gene expression was most evident in \textit{Csf1r}\textsuperscript{+/-} ECs treated with \textit{Csf1r}\textsuperscript{+/-} MCM, reflecting the ALSP scenario (Figure 4.20). \textit{Csf1r}\textsuperscript{+/-} EC expression of \textit{Cld5}, \textit{Ocln} and \textit{MarvelD2} was unchanged by MCM treatment, with only a slight increase in \textit{MarvelD2} observed in response to \textit{Csf1r}\textsuperscript{+/-} MCM that did not reach significance (Figure 4.20, b-d). Strikingly, in both \textit{Csf1r}\textsuperscript{+/-} and \textit{Csf1r}\textsuperscript{+/-} ECs there was no change in \textit{Tjp1} expression. Changes specific to \textit{Csf1r}\textsuperscript{+/-} ECs were observed as \textit{Cld5}, \textit{Ocln} and \textit{MarvelD2} were significantly downregulated in \textit{Csf1r}\textsuperscript{+/-} ECs in response to \textit{Csf1r}\textsuperscript{+/-} MCM. This downregulation resulted in gene expression of each being significantly lower than the expression values observed in \textit{Csf1r}\textsuperscript{+/-} ECs under the same treatment conditions. It should be noted that the transcriptional state of \textit{Cld5} in \textit{Csf1r}\textsuperscript{+/-} ECs did not correlate with the apparent increase at the protein level, and together this might tentatively suggest an increase in post-transcriptional processing of \textit{Cld5} mRNA.
Figure 4.20: Transcriptional changes of tight junction genes in Csf1r<sup>+/+</sup> and Csf1r<sup>+-</sup> endothelial cells treated with control media (Unt), or conditioned media from Csf1r<sup>+/+</sup> (+/+) or Csf1r<sup>+-</sup> (+/-) microglia. For each gene, the same data is presented in two formats, either comparing changes in gene expression within genotypes (left) or between genotypes (right). (n = 2 independent microglia media conditionings and MBEC treatments, two-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.005, ****p < 0.0001.)
Concerning transcriptional changes in components of the CSF-1R pathway, we again identified changes unique to the ALSP scenario (Figure 4.21). Csf1r expression in untreated Csf1r<sup>−/−</sup> ECs was roughly at 50% compared to Csf1r<sup>+/+</sup> ECs. This confirmed what was also observed in Csf1r<sup>−/−</sup> microglia, that the heterozygous state reduces Csf1r expression accordingly and without compensatory transcriptional upregulation (Figure 4.21, a). While Csf1r<sup>−/−</sup> ECs did not display any changes in Csf1r expression, transcript levels of Csf1r in Csf1r<sup>+/−</sup> ECs was significantly reduced in response to Csf1r<sup>+/+</sup> MCM. This reduction equalised Csf1r expression in both sets of ECs as it did not affect Csf1r<sup>−/−</sup> ECs. In contrast, Csf1r<sup>+/+</sup> MCM similarly reduced expression in both sets of ECs, maintaining the differential expression observed in untreated cells. As Csf1r<sup>−/−</sup> MCM could selectively decrease Csf1r expression in homozygous but not heterozygous ECs, it is possible that Csf1r<sup>+/−</sup> ECs are responsive to a secreted factor produced by Csf1r<sup>+/+</sup> but not Csf1r<sup>−/−</sup> microglia, and that ECs heterozygous for Csf1r are unresponsive to this same stimulus. This decrease alternatively could be due to activation of a signalling pathway that is constitutively active in heterozygous ECs, making them unresponsive to further stimuli. Expression of the alternative IL-34 receptor did not display this same genotype-specific response, instead Ptprz1 expression decreased in both sets of ECs in response to MCM treatment (Figure 4.21, b). Although Csf1r<sup>−/−</sup> ECs had uniformly lower Ptprz1 expression across each condition, both Csf1r<sup>+/−</sup> and Csf1r<sup>+/+</sup> MCM induced comparable Ptprz1 downregulation suggesting that endothelial Csf1r alone is responsible for regulating endothelial Ptprz1 expression.

Examining the endothelial expression of CSF-1R ligands, both endothelial and microglial Csf1r appeared to influence gene expression. In Csf1r<sup>−/−</sup> ECs, Csf1 expression was unresponsive to either MCM treatment (Figure 4.21, c). In response to Csf1r<sup>+/−</sup> MCM, however, Csf1 expression was upregulated in homozygous ECs resulting in significantly higher expression compared to the unaffected Csf1r<sup>−/−</sup> ECs. Csf1r<sup>+/−</sup> MCM similarly could only upregulate Csf1 in homozygous ECs, although this weaker increase equalised expression between homozygous and heterozygous ECs. Changes in Il34 expression similarly differed between ECs, with Csf1r<sup>−/−</sup> ECs displaying the same Il34 expression changes as were seen in Csf1. Only Csf1r<sup>+/−</sup> MCM induced changes in Il34 expression in Csf1r<sup>−/−</sup> ECs, increasing transcript levels. Csf1r<sup>+/−</sup> MCM had no effect on Csf1r<sup>−/−</sup> ECs (Figure 4.21, d). In contrast, Csf1r<sup>+/−</sup> ECs were not only differentially responsive to both MCM treatments but displayed a 4-fold higher baseline Il34 expression. This elevated Il34 expression was downregulated by Csf1r<sup>+/−</sup> MCM, but exacerbated by Csf1r<sup>+/−</sup> MCM to expression values 6-fold higher than those of the control. As with tight junction gene expression, Csf1r<sup>+/−</sup> MCM affected Il34 expression in only the heterozygous ECs in the so-called ALSP scenario.
Results from a two-way ANOVA of this data indicate both endothelial and microglial \textit{Csf1r} genotype is a significant factor in the EC response to MCM (\textbf{Table 4.2}). Two exceptions to this are \textit{MarvelD2} and \textit{Csf1}. The effects on \textit{MarvelD2} transcription appear to be due purely to interactive effects rather than due to a predominant differential EC response or effect of MCM treatment. This may be due to the relatively higher standard error within this data set. For \textit{Csf1} the absence of an endothelial effect is likely due to the consistent \textit{Csf1} expression values in all but the \textit{Csf1r}^{+/−} MCM treated \textit{Csf1r}^{+/−} ECs. There is still a \textit{Csf1} upregulatory response unique to ECs homozygous for \textit{Csf1r} evident within the data, hence the significant interaction. Together, these results strongly support CSF-1R as a regulator of microglial-endothelial crosstalk in both cell types. Despite the current focus on CSF-1R in microglia, we show that endothelial CSF-1R can influence how the endothelial cell responds to cues from microglia.
Figure 4.21: Transcriptional changes of CSF-1R pathways components in $Csf1r^{+/+}$ and $Csf1r^{-/-}$ endothelial cells treated with control media (Unt), or conditioned media from $Csf1r^{+/+}$ (+/+) or $Csf1r^{-/-}$ (+/-) microglia. For each gene, the same data is presented in two formats, either comparing changes in gene expression within genotypes (left) or between genotypes (right). (n = 2 independent microglia media conditionings and MBEC treatments, two-way ANOVA with Dunnett’s post-test for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.005, ****p < 0.0001.)
<table>
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<th>Endothelial Genotype</th>
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Table 4.2: p-Values from two-way ANOVA of transcriptional changes in Csf1r/+ and Csf1r/- endothelial cells treated with control media, or conditioned media from Csf1r/+ or Csf1r/- microglia. For each gene, the source of variation was analyzed to determine whether microglial or endothelial CSF-1R state drove expression changes. (p-Values in the bolded text are significant, denoted by adjacent asterisks. N = 2 independent microglia media conditionings and MBEC treatments, two-way ANOVA, *p < 0.05, **p < 0.005, ***p < 0.005, ****p < 0.0001.)
4.4 Discussion

The objective of the work presented in this chapter was to explore a putative role for CSF-1R in endothelial cells. These results indicate that endothelial CSF-1R signalling can directly regulate tight junction expression and inhibition of CSF-1R can increase endothelial monolayer permeability. Here, we have also presented a role for CSF-1R in microglial-endothelial crosstalk, with endothelial CSF-1R and tight junction expression responding to soluble factors released by microglia. We furthermore validate the effects of CSF-1R loss in microglia and endothelial cells and its impact on the outcomes of microglial-endothelial crosstalk.

PLX3397 treatment of the b.End3 cell line and primary mouse brain endothelial cells yielded reductions in tight junction expression at both the protein and transcript level. Occludin and claudin-5 were significantly downregulated in b.End3 cultures, with protein and transcript reducing as PLX3397 concentration rose. Although protein analyses of the same treatment in MBECs was limited to a single replicate, an apparent reduction in occludin and claudin-5 could be observed. *Cld5* and *Ocln* transcript levels were significantly reduced in PLX3397 treated MBECs, while *Tjp1*, *Csf1r*, *Csf1* and *Ptprz1* were each increased under the same conditions. These increases in CSF-1R pathway components are likely compensatory responses to PLX3397-induced receptor degradation. The changes in tight junction components, however, had a functional impact on the endothelial cell permeability. Both b.End3 and MBEC cultures have increased permeability to 4 kDa FITC-Dextran when treated with PLX3397, with increased permeability observed under treatment conditions that also induce claudin-5 and occludin downregulation. These data together suggest that CSF-1R inhibition directly increases endothelial monolayer permeability, and although tight junction proteins were reduced we did not confirm whether this permeability was limited to the paracellular pathway. An increase in transcytosis or increased pinocytosis could also contribute to the increased permeability.

The decrease in junctional protein expression in response to CSF-1R inhibition led us to next determine whether CSF-1R activation could drive a corresponding upregulation of junctional proteins. Surprisingly, occludin and claudin-5 expression was reduced by CSF-1 treatment with decreased protein levels at 24 h. Junctional protein expression had recovered by 48 h, however, perhaps due to the accompanying transcriptional increase *Tjp1*. Intriguingly, IL-34 did not have an identical effect on gene expression as CSF-1. IL-34 reduced *Cld5* transcription at both time points and had no effect on tight junction protein levels. CSF-1R activation by CSF-1 may induce a transient downregulation of tight junction protein followed by recovery through increased transcription. Such a model would facilitate CSF-1 expressing immune cells to gain entry into the...
brain, with recruited macrophages transiently and locally increasing BBB permeability for paracellular transmigration. The opposing effects of CSF-1 and IL-34, and dosage specific effects, might be due to the differences in ligand-receptor affinity. While CSF-1 treatment activates CSF-1R alone, IL-34 has alternate receptors which could modulate its affinity for CSF-1R. Furthermore, \textit{trans} effects from the IL-34 receptor PTP-ζ could contribute to the CSF-1R signalling response through phosphatase activity. Most striking are the parallels in tight junction response to both CSF-1R activation and inhibition, with a reduction of occludin and claudin-5 protein expression and transcriptional upregulation of \textit{Tjp1} seen in both scenarios. An extended timecourse would reveal whether the critical difference is the reversibility of CSF-1R mediated tight junction breakdown versus the sustained tight junction reductions seen in response to PLX3397.

With the information that CSF-1R signalling occurs in endothelial cells, we next examined whether microglia were affected by an incomplete loss of CSF-1R activity. The dependence of microglia on CSF-1R for viability is well established in the literature, therefore incomplete loss of CSF-1R activity in these cells may lead to altered microglial function. Within the ALSP brain, microglia are haploinsufficient for CSF-1R and may be contributing to the observed BBB permeability through these altered functions. We examined whether CSF-1R inhibition affected microglial-endothelial crosstalk and found that BV2 CM could upregulate endothelial CSF-1R. This effect was reduced by pre-treatment of microglia with PLX3397 and completely inhibited by treatment with a higher dose of the inhibitor. Together this indicates that endothelial CSF-1R expression was responsive to a CSF-1R dependant secreted stimulus from microglia. Furthermore, tight junction remodelling in response to MCM differed depending on PLX3397 pre-treatment. Although claudin-5 protein levels decreased in response to any media treatment, conditioned media from untreated microglia, or those treated with the lower PLX3397 dose, could upregulate CSF-1R as well as \textit{Ocln} transcription. Microglia treated with higher doses of PLX3397 were unable to increase endothelial CSF-1R, and additionally downregulated occludin and ZO-1 at the protein and transcript level. Whether these effects were due to the increased endothelial CSF-1R or the effects of other stimuli in the conditioned media was not examined. Moving forward, carrying out an examination of differentially secreted cytokines and signalling molecules, including CSF-1 and IL-34, will provide insight into the altered activities of CSF-1R inhibited microglia.

The increase in endothelial CSF-1R in response to BV2 CM indicates that vessel associating microglia may prime the endothelium for CSF-1R activation. Endothelial cells were pre-treated with conditioned media from untreated or CSF-1R inhibited microglia, and then treated with CSF-
1 or IL-34 to determine whether they had enhanced responses in tight junction expression after CSF-1R activation. Despite some small effects, the results strongly supported the null hypothesis. Although tight junction changes were observed, they could be attributed to the effects of the BV2 CM rather than an improved response to ligand treatment. This does not necessarily imply that CSF-1R signalling was not upregulated, but rather that tight junction remodelling was not an increased signalling output. Further examination of cell elongation, proliferation, activation or migration may reveal more classically associated roles for CSF-1R within endothelial cells.

PLX3397 and immortalised microglial cell lines were of use in screening for CSF-1R related microglial-endothelial crosstalk, however, it is CSF-1R haploinsufficiency which produces the BBB pathologies of ALSP. Switching from pharmacological CSF-1R inhibition to genetic inactivation of Csf1r, we produced mice heterozygous for Csf1r in endothelial cells or macrophages and repeated the microglial conditioned medium experiment. This revealed unique endothelial responses to microglial conditioned media dependent on both the endothelial and microglial Csf1r genotype. In response to MCM produced by microglia heterozygous for Csf1r, expression of Cld5, Ocln and MarvelD2 was decreased in Csf1r+/− endothelial cells. This effect was not observed in Csf1r+/− endothelial cells under any conditions or in heterozygous endothelial cells treated with Csf1r+/− MCM. This context of both the microglia and endothelial cells being heterozygous for Csf1r is representative of the conditions in the ALSP brain. That these decreases in tight junction expression were observed only in this scenario is indicative of potentially similar crosstalk being present within the ALSP brain. As microglia are recruited to the vasculature, a dysfunctional downregulation of endothelial tight junction occurs which is absent in the healthy brain.

Transcription of CSF-1R pathway components also responded differentially depending on endothelial and microglial genotypes. In homozygous endothelial cells, Csf1r itself was downregulated by Csf1r+/+ MCM but not Csf1r+/− MCM. Increases in Il34 and Csf1 were observed when Csf1r+/− ECs were treated with CM from microglia of the same genotype, as such it could be that microglia stimulate CSF-1R ligand expression in nearby endothelial cells. Csf1r+/− ECs were unresponsive regarding Csf1, potentially due to compensatory overexpression of the higher-affinity CSF-1R ligand Il34. This highly expressed gene was severely downregulated by Csf1r+/+ MCM and further upregulated by Csf1r+/− MCM. Microglia may, therefore, be communicating a low or high need respectively for CSF-1R activation to ECs, and the endothelial CSF-1R state may govern the severity of this response.

This study provides a characterisation of the microglial-endothelial crosstalk that may be present within the ALSP brain as well as in individuals with native CSF1R. We show that
heterozygosity for Cf1r and an accompanying reduction in Cf1r transcript is sufficient to disrupt microglial-endothelial crosstalk. Although we did not examine whether Cf1r loss impacts microglial or endothelial activation, we show that Cf1r–/– MCM downregulates Clsd5, Ocln and MarvelD2 only in endothelial cells which are themselves heterozygous for Cf1r. This by itself indicates that although resting microglia may not sustain endothelial tight junctions, the microglial secretome can influence the BBB in a CsF-1R dependent manner. Cf1r loss alters the microglial secretome, making it damaging to BBB homeostasis. Furthermore, endothelial cells heterozygous for Cf1r are particularly susceptible to this change in the microglial secretome, proposing a dual role for CSF-1R in both endothelial cell maintenance and microglial homeostasis. In terms of the CsF-1R pathway itself, this altered microglial secretome further reduces endothelial Cf1r expression and appears to selectively enhance the elevated Il34 expression present in Cf1r–/– ECs. This shift to Il34 expression coincides with Cf1r–/– ECs being unresponsive to the Cf1 upregulatory effects of Cf1r+/+ MCM.

There was no difference between tight junction expression in untreated Cf1r+/+ and Cf1r–/– ECs, only in response to Cf1r–/– MCM are these changes induced. Similarly, Cf1r–/– MCM was able to rescue Il34 expression in heterozygous endothelial cells without downregulating Cf1r itself. These results may lend strength to the benefit of HSCT for ALSP patients. As discussed previously, engraftment of HSCs into the brain to produce microglia-like cells is uncommon in mouse models unless the skull is also irradiated. However, if a small population of transplanted CSF1R+/+ cells engraft within the ALSP brain it could lead to a local improvement through gradually changing the microglial secretome within the brain. Microglia, or microglia-like cells, that are homoygous for functional CSF1R could out-compete and replace the native microglial population during the gradual process of microglial cell turnover. This, in concert with improved peripheral myeloid cell populations and restored macrophage phagocytosis, could protect the heterozygous vasculature through correcting Il34 expression and preventing CLD5, OCLN, CSF1R and MARVELD2 downregulation during microglial-endothelial association.
Chapter 5:

*Csf1r* Heterozygosity Disrupts BBB Homeostasis, NVU Response To CSF-1R Activation, And Outcome of Hippocampal Aβ.
5.1 Abstract

The results of the work presented in previous chapters indicate that CSF-1R regulates the endothelial tight junction through direct activation combined with CSF-1R dependent microglial cross talk. As these data were from in vitro experiments, our next aim was to translate these human and in vitro findings into an in vivo model. First, the response of the NVU, as well as vessel associating cells, to acute CSF-1R activation was examined through intravenous injection of recombinant CSF-1 and IL-34. Transcriptional changes in the cerebrovascular microvessels were examined using an enzyme-free method of microvessel isolation, allowing the transcriptome of the intact NVU and directly associating cells to be investigated. Moreover, it was determined whether these changes were driven by endothelial or macrophage CSF-1R activation through the use of wild type as well as cell-specific Csf1r deficient mice. Finally, to determine whether CSF-1R in endothelial cells or macrophages mediated the acute response to Aβ accumulation, unilateral intrahippocampal injections of Aβ42 were performed and mice assessed behaviorally and using immunohistochemistry.

5.2 Introduction

5.2.1 Transport Of Aβ Across The CNS Endothelium

In a healthy individual, the rate of amyloid clearance exceeds that of Aβ production. Measurement of Aβ synthesis in comparison to CSF-mediated clearance estimated that the hourly rate of Aβ efflux was 0.7 % higher than the rate of Aβ production\(^\text{340}\). As such, Aβ accumulations within the brain are generally unlikely under healthy conditions, but the margin for error in regulating these processes is narrow. Three main mechanisms exist in the brain to maintain this rate of clearance, extracellular enzymatic degradation, intracellular lysosomal degradation and clearance via the cerebrovasculature. Research thus far has implicated a range of proteases in Aβ clearance, classed as so-called Aβ-degrading enzymes (ADEs). ADEs include metalloproteases such as MMP-9 and MMP-2\(^\text{341}\) as well as serine proteases such as plasmin\(^\text{342}\) and myelin basic protein (MBP)\(^\text{343}\). These enzymes cleave Aβ into smaller isoforms, reducing neurotoxicity and facilitating clearance via other pathways such as microglial, neuronal or astrocyte phagocytosis. These two clearance pathways function to prevent toxic Aβ oligomerisation and the development of Aβ plaques. In ALSP, however, Aβ plaques are not the observed pathology. Instead, Aβ accumulates at and around the cerebrovasculature, indicating dysfunction of the vascular Aβ clearance pathway. Vascular clearance of Aβ is performed via two mechanisms, the first being clearance through exchange between interstitial fluid (ISF) and CSF. ISF-mediated amyloid clearance involves the so-called glymphatic hypothesis of drainage.
pathways within the Virchow-Robins perivascular space. The perivascular clearance mechanism of the brain was first discovered following injection of 3 kDa fluorescent dextran and ovalbumin tracers into the grey matter of the brain. Over the course of 3 hours, the dye was removed from the parenchyma of the grey matter, accumulating within the basement membranes of the capillaries and arteries. Furthermore, the tracers were present in the perivascular macrophages, but not basement membrane, of the leptomeninges indicating passage via the leptomeningeal vessels themselves during clearance from the brain. This clearance pathway is driven by the pulsation of the vascular wall, as well as by interactions with astrocyte endfeet. as it is purported to be dependent on AQP4 expression. Drainage of Aβ through this glymphatic system has been reported to be 6-fold slower than through LRP-1 mediated transendothelial clearance, although glymphatic clearance is increased following downregulation of LRP-1 or the ADE nepriysilin.

The second mechanism through with the vasculature clears Aβ is active transport across the endothelium. As mentioned in Chapter 3, there are three principal transport proteins responsible for the movement of Aβ across the CNS endothelium, LRP-1, RAGE and P-gp. Amyloid uptake into the CNS is largely dependent on RAGE, a transmembrane receptor belonging to the immunoglobulin superfamily, and is expressed in microglia, neurons and endothelial cells. Circulating soluble Aβ in the plasma is transported into the CNS by RAGE, which upon ligand binding can also activate proinflammatory NF-κB signalling in the endothelium. As RAGE expression can be upregulated by neuroinflammation, Aβ deposition at the BBB can result in a positive feedback loop as RAGE sustains its own expression through endothelial cell activation. This positive feedback has been observed in the triple transgenic mouse model of AD (3xTg-AD), in which RAGE expression increases with age and co-localises with increased intracellular APP and Aβ. The impact of RAGE on cognitive decline was also observed in the Appsw/0 mouse model of AD. Treatment of mice with a RAGE-specific inhibitor reduced microglial activation and neuroinflammation within the brain, in addition to BACE-mediated production of Aβ itself. RAGE inhibition also reduced levels of Aβ within the brain and normalised cognitive dysfunction in aged Appsw/0 mice, providing evidence of the receptor being a promising target for AD therapeutics.

While RAGE mediates Aβ uptake, LRP-1 and P-gp are the main Aβ efflux proteins at the BBB. P-gp is enriched on the luminal face of the BBB endothelium but is also expressed at the abluminal face, allowing it to bind Aβ within the brain and transport it across the endothelium. Microvessel P-gp expression is reduced in areas of Aβ plaque, as well as in vessels affected by
P-gp expression also decreases with age, with age-related downregulation being more severe in individuals with AD than in healthy age-matched controls. In the Mdr1a knockout mouse, the loss of P-gp expression led to a significant increase in accumulation of intravenously injected Aβ within the brain. When crossed to the Tg2576 mouse model of AD, loss of one P-gp isoform resulted in an acceleration of spontaneous Aβ accumulation. Upon examination of the Aβ isoforms affected by P-gp loss, only levels of the soluble Aβ_{40} isoform were increased, indicating that the clearance of the insoluble Aβ_{42} isoform was not driven by P-gp, but may be compensated for by LRP-1. Double knockout of both murine P-gp isoforms resulted in reduced clearance of stereotaxically injected Aβ_{40}, as well as a similar increase in cerebral Aβ load in the Tg2576 background. In these studies, it was found that expression of LRP-1 was downregulated in parallel with P-gp, and as such these increases in Aβ could not be solely attributed to P-gp loss. As with P-gp, endothelial LRP-1 expression decreases with age. LRP-1 plays a pivotal role in Aβ clearance, having been implicated in the Aβ uptake and clearance of endothelial cells, pericytes and astrocytes. Although LRP-1 is expressed in multiple cell types of the brain, the central role of endothelial LRP-1 in Aβ efflux was demonstrated using a brain endothelial-specific inducible Lrp1 knockout. As with experiments involving the loss of P-gp, loss of endothelial LRP-1 resulted in the reduced efflux of injected Aβ_{42} in wild-type mice. In the 5xFAD mouse model of AD, loss of endothelial LRP-1 decreased plasma concentrations of Aβ, while elevating the levels of soluble Aβ in the brain and decreasing cognitive function. LRP-1 is expressed in two isoforms, membrane-bound full-length LRP-1 and a soluble cleavage product (sLRP-1) which is shed into the plasma and CSF where it sequesters circulating Aβ for later degradation in the liver, kidney and spleen. Full-length LRP-1 is expressed abluminally in BBB endothelial cells, internalising CNS Aβ and shuttling it to the luminal face for secretion or sLRP-1 shedding into the circulatory system. This process involves apolipoprotein E (ApoE) a well known genetic risk factor for sporadic AD, with the ApoE4 variant being one of the strongest genetic risk factors for the disease. Brain pericyte uptake and degradation of Aβ is reduced by pharmacological or genetic inhibition of ApoE, and this loss of Aβ uptake and clearance could be rescued by introduction of human ApoE3, but not the ApoE4 risk variant. As with P-gp, inhibition of LRP-1 resulted in decreased clearance of stereotaxically injected Aβ, and this reduction in Aβ clearance was exacerbated in an ApoE^{−/−} background. Furthermore, the age-associated decrease in endothelial LRP-1 expression correlated with the regional accumulation of Aβ. This change is critical in the understanding of Aβ flux in the aged or diseased brain. As LRP-1 and P-gp are decreased concomitant with age, Aβ accumulates locally in regions with decreased efflux expression and will upregulate RAGE expression, further exacerbating Aβ build-up within the brain.
5.3 Results

5.3.1 Cell-Specific Heterozygous Loss Of Csf1r Alters Baseline Gene Expression And Ligand-Induced Changes In The Cerebrovasculature

Although *in vitro* administration of recombinant IL-34 and CSF-1 yielded few changes in endothelial monocultures, the NVU is comprised of several cell types which induce more complex endothelial expression profiles. This may include upregulation of CSF-1R or its downstream signalling components. To determine the *in vivo* effects of CSF-1R ligands on the NVU, intravenous injections of recombinant IL-34 and CSF-1 were performed, with brain microvessels purified by density gradient centrifugation to examine the changes at the BBB at 24 h. Cerebral microvessel isolation protocols often will include a single or multiple collagenase/dispase digestions to displace cells adhering to the vasculature. These include NVU components such as pericytes and astrocytic endfeet as well as vessel associating macrophages. To capture the full effects of CSF-1R activation on the CNS vasculature a newer protocol published by Lee *et al.* (2019) was utilised which isolates intact microvessels as well as associating cells. Peripheral injection of recombinant IL-34 and CSF-1 would result in ligand interaction with both with the luminal face of the endothelium as well as circulating and tissue-resident myeloid cells. Therefore, the responses observed at the NVU could be attributed to either cell population. As we had established that the CSF-1R state of both microglia and endothelial cells could interact during microglial-endothelial crosstalk, the cell-specific contributions to the observed changes needed to be determined. Peripheral recombinant ligand injections were performed in C57BL6 (Wild Type) mice alongside two mouse models with either endothelial cell (EC) or macrophage (MΦ) Csf1r heterozygosity, the Csf1r<sup>flx/wt</sup>;Tie2-Cre<sup>+</sup> (Csf1r<sup>+/−</sup> EC) and Csf1r<sup>flx/wt</sup>;Cx3cr1-Cre<sup>+</sup> (Csf1r<sup>+/−</sup> MΦ) mouse respectively. Transcriptional changes detected in these transgenic strains were compared alongside those observed in the wild type mice, with analysis incorporating a two-way model to allow for comparison of gene expression between genotypes as well as in response to recombinant ligand injection.
5.3.1.1 Tight Junction Gene Expression is Dysregulated in Csf1r-Deficient Mouse Microvessels

Examining first the changes in microvessel TJ gene expression, sensitivity to CSF-1 was observed in the wild type and Csf1r<sup>-/-</sup> EC microvessels, with CSF-1 driven upregulation of Tjp1 (Figure 5.1, a, d). There was an additional CSF-1 mediated upregulation of CluS expression in the Csf1r<sup>-/-</sup> EC mouse which was notably absent in the two other strains (Figure 5.1, c). Intriguingly the microvessels of the Csf1r<sup>-/-</sup> MΦ mouse, which would have endothelial cells expressing two copies of Csf1r, did not have increased Tjp1 expression in response to CSF-1, suggesting it may be a macrophage-mediated effect. While wild-type and Csf1r<sup>-/-</sup> EC TJ gene expression were unresponsive to IL-34, expression of both Ocln and CluS increased in Csf1r<sup>-/-</sup> MΦ capillaires in response to IL-34 (Figure 5.1, b, c). The unique regulatory effects of recombinant CSF-1 and IL-34 were demonstrated here in the Csf1r<sup>-/-</sup> MΦ mouse, with Ocln downregulated by CSF-1, despite IL-34 mediated CSF-1R activation inducing upregulation of the same gene.

These strain-specific differences were likely due to altered homeostatic gene expression in the cerebrovasculature, which could be observed through a comparison of the three treated mouse strains. At baseline, in vehicle-injected animals, Tjp1 expression was equal across the three mouse strains, however, in CSF-1 treated animals a change in responsivity was revealed. While both wild type and Csf1r<sup>-/-</sup> EC mice displayed an increase in Tjp1 expression in response to CSF-1, this upregulation was significantly stronger in Csf1r<sup>-/-</sup> EC microvessels (Figure 5.1, d). This heightened sensitivity of Csf1r<sup>-/-</sup> EC Tjp1 to CSF-1 contrasts to the complete lack of a response in the Csf1r<sup>-/-</sup> MΦ mouse, with Tjp1 expression remaining static under all conditions. Concerning Ocln, the untreated Csf1r<sup>-/-</sup> MΦ mouse had elevated baseline expression, which was rescued to wild type levels by CSF-1 but further increased by IL-34 (Figure 5.1, e). A similar dysregulation of the Csf1r<sup>-/-</sup> MΦ cerebrovasculature was seen in the increased CluS expression, which was maintained following CSF-1 treatment and, as with Ocln, further upregulated by IL-34 (Figure 5.1, f). In contrast, microvessels of the Csf1r<sup>-/-</sup> EC mouse had reduced CluS expression at baseline which was rescued to wild type levels by CSF-1, not unlike the Ocln response of the Csf1r<sup>-/-</sup> MΦ mouse. Although CluS expression in IL-34 treated Csf1r<sup>-/-</sup> EC microvessels was significantly lower than wild type, this was likely due to the reduced baseline being unaffected by IL-34 rather than a specific response to the ligand.

Overall, the examined TJ gene expression in the wild type BBB was entirely unresponsive to IL-34, with CSF-1 mediated CSF-1R activation upregulating Tjp1 alone. Loss of either endothelial or
macrophage Csf1r homozygosity resulted in dysregulated baseline TJ gene expression, in addition to altered responsivity to IL-34 and CSF-1.
Figure 5.1: Transcription of tight junction genes in brain microvessels isolated from injected C57BL/6 and Csf1r deficient mice. For each gene, the same data is presented in two formats, either comparing changes in gene expression within genotypes (right) or between genotypes (left). RNA was isolated from brain microvessel isolates of C57BL/6 (Wild Type), Csf1r<sup>flx/wt</sup>;Tie2-Cre<sup>-</sup> (Csf1r<sup>+/−</sup> EC) or Csf1r<sup>flx/wt</sup>;Cx3cr1-Cre<sup>-</sup> (Csf1r<sup>+/−</sup> MΦ) mice injected with Vehicle (Veh, V), CSF-1 (C, 100 μg/kg) or IL-34 (I, 100 μg/kg). (Wild Type n = 4 mice per group. Csf1r<sup>+/−</sup> EC n = 2 for V, n = 3 for C and I, Csf1r<sup>+/−</sup> MΦ n = 4 for V and I, n = 3 for C, two-way ANOVA with Dunnett’s test for multiple comparisons, *p < 0.05, **p < 0.0005, ****p < 0.0001.)
5.3.1.2 Microvessel Csf1r Expression Is Decreased By Endothelial And Macrophage Csf1r Heterozygosity

Transcriptional changes in the CSF-1R pathway itself were also observed in microvessel analyses, with changes in ligand responsivity and baseline expression evident in the Csf1r deficient mice. Examining gene expression of the ligands themselves, wild type Csf1 and Il34 were unresponsive to treatment with either ligand (Figure 5.2, a, b). In both Csf1r heterozygotes, there was an upregulation of Csf1 expression following ligand treatment. As observed previously in the response of Tjp1 gene expression, this increase was observed in response to CSF-1 but not IL-34. In the Csf1r<sup>−/+</sup> MΦ mouse, there was an additional acquired responsivity of Il34 to IL-34, with the ligand increasing its own expression (Figure 5.2, b). In the wild type cerebrovasculature, expression of Csf1r was upregulated by both CSF-1 and IL-34 (Figure 5.2, c, d). Strikingly, there was a loss of ligand-mediated upregulation of Csf1r expression in both the Csf1r<sup>−/+</sup> MΦ and Csf1r<sup>−/−</sup> EC microvessels, despite only the Csf1r<sup>−/+</sup> EC mouse having an endothelium heterozygous for Csf1r (Figure 5.2, c). Gene expression of Ptprz1 was similarly unresponsive in the Csf1r<sup>−/+</sup> EC microvessels, while a slight CSF-1 mediated upregulation was observed in the Csf1r<sup>−/−</sup> MΦ mouse.

Switching to the inter-genotype analyses, an elevation in baseline Csf1 expression was seen in the Csf1r<sup>−/+</sup> MΦ microvessels, which reached significance following either CSF-1 or IL-34 treatment (Figure 5.2, e). Baseline Il34 expression was roughly equivalent across the three mouse strains, with the Csf1r<sup>−/+</sup> EC microvessels having a slightly reduced level of expression. This decrease reached significance in the presence of CSF-1. Due to the unique response of Il34 in the Csf1r<sup>−/+</sup> MΦ microvessels, Il34 expression was significantly higher relative to wild type expression levels in the IL-34 treatment group (Figure 5.2, f). The unresponsive expression of Csf1r in the Csf1r<sup>−/+</sup> MΦ microvessels was significantly reduced in untreated mice, while expression in the Csf1r<sup>−/+</sup> EC microvessels appeared to be conserved (Figure 5.2, g). Regardless of the conserved basal expression, Csf1r expression was revealed to be unresponsive in both transgenic lines of mice, resulting in significantly lower expression in the presence of either cytokine (Figure 5.2, g). At baseline, Ptprz1 expression was significantly elevated in the Csf1r<sup>−/+</sup> MΦ microvessels and this increase was conserved across all treatment groups (Figure 5.2, h).
Figure 5.2: Transcription of CSF-1R pathway genes in brain microvessels isolated from injected C57BL/6 and Csf1r deficient mice. For each gene, the same data is presented in two formats, either comparing changes in gene expression within genotypes (right) or between genotypes (left). RNA was isolated from brain microvessel isolates of C57BL/6 (Wild Type), Csf1rflx/wt;Tie2-Cre+ (Csf1r+/− EC) or Csf1rflx/wt;Cx3cr1-Cre+ (Csf1r+/− MΦ) mice injected with Vehicle (Veh, V), CSF-1 (C, 100 μg/kg) or IL-34 (I, 100 μg/kg). (Wild Type n = 4 mice per group. Csf1r+/− EC n = 2 for V, n = 3 for C and I, Csf1r+/− MΦ n = 4 for V and I, n = 3 for C, two-way ANOVA with Dunnett’s test for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.0005.)
5.3.1.3 Gene Expression of Aβ Influx Transporters Is Uniquely Responsive to CSF-1R Activation In Csf1r-Deficient Microvessels

While tight junction expression governs the paracellular pathway, transcellular passage across the BBB is heavily dependent on transport receptors. Expression of three receptors implicated in the transport of Aβ, LRP-1, RAGE and MDR1a were examined across CSF-1R ligand injected mice. The wild type cerebrovasculature appeared largely unresponsive to CSF-1 or IL-34 treatment, however, loss of Csf1r homozygosity resulted in baseline and ligand-induced changes in gene expression (Figure 5.3, a-c). CSF-1R mediated regulation of Aβ transporter gene expression was limited to Mdr1a in the Csf1r+/− MΦ mouse, which was upregulated by IL-34 (Figure 5.3, c). In contrast, Mdr1a expression was unresponsive to treatment in the Csf1r+/− EC microvessels while both Lrp1 and Rage displayed sensitivity to CSF-1. Lrp1 expression in the Csf1r+/− EC microvessels was inhibited by CSF-1, while Rage was strongly upregulated to 7-fold that of the wild type baseline (Figure 5.3, a, b).

Despite few ligand-induced changes, particularly in the Csf1r+/− MΦ microvessels, significant changes in baseline expression were observed between the mouse strains. Baseline Lrp1 expression in the Csf1r+/− EC microvessels was significantly higher than that of the wild type, and this was sustained in the presence of IL-34. The significant decrease of Lrp1 expression in response to CSF-1 brought expression levels to near wild type, however, equalisation was not achieved (Figure 5.3, d). Rage expression in the Csf1r+/− MΦ microvessels was also significantly higher than wild type under each condition, as was Rage expression in CSF-1 treated Csf1r+/− EC microvessels (Figure 5.3, e). Csf1r+/− EC expression of Mdr1a was significantly lower than wild type at baseline and following CSF-1 treatment, while the decreased expression lost significance in the IL-34 treatment group (Figure 5.3, f). In contrast, baseline Mdr1a expression in the Csf1r+/− MΦ microvessels was normal and unresponsive to CSF-1. Following IL-34 treatment, Mdr1a expression in Csf1r+/− MΦ microvessels was upregulated to become significantly higher than wild type.
Figure 5.3: Transcription of Aβ transport receptor genes in brain microvessels isolated from injected C57BL/6 and Csf1r deficient mice. For each gene, the same data is presented in two formats, either comparing changes in gene expression within genotypes (right) or between genotypes (left). RNA was isolated from brain microvessel isolates of C57BL/6 (Wild Type), Csf1r^{flx/wt};Tie2-Cre^{+} (Csf1r^{+/−} EC) or Csf1r^{flx/wt};Cx3cr1-Cre^{+} (Csf1r^{+/−} MΦ) mice injected with Vehicle (Veh, V), CSF-1 (C, 100 μg/kg) or IL-34 (I, 100 μg/kg). (Wild Type n = 4 mice per group. Csf1r^{+/−} EC n = 2 for V, n = 3 for C and I, Csf1r^{+/−} MΦ n = 4 for V and I, n = 3 for C, two-way ANOVA with Dunnett’s test for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.)
5.3.1.4 Elevated Macrophage Activity At The Cerebrovasculature In Csf1r-Deficient Microvessels

Finally, the expression of macrophage activity genes was examined in addition to the expression of *Icam1*. In the wild type cerebrovasculature, CSF-1 treatment induced an increase in *Icam1* and *Cd68* expression (Figure 5.4, a, b). As with *Tjp1*, the CSF-1 mediated increase of *Icam1* was conserved in the *Csf1r*+/− EC microvessels, but absent from the *Csf1r*+/− MΦ microvessels, indicative of it being dependent on macrophage *Csf1r* homozygosity (Figure 5.4, a). The CSF-1 mediated increase in *Cd68* was also absent from the transgenic mouse microvessels, although this may in part be due to the slightly higher basal expression in both *Csf1r*-deficient strains (Figure 5.4, b).

Although there was a sharp downregulation of both *Cd68* and *Adgre1* in response to IL-34 in the *Csf1r*+/− EC microvessels, these changes did not achieve significance (Figure 5.4, b, c). Only *Csf1r*+/− MΦ microvessel *Adgre1* expression displayed an acquired response to ligand, increasing in response to CSF-1, but not IL-34 (Figure 5.4, c). Expression of *Iba1* was relatively unresponsive to CSF-1R ligand treatment for all mice (Figure 5.4, d).

While there was little ligand-induced regulation of macrophage activity genes, major differences in gene expression were observed between wild type and *Csf1r*-deficient microvessels. *Icam1* expression was significantly higher at baseline and in the presence of IL-34 in the *Csf1r*+/− MΦ microvessels, and the observed CSF-1 mediated upregulation of *Icam1* was significantly stronger in *Csf1r*+/− EC microvessels (Figure 5.4, e). *Cd68* downregulation in the *Csf1r*+/− EC microvessels was significant in the IL-34 treatment group, although the decrease in *Adgre1* expression was not (Figure 5.4, e, f). For both *Adgre1* and *Iba1* expression, *Csf1r*+/− MΦ microvessels had consistently higher expression across all three treatment conditions. Although enhanced by CSF-1 treatment in the context of *Adgre1* expression, these increases were primarily due to elevated baseline expression in the microvessels of these mice with expression of *Adgre1* and *Icam1* 10 and 3-fold higher than wild type respectively (Figure 5.4, e, f).
Figure 5.4: Transcription of macrophage adhesion and activity genes in brain microvessels isolated from injected C57BL/6 and Csf1r deficient mice. For each gene, the same data is presented in two formats, either comparing changes in gene expression within genotypes (right) or between genotypes (left). RNA was isolated from brain microvessel isolates of C57BL/6 (Wild Type), Csf1r^{flx/wt};Tie2-Cre^+ (Csf1r^{+/-} EC) or Csf1r^{flx/wt};Cx3cr1-Cre^+ (Csf1r^{+/-} MΦ) mice injected with Vehicle (Veh, V), CSF-1 (C, 100 μg/kg) or IL-34 (I, 100 μg/kg). (Wild Type n = 4 mice per group. Csf1r^{+/-} EC n = 2 for V, n = 3 for C and I, Csf1r^{+/-} MΦ n = 4 for V and I, n = 3 for C, two-way ANOVA with Dunnett’s test for multiple comparisons, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.)
5.3.2 Csf1r Heterozygosity Increases Aβ42 Retention And Reduces Macrophage Response Following Intrahippocampal Injection

With the CSF-1R pathway shown to modify tight junction expression, cellular adherence, Aβ transport receptors and macrophage activity, we next sought to examine how Csf1r deficiency impacted the acute response to Aβ. Both Csf1r\textsubscript{flx/wt};Tie2-Cre\textsuperscript{+} (Csf1r\textsuperscript{+/−} EC) or Csf1r\textsubscript{flx/wt};Cx3cr1-Cre\textsuperscript{+} (Csf1r\textsuperscript{+/−} MΦ) mice were administered a unilateral intrahippocampal injection of AggreSure\textsuperscript{TM} Aβ42. Behavioural assessment was performed to determine strain-specific deficits in short term recognition memory (Novel Object Recognition Test) and working spatial memory (Plus Maze and T-Maze). These behavioural assessments were performed at 3 days post-injection, and brains were taken for immunohistochemical analyses. An extended duration was possible for the Csf1r\textsuperscript{+/−} EC mice, allowing for further behavioural assessment at 14 and 28 days post-injection.

At 3 days post-injection, neither strain displayed any significant changes in Plus Maze performance when compared to injected controls (Figure 5.5). On extending the timecourse for the Csf1r\textsuperscript{+/−} EC mice, however, strain-specific changes were identified in the Plus Maze (Figure 5.5, b, d). By day 28 post-injection, the Csf1r\textsuperscript{+/−} EC mice had a significantly higher rate of error within the maze, being defined as consecutively entering the same arm of the maze twice. This increase in errors may represent a loss of working spatial memory due to a neuronal change during the final two weeks of Aβ inoculation. The number of arm entries was also significantly changed in the Csf1r\textsuperscript{+/−} EC mice, with the Csf1r\textsuperscript{+/−} EC mice performing significantly fewer arm entries when compared to performance on day 3 post-injection, and the wild type injected controls on day 14 (Figure 5.5, b). By day 28 post-injection, the wild type injected controls had deteriorated to match the performance of the Csf1r\textsuperscript{+/−} EC mice, with both strains performing significantly fewer arm entries than on day 3. The drop in Csf1r\textsuperscript{+/−} EC entries preceded that of the wild type controls, an indication that an endothelium heterozygous for Csf1r may increase the rate of Aβ-induced deterioration within the brain. As the alternation score, which is a measure of working spatial memory, was unchanged, these reduced arm entries could be the product of an overall reduction in mouse activity or ambulation. Neither Csf1r-deficient strain had a deficit in short term recognition memory, as indicated by the lack of significant changes in the Novel Object Recognition Test. Interestingly, while behavioural deficits were only present at later time points for the Csf1r\textsuperscript{+/−} EC mice, injected Csf1r\textsuperscript{+/−} MΦ mice underperformed in the T-Maze at 3 days post-injection (Figure 5.6, b). The T-Maze assesses spontaneous alternation between two trials, with an intertrial time of 10 minutes. The Plus Maze, however, measures ongoing working memory over an 8-minute trial. While the Csf1r\textsuperscript{+/−} MΦ mice achieved a lower T-Maze alternation score...
relative to controls, there was no significant change in Plus-Maze alternation scores. The continuous nature of the Plus-Maze alternation versus the retention based performance of the T-Maze may account for these differences. Overall, these data indicate the presence of exacerbated behavioural deficits in Csf1r-deficient mice following intrahippocampal Aβ injection.

To examine the molecular response to Aβ injection, brains were taken at 3 days post-injection and hippocampal sections stained for tight junctions and macrophage markers. Immunohistochemical analyses of Claudin-5 and ZO-1 at 3 days post-injection yielded no significant changes in either mouse (Figure 5.7-5.15). A significant increase in GFAP intensity was observed in both groups of the Csf1r<sup>−/−</sup> MΦ cohort, although GFAP intensity did not differ significantly between wild type and Csf1r<sup>−/−</sup> MΦ injected mice (Figure 5.9, e). Despite the lack of changes in junctional protein expression, when the macrophage response to Aβ injection was examined there was a striking loss of responsivity in the Csf1r<sup>−/−</sup> MΦ mouse. In wild type and Csf1r<sup>−/−</sup> EC mice, there is a localisation of IB4<sup>+</sup> and F4/80<sup>+</sup> cells to the Aβ plaque which is severely diminished in the Csf1r<sup>−/−</sup> MΦ hippocampus (Figure 5.10). Quantification of IB4, which stains the endothelial glycocalyx as well as macrophages, and F4/80 immunopositivity revealed a significant decrease in the Csf1r<sup>−/−</sup> MΦ sections which was not observed in the Csf1r<sup>−/−</sup> EC mouse (Figure 5.11, c, e). While these responses were conserved in the Csf1r<sup>−/−</sup> EC brain, there was a significantly higher level of Aβ in the Csf1r<sup>−/−</sup> EC brain (Figure 5.11, b).

In the Csf1r<sup>−/−</sup> MΦ mice, we see an early behavioural phenotype with a lower alternation score in the T-maze as well as a reduced macrophage response to Aβ injection. This cellular response is unaffected in the Csf1r<sup>−/−</sup> EC mouse, indicating it to be at least partially driven by macrophage CSF-1R mediated signalling. Although this response is conserved, Csf1r<sup>−/−</sup> EC mice had higher levels of hippocampal Aβ at 3 days post-injection, without a corresponding behavioural phenotype. Indeed, the Csf1r<sup>−/−</sup> EC mouse only displayed strain-specific behavioural deficits at 14 days post-injection. These data propose that the acute response to Aβ injection, involving recruitment of macrophages to regions of Aβ, is in part driven by CSF-1R expressed in macrophages. Endothelial CSF-1R might instead have an effect on Aβ retention or vascular clearance, with elevated Aβ levels resulting in the accelerated onset of Plus Maze behavioural deficits.
Figure 5.5: Plus Maze behavioural data of wild type and Csf1r deficient mice following intrahippocampal injection of Aβ42. Behavioural outputs of errors (a, b), number of arm entries (c, d) and overall score (e, f) from the Plus Maze test for Csf1r\textsuperscript{flx/wt};Cre\textsuperscript{-} (Wild Type), Csf1r\textsuperscript{flx/wt};Tie2-Cre\textsuperscript{-} (Csf1r\textsuperscript{+/−} EC) or Csf1r\textsuperscript{flx/wt};Cx3cr1-Cre\textsuperscript{-} (Csf1r\textsuperscript{+/−} MΦ) mice following a unilateral intrahippocampal injection of Aβ42. Tests were performed at 3 days post-injection (D + 3) for both strains, and additionally at 14 days post-injection (D + 14) and 28 days post-injection (D + 28) for Csf1r\textsuperscript{+/−} EC mice. (n = 5 mice per genotype. Csf1r\textsuperscript{+/−} MΦ data analysed by unpaired t-test with Welch’s correction. Csf1r\textsuperscript{+/−} EC data analysed by 2-way repeated-measures ANOVA with Sidak’s test for multiple comparisons. Asterisks (*) indicate comparison to baseline, obliques (#) indicate comparison between wild type and Csf1r deficient mice at the same timepoint, # or *p < 0.05, ***p < 0.0005, ****p < 0.0001.)
Figure 5.6: Novel Object Recognition Test and T-Test behavioural data of wild type and Csf1r deficient mice following intrahippocampal injection of Aβ42. Behavioural outputs of NOR discrimination index (a, b) and T-maze alternation score (c, d) for Csf1r<sup>flx/wt</sup>;Cre<sup>+</sup> (Wild Type), Csf1r<sup>flx/wt</sup>;Tie2-Cre<sup>+</sup> (Csf1r<sup>+/−</sup> EC) or Csf1r<sup>flx/wt</sup>;Cx3cr1-Cre<sup>+</sup> (Csf1r<sup>+/−</sup> MΦ) mice following a unilateral intrahippocampal injection of Aβ42. Tests were performed at 3 days post-injection (D + 3) for both strains, and additionally at 14 days post-injection (D + 14) and 28 days post-injection (D + 28) for Csf1r<sup>+/−</sup> EC mice. (n = 5 mice per genotype. Csf1r<sup>+/−</sup> MΦ data analysed by unpaired t-test with Welch’s correction. Csf1r<sup>+/−</sup> EC data analysed by two-way repeated-measures ANOVA with Sidak’s test for multiple comparisons. Asterisks (*) indicate comparison to baseline, obliques (#) indicate comparison between wild type and Csf1r deficient mice at the same timepoint.)
Figure 5.7: ZO-1 and GFAP immunohistochemistry of wild-type and Csf1r deficient mice after unilateral intrahippocampal injection of Aβ42. IB4 (green), GFAP (red) and ZO-1 (white) staining of mouse hippocampal sections. (a) IHC of Csf1r^{flx/wt};Cre^+ (Wild Type, top panel) and Csf1r^{flx/wt};Cx3cr1-Cre^+ (Csf1r^{-/-} MΦ, lower panel) mouse sections. (b) IHC of Csf1r^{flx/wt};Cre^+ (Wild Type, top panel) and Csf1r^{flx/wt};Tie2-Cre^+ (Csf1r^{-/-} EC, lower panel) mouse sections. Images representative of n = 4 injected mice per genotype, scale bars indicate 50 μm.
Figure 5.8: Claudin-5 immunohistochemistry of C57BL/6 and Csf1r deficient mice after unilateral intrahippocampal injection of Aβ42. IB4 (green) and Claudin-5 (red) staining of mouse hippocampal sections. (a) IHC of Csf1r<sup>flx/wt;Cre</sup> (Wild Type, top panel) and Csf1r<sup>flx/wt; Cx3cr1-Cre</sup> (Csf1r<sup>++/-</sup> MΦ, lower panel) mouse sections. (b) IHC of Csf1r<sup>flx/wt;Cre</sup> (Wild Type, top panel) and Csf1r<sup>flx/wt;Tie2-Cre</sup> (Csf1r<sup>++/-</sup> EC, lower panel) mouse sections. Images representative of n = 4 injected mice per genotype, scale bars indicate 50 μm.
Figure 5.9: Quantification of tight junction and GFAP immunopositivity following intrahippocampal injection of Aβ₄₂. Quantification of immunopositivity in both injected and uninjected hippocampi of Csf1r¹⁺/⁻;Cre⁺ (Wild Type), Csf1r¹⁺/⁻;Tie2-Cre⁺ (Csf1r⁺/⁻ EC) or Csf1r¹⁺/⁻;Cx3cr1-Cre⁺ (Csf1r⁺/⁻ MΦ) mice. (n = 4 mice per group, with two sections per mouse quantified. Two-way ANOVA with Sidak’s test for multiple comparisons. Asterisks (*) indicate comparison to immunopositivity values of the uninjected hippocampus, obliques (#) indicate comparison between wild type and Csf1r deficient mice, * p < 0.05, **** p < 0.0001).
Figure 5.10: Amyloid-β and F4/80 immunohistochemistry of wild-type and Csf1r deficient mice after unilateral intrahippocampal injection of Aβ42. IB4 (green), Amyloid-β (red) and F4/80 (white) staining of mouse hippocampal sections. (a) IHC of Csf1rflx/wt;Cre (Wild Type, top panel) and Csf1rflx/wt;Cx3cr1-Cre (Csf1rflx/mut; MΦ, lower panel) mouse sections. (b) IHC of Csf1rflx/wt;Cre (Wild Type, top panel) and Csf1rflx/wt;Tie2-Cre (Csf1rflx/EC, lower panel) mouse sections. Images representative of n = 5 injected mice per genotype, scale bars indicate 50 μm.
Figure 5.11: Quantification of Amyloid-β, F4/80 and IB4 immunopositivity following intrahippocampal injection of Aβ42. Quantification of immunopositivity in both injected and uninjected hippocampi of Csf1rflx/wt;Cre (Wild Type), Csf1rflx/wt;Tie2-Cre (Csf1rflx/wt;Csf1rflx/wt;EC) or Csf1rflx/wt;Cx3cr1-Cre (Csf1rflx/wt;MΦ) mice. (n = 5 mice per group, with two sections per mouse quantified. Two-way ANOVA with Sidak’s test for multiple comparisons. Asterisks (*) indicate comparison to immunopositivity values of the uninjected hippocampus, obliques (#) indicate comparison between wild type and Csf1r deficient mice, # or *p < 0.005, ## or **p < 0.005, ### or ***p < 0.0005, #### or ****p < 0.0001)
5.4 Discussion

The findings of this chapter indicate that the BBB displays ligand-specific sensitivities regarding CSF-1R activation. This is not simply limited to whether or not the vasculature responds, but can also result in CSF-1R activation driving opposite responses in gene expression. These responses to CSF-1R activation are further altered by heterozygous loss of endothelial or macrophage Csf1r.

The first objective of this chapter was to first examine these effects of CSF-1R activation on the BBB in vivo. An immediate observation in the brain microvessels was a more potent response to CSF-1 in comparison to IL-34, with IL-34 treatment failing to induce any significant changes in gene expression in wild type mice despite it’s reported higher binding affinity to CSF-1R. Indeed, with the exception of ligand-mediated upregulation of Csf1r expression, significant gene expression changes in response to IL-34 were unique to the Csf1r+/− MΦ mouse. In the context of TJ gene regulation, administration of recombinant CSF-1 and IL-34 had few effects on Cld5 and Ocln expression within the wild type cerebrovasculature. A CSF-1 dependent increase in Tjp1 and Icam1 expression was observed to be dependent on macrophage expression of Csf1r. The heterozygous loss of Csf1r in macrophages, but not endothelial cells, resulted in unresponsive Tjp1 and Icam1 expression in brain microvessels. Heterozygous loss of endothelial Csf1r does, however, result in a stronger response of Tjp1 and Icam1 to this macrophage-crosstalk supporting the requirement for endothelial CSF-1R for functional crosstalk. Regulation of Ocln similarly could tolerate the heterozygous loss of endothelial Csf1r, although gene expression of the critical TJ Cld5 was decreased to roughly 50% of wild type expression levels. In contrast, expression of both Ocln and Cld5 was upregulated in the microvessels of the Csf1r-deficient macrophage mouse, and these genes acquired a unique IL-34 sensitivity. While IL-34 further amplifies the dysfunctional gene expression in the Csf1r+/− MΦ mouse microvessels, CSF-1 restores to wild type levels the expression of Ocln and Cld5 in the Csf1r+/− MΦ Csf1r+/− EC microvessels respectively. The mechanism through which this restoration of normal gene expression occurs might be the upregulation of Csf1 itself. As the upregulatory response of Csf1r expression to CSF-1 and IL-34 is abolished in the Csf1r-deficient mice, producing an abundance of additional ligand may compensate for lower CSF-1R activity. Notably, CSF-1 did not upregulate Il34 expression which would potentially extend the IL-34 mediated dysfunctional TJ expression observed in Csf1r+/− MΦ microvessels. This acquired IL-34 sensitivity in the cerebrovasculature of the Csf1r+/− MΦ mouse might be a factor in the sparing of the cerebellum in ALSP. As IL-34 is the predominantly expressed CSF-1R ligand in the non-cerebellar regions of the brain, in addition to being secreted by the neurons themselves, it may locally induce pathogenic activity in the CNS macrophages.
heterozygous for CSF1R<sup>226,368</sup>. Furthermore, IL-34 was able to induce its own upregulation only in the Csf1r<sup>-/-</sup> MΦ mouse, suggesting it to be a unique response of macrophages heterozygous for Csf1r which could develop into a feed-forward loop of Il34 expression. Overall, it appears that increased ligand expression may be a mechanism through which the response to peripheral CSF-1 or IL-34 is amplified in a CSF-1R-low environment. This shifting between CSF-1 and IL-34 driven signalling may be a biological mechanism for modulating the responsivity of CSF-1R itself. Prioritising CSF-1 expression would directly increase CSF-1R activity while shifting to an IL-34 centred expression profile would result in a more complex activation of CSF-1R as IL-34 bioavailability can be modulated by expression of syndecans and PTP-ζ. Cells with different syndecan and PTP-ζ expression profiles would be differentially responsive to IL-34, but equally responsive to CSF-1, allowing IL-34 to perform a more finely tuned or selective activation of cells expressing CSF-1R.

Although reduced Csf1r expression was confirmed in pure monocultures of primary endothelial cells isolated from the Csf1r<sup>-/-</sup> EC mouse brain (4.3.5), Csf1r expression was rescued in the resting microvessels. This expression remained unresponsive to ligand treatment but indicates a compensatory mechanism that may not be intrinsic to the endothelial cell itself. Crosstalk from macrophages homozygous for Csf1r may be responsible for this rescue, as the opposite is observed in the microvessels of the Csf1r<sup>-/-</sup> MΦ mouse. Irrespective of the endothelial Csf1r genotype, Csf1r expression is significantly reduced in the resting microvessels of this mouse line. Crosstalk from Csf1r heterozygous macrophages may be detrimental to the NVU, with dysfunctional TJ and Csf1r gene expression induced through paracellular signalling.

As shown in Chapter 3, the monoallelic loss of CSF-1R functionality can lead to CAA. In these Csf1r-deficient mouse strains we see alterations in the baseline expression of three Aβ transport proteins, in addition to acquired CSF-1 and IL-34 responsive gene expression. In the Csf1r<sup>-/-</sup> EC mouse, expression of Mdr1a is significantly decreased and wholly unresponsive to ligand treatment. In the same mouse, there is an increase in microvessel Lrp1, possibly a compensatory mechanism to increase the rate of substrate efflux from the brain. As P-gp functions as an efflux transporter for a broad range of substrates, this could result in Aβ accumulation at the endothelium as well as a reduction in the brain’s capacity to clear harmful substrates via the circulatory system. A notable scenario here is that in the presence of CSF-1, Aβ efflux transporter expression is reduced while there is a simultaneous increase in gene expression of the Rage Aβ influx receptor. This may contribute to the build-up vascular Aβ by reducing endothelial LRP-1 and P-gp mediated clearance in response to systemic sCSF-1 or secretion by macrophages.
localising to regions of CAA, which would contribute to the feed-forward CSF-1 mediated increase of Csf1 expression. There is a similar constitutive upregulation of Rage expression in the Csf1r\textsuperscript{+/-} MΦ microvessels, indicative of detrimental effects of Csf1r\textsuperscript{+/-} macrophages concerning cerebrovascular Aβ clearance. As Rage is increased in response to endothelial cell activation, vascular-associating macrophages may be in a pro-inflammatory activation state, producing the altered gene expression profiled observed in the Csf1r\textsuperscript{+/-} MΦ microvessels.

In wild type mice, activation of CSF-1R through intravenous administration of CSF-1 appears to induce conditions that promote macrophage association with the vasculature. Upregulation of \textit{lcam1}, a cell adhesion molecule expressed by activated endothelial cells, would facilitate peripheral cell adhesion to the endothelium and promote further transmigration across the BBB. The increase in microvessel Tjp1 and \textit{lcam1} expression may indicate remodelling of the tight junction-cytoskeleton complex to promote infiltration of circulating immune cells. \textit{lcam1} is expressed in endothelial cells, microglia and reactive astrocytes in the brain and functions to facilitate transmigration of leukocytes into the brain\textsuperscript{369-371}. The CSF-1 driven increases in \textit{lcam1} and \textit{Cd68} tentatively indicate an increase in macrophage localisation and activity in CSF-1 treated brain microvessels. Although \textit{Cd68} expression is not unique to peripheral macrophages, the absence of a corresponding increase in lba1 suggests that this increase stems from the recruitment of peripheral macrophages, or perhaps activation of the perivascular macrophage population. This localisation may either be due to immune cell recruitment by the neurovasculature or a product of systemic CSF-1 administration inducing macrophage transport to the CNS endothelium. Alterations in macrophage recruitment and activity were also evident in the Csf1r-deficient mouse microvessels, in particular the Csf1r\textsuperscript{+/-} MΦ mouse. In the context of \textit{lcam1} expression, responsivity is lost completely in the Csf1r\textsuperscript{+/-} MΦ mouse while CSF-1 mediated increases are conserved, if amplified, in the Csf1r\textsuperscript{+/-} EC mouse. The elevated \textit{lcam1} expression in the Csf1r\textsuperscript{+/-} MΦ mouse corresponds to the elevated expression of Adgre1 and lba1 in the same strain, suggestive of microglial involvement. Intriguingly, in the Csf1r\textsuperscript{+/-} EC microvessels, there is a CSF-1 mediated increase in Cd68 and Adgre1 corresponding to the lcam1 upregulation, but lba1 is unresponsive. The enhanced lcam1 upregulatory capacity of CSF-1 in the Csf1r\textsuperscript{+/-} EC mouse proposes that the Csf1r-deficient vasculature may be primed for lcam1 increase. Moreover, the Csf1r\textsuperscript{+/-} MΦ mouse displays constitutively elevated, and ligand-independent, increases in lcam1, Adgre1 and lba1. These effects, in addition to the Csf1r\textsuperscript{+/-} MΦ dysregulated microvessel gene expression discussed thus far, may stem from an overactive, dysregulated or chronically active macrophage population localising to the cerebrovasculature.
We have previously shown CAA to be a pathology of ALSP in kindreds where the disease is driven by the presence of a null CSF1R variant. To examine whether endothelial or macrophage Csf1r heterozygosity could have an impact on the brain’s response to Aβ deposition in vivo, intrahippocampal injections of Aβ42 were performed in the Csf1r+/− EC and Csf1r+/− MΦ mice. Changes in the microvessels of these mice, including in macrophage activity and the expression of Aβ transport proteins, indicated that Aβ retention and the peripheral immune response may be affected. Examination of tight junctions claudin-5 and ZO-1 revealed no significant changes in expression, with astrocyte activation differing slightly between treatment groups.

Despite the elevated and static expression of icam1 and macrophage activity genes in the Csf1r+/− MΦ microvessels, the macrophage response was severely diminished. This discrepancy may be due to geographic effects of Csf1r loss in the mouse brain. As microvessels were isolated from the total grey matter of the brain, the transcriptional changes observed are representative of the net increase or decrease throughout the brain. Region-specific isolations may yield further insight into the geographic transcriptional effects of Csf1r loss. In hippocampal regions of Aβ42 deposition of wild type and Csf1r+/− EC mice, there was an increase in F4/80 and IB4 positive cells associating with injected Aβ. This response was absent in the Csf1r+/− MΦ mouse, with weak F4/80 and IB4 immunopositivity. Loss of one copy of Csf1r appeared to severely diminish the capacity of these macrophages to localise to sites of amyloid accumulation. This may have been due to a deficit in localisation or BBB transmigration, although there was no apparent vascular accumulation and stalling of IB4 or F4/80 positive cells. As seen in Chapter 3, macrophages produced from ALSP PMBCs and bone marrow from the Csf1r+/− MΦ mouse have reduced phagocytic capacity. Additionally, the circulatory leukocyte population in ALSP donors was lacking in mature myeloid cells, and as discussed earlier in this thesis, CSF-1R dependent peripheral macrophages have been shown to contribute to vascular amyloid clearance. Together this suggests that the peripheral CSF-1R response may play a role in hippocampal Aβ detection and clearance in ALSP. Failure to recruit active myeloid clearance machinery to regions of Aβ deposition may facilitate the development of CAA in ALSP as increased pressure is put on the vasculature for Aβ42 clearance. Behaviourally, the Csf1r+/− MΦ mice had a poorer performance in the T-Maze, having a lower alternation score and poorer short term working memory.

In contrast, the Csf1r+/− EC mouse performed on par with the wild type controls at 3 days post-injection. In this Csf1r-deficient mouse, there was greater retention of Aβ42 in the hippocampus compared to controls. Loss of endothelial Csf1r led to an apparent decrease in the ability to clear Aβ42, and this may have led to the earlier deterioration in Plus-Maze performance in this strain.
By day 14, the Csf1r+/− EC mouse had fewer arm entries than injected controls, indicating a behavioural deficit in exploration and short term working memory. The decrease in arm entries was seen in both the Csf1r+/− EC and injected controls by day 28, suggesting an accelerated decline in the Csf1r-deficient strain. Furthermore, the injected wild type controls made significantly fewer errors in the Plus Maze at day 28, indicating that even four weeks post-injection the Csf1r+/− EC mice still displayed greater behavioural deficits.

With a role for CSF-1R validated in endothelial cells in vitro, the objective of this chapter was to discern whether the endothelium was responsive to CSF-1R activation, and genetic inactivation, in vivo. Furthermore, as ALSP is widely considered a microgliopathy, determining whether loss endothelial Csf1r resulted in BBB changes was critical should the BBB be considered a possible therapeutic target. To achieve these objectives, we have examined the transcriptional activity in microvessels of wild type, endothelial Csf1r-deficient and macrophage Csf1r-deficient mouse models both at rest and in response to ligand administration. The data in this chapter indicate that the coordinated actions of the endothelium and macrophage populations maintain the BBB. Heterozygous loss of Csf1r in either cell type results in changes in the expression of tight junctions, Aβ transport proteins and macrophage activity markers. The CSF-1R pathway itself is also transcriptionally altered in both transgenic mouse strains, supporting the requirement for both endothelial and macrophage CSF-1R activity for effective cross-talk. Furthermore, alterations in lcam1 in response to CSF-1 appears to correlate with the association of Cd68 and Adgre1 expressing macrophages. The data presented here reveal differing actions of IL-34 and CSF-1 on the brain microvessels, with CSF-1R activation and signalling output dependent on the ligand provided and Csf1r genotype. A critical finding here is that heterozygous loss of macrophage Csf1r results in the brain microvessels having downregulated Csf1r transcription. As discussed in Chapter 4, cross-talk between microglia and endothelial cells is both a process dependent on the CSF-1R state of these cells, as well as a means by which CSF-1R itself, its pathway components and endothelial tight junctions are transcriptionally regulated. This data supports the view that CSF-1R mediated cerebral dysfunction, as seen in ALSP, is mediated not simply through BBB dysfunction or abnormal microglial activity, but through alterations in cross-talk between the cerebrovasculature and surrounding macrophage population. This was further shown by the different outcomes of Aβ injection in the Csf1r-deficient mouse models. In the absence of homozygous macrophage Csf1r, the localisation of macrophages to the region of Aβ deposition is severely attenuated. In the absence of endothelial Csf1r, this innate immune response is conserved, however, there is an earlier onset of late-stage behavioural deficits as well as greater retention of Aβ at 3 days post-injection. These effects together would result in slower
Aβ clearance from the brain over time, potentially through increased expression of Aβ influx transport proteins with corresponding decreases in Aβ efflux protein expression. Even upon reaching a threshold of Aβ accumulation sufficient to activate peripheral macrophage clearance, reduced localisation of poorly phagocytic macrophages would be inefficient in clearing Aβ accumulates at the vasculature. These effects, in combination with altered CSF-1R, CSF-1 and IL-34 expression and activation outcomes may ultimately result in the generation of CAA in ALSP. Further experiments in the global Csf1r+/− mouse will reveal whether these changes are present in a brain with cross-talk between endothelial cells and macrophages of equivalent Csf1r states.
Chapter 6:

General Discussion
6.1 General Discussion

Prior to the discovery of CSF1R mutations being the causative variants in ALSP, diagnosis of the disease was largely dependent on neuropathological findings, with accurate diagnoses often only being given post-mortem. Although still rare enough to be classed as an orphan disease, the incidence of ALSP has increased due to the ability to rely on genetic diagnoses. It has been estimated that 10% of adult-onset leukoencephalopathies are caused by mutations in CSF1R, and this is expected to increase due to improvements in diagnostic accuracy and awareness of the condition. Research concerning CSF-1R has historically focused on its functional role in myeloid lineage cell development and the activities of tumour associating macrophages in cancer. Following the discovery of its necessity for microglial viability, this focus expanded to include documenting the expression of CSF-1R and its ligands in the development of the CNS, and targeting CSF-1R as a means to deplete the CNS microglial population. Due to this microglial dependency on CSF-1R, ALSP has more recently been considered a microgliopathy, with neurological dysfunction stemming from the aberrant functions of the CSF1R-heterozygous microglia. The objective of this doctoral work was to challenge this understanding, proposing that CSF-1R expression additionally is required for BBB maintenance. Furthermore, we hypothesise that CSF-1R regulates vascular crosstalk with leukocytes, mediating the response of peripheral cells to cerebral Aβ as well as microglial regulation of the endothelial tight junction.

Although BBB involvement has been reported in many diseases of the CNS, here we present the first documented cases of BBB dysfunction in ALSP and additionally identify CSF-1R as a regulator of endothelial cell function. In collaboration with clinical neuropathologist partners, two genetically distinct CSF1R variants were shown to lead to ALSP accompanied by BBB dysfunction. Examination of post-mortem cortical tissue revealed extravasation of both IgG and fibrinogen, as well as extensive CAA and perivascular astrogliosis. Monogenic variants capable of producing CAA are exceedingly rare and generally are caused by mutations in APP itself, directly affecting protein folding and rates of fibrilisation. Furthermore, we confirmed in vivo BBB dysfunction in a carrier of one of these variants coinciding with clinical onset of ALSP, prior to the symmetrical incidence of white matter lesions. The unusual incidence of CAA, accompanied by BBB dysfunction, in a CSF-1R driven disease indicated a possible role for the receptor in cerebrovascular function and indeed clearance of cerebral amyloid. Through in vitro assessment of these CAA-associating CSF-1R variants, it was determined that these individuals could be effectively considered haploinsufficient for CSF-1R. These variant isoforms of CSF-1R were not only incapable of signalling through ERK1/2, but they were also actively targeted for degradation.
via the UPS. While native and variant CSF-1R were found to each be targets for autophagy-mediated protein turnover, this UPS-mediated degradation was unique to the variant isoforms of the receptor. As variant CSF-1R localises to the cell membrane, this increased degradation is possibly a means through which the cell inhibits native:variant CSF-1R dimerisation at the cell surface, which would lead to inactivation of native CSF-1R molecules. Confirmation of reduced, rather than aberrant, CSF-1R signalling permitted us to further examine CAA-associated CSF-1R signalling through CSF-1R inhibition and CSf1r-heterozygous mouse models. Indeed, direct inhibition of endothelial CSF-1R resulted in a loss in TJ expression in vitro, with TJ components occludin and claudin-5 decreased at the protein and RNA level. This reduction in TJ expression translated to a functional increase in endothelial monolayer permeability to a 4 kDa tracer, demonstrating a CSF-1R dependency for endothelial function previously only demonstrated under pro-inflammatory conditions in vitro. Despite robust downregulation of TJ components under CSF-1R inhibitory conditions, a corresponding upregulation of TJ expression of the same magnitude was not observed following in vitro CSF-1R activation in endothelial cells. Treatment with CSF-1 resulted in an acute decrease in claudin-5 and occludin expression, although this was recovered within 48 h of treatment. This decrease was accompanied by some transcriptional upregulation of TJ gene expression, however, it argued against CSF-1R functioning as a major regulator of endothelial TJ expression. Furthermore, IL-34 had little effect on TJ expression, demonstrating a ligand-specific response within the endothelium which would later also be evident following in vivo administration of CSF-1 and IL-34. This weak response of endothelial cells to CSF-1R activation would prevent secreted circulating CSF-1 and cerebral IL-34 from directly remodelling the endothelial TJ during infection or CNS stress. It did not, however, lend support to the loss of CSF-1R signalling being the primary vascular dysfunction in ALSP. Considering the essential role of CSF-1R in microglia, we next sought to determine whether CSF-1R loss could impact microglial interactions with the CNS endothelium. Using microglia conditioned media treatments with concomitant CSF-1R inhibition, a CSF-1R dependent effect of microglia on endothelial cell cultures was identified in vitro. Microglia conditioned media could increase endothelial cell expression of CSF-1R, and additionally induce changes in TJ expression, namely downregulation claudin-5 protein expression and upregulating Ocln gene expression. This capacity to increase endothelial CSF-1R and Ocln expression was lost upon pre-treatment of microglia with PLX3397, demonstrating these changes to be dependent on microglial CSF-1R.

A critical downside of the molecular work discussed above had been the genotypes of the biological materials used, with microglia and endothelial cells being homozygous for wild type Csf1r. While the results obtained demonstrated the role of CSF-1R in endothelial cell function and
microglial-endothelial crosstalk, they were by no means representative of the ALSP CNS, and by extension, the conditions conducive to the development of CAA. To address this, two transgenicCre-\textit{Csf1r}\textsuperscript{loxP}\textsuperscript{WT} mouse lines were bred which induced the loss of one allele of \textit{Csf1r} expression in either endothelial cells or macrophages. Primary microglia and endothelial cells were isolated from these mice, and the microglia conditioned media experiment repeated, examining transcriptional changes in endothelial cells. This revealed effects of microglial conditioned media on endothelial cells which were dependent on \textit{Csf1r} expression in both cell types. Notably, unique susceptibilities of endothelial cells to microglial cross-talk were observed in the “ALSP” context wherein \textit{Csf1r}\textsuperscript{+/−} endothelial cells were treated with conditioned media produced by microglia heterozygous for \textit{Csf1r}. Under these conditions, there was significant downregulation of \textit{Cld5}, \textit{Ocln} and \textit{Marvedl2} gene expression, while endothelial cells homozygous for \textit{Csf1r} were unaffected by the same treatment. This susceptibility of \textit{Csf1r}\textsuperscript{+/−} endothelial cells to \textit{Csf1r}\textsuperscript{+/−} microglial conditioned media was also evident in the transcriptional response of \textit{Csf1r} itself. Furthermore, baseline gene expression of CSF-1R pathways components \textit{Csf1r}, \textit{Ptprz1}, \textit{Csf1} and \textit{Il34} was dysregulated in \textit{Csf1r}\textsuperscript{+/−} endothelial cells, with decreased expression of both receptors and a corresponding increase in ligand expression. These results indicated that in monoculture, tight junction gene expression was relatively unaffected by heterozygous loss of endothelial \textit{Csf1r}, however, alterations in the expression of the CSF-1R pathway itself led to a heightened sensitivity to secreted factors from \textit{Csf1r}\textsuperscript{+/−} microglia, ultimately resulting in decreased TJ gene expression in response to treatment. Endothelial \textit{Csf1r} loss also abolished the \textit{Csf1r}-inhibitory and \textit{Csf1}-upregulatory effects of \textit{Csf1r}\textsuperscript{+/−} microglia conditioned media, possibly due to the already altered baseline expression of these genes in the \textit{Csf1r}\textsuperscript{+/−} endothelial cells. In contrast to the changes observed in BV2-bEnd.3 cell culture experiments, the effects of the microglial secretome on endothelial tight junction and \textit{Csf1r} gene expression were more dynamic when \textit{Csf1r} gene dosage was modified. While CSF-1R activation \textit{in vitro} had a weak effect on tight junction expression, even in the presence of BV2 conditioned media, the receptor appears to regulate both the capacity of microglia to modify endothelial gene expression and the responsivity of the endothelial cell itself to these signals.

Macrophages, including CNS microglia, appear to have a protective function regarding CAA development and Aβ clearance at the cerebrovasculature. Ablation of patrolling (Ly6C\textsuperscript{lo}CX3CR1\textsuperscript{hi}CCR2\textsuperscript{−}) monocytes, perivascular CD163 macrophages or CNS microglia themselves can accelerate vascular Aβ deposition\textsuperscript{283,316}. In the case of microglial depletion, the mechanism of cerebral Aβ accumulation is actively transitioned from plaque development to vascular deposition\textsuperscript{272}. While the enzymatic and lysosomal macrophage activities facilitate
parenchymal Aβ clearance, the removal of perivascular Aβ places the macrophage in an ideal position for paracrine endothelial communication. Increasing paracellular clearance through tight junction modification or transcellular clearance via LRP-1 or P-gp would complement the phagosomal and enzymatic macrophage Aβ pathways. The in vitro experiments detailed in this work indicate that endothelial gene expression of tight junction and CSF-1R pathway components is responsive to paracrine microglial signalling, with the CSF-1R state of both cell types further shaping the outcome of this signalling. The integrity of these interactions between microglia and the CNS endothelium may be compromised in the ALSP brain. Loss of functional CSF-1R would disrupt cross-talk at the vasculature, resulting in dysregulated BBB responses to neighbouring macrophages, and additionally to CSF-1 and IL-34 secretion. Through isolating cerebral microvessels from wild type and Csf1r-deficient mice, we confirmed that loss of endothelial or macrophage Csf1r could indeed impact BBB gene expression in vivo. In the resting cerebrovasculature, induction of endothelial Csf1r heterozygosity resulted in a significant reduction in the expression of Cld5 and Mdr1a, while Lrp1 expression was increased. Macrophage Csf1r heterozygosity led to elevated Cld5, Ocln and Rage expression and additionally decreased vascular Csf1r. The changes in this mouse were accompanied by an overall upregulation of Icam1, Adgre1 and Iba1 at the cerebrovasculature, indicating dysregulated macrophage association with the vasculature. Intravenous injection of recombinant CSF-1R ligands uncovered alternate outcomes of CSF-1R activation in the microvessels of Csf1r-deficient mice. In the microvessels of the endothelial Csf1r heterozygote, increased sensitivity to CSF-1 was evident whereas acquired IL-34 responsivity was seen in the context of macrophage Csf1r loss. Transcriptional changes in Tjp1, Rage and Icam1 in response to CSF-1 were amplified by the loss of endothelial Csf1r, and despite baseline Csf1r expression being unaffected, Csf1r gene expression lost responsivity to ligand-mediated upregulation. Loss of macrophage Csf1r resulted in the expression of Tjp1, Icam1, Cd68 and Csf1r itself becoming unresponsive to ligand treatment, while there were acquired responses in Ocln, Il34 and Mdr1a to CSF-1R ligation. As previously seen in the in vitro microglia conditioning experiment with cells isolated from these mice, ligand expression appeared to be preferentially elevated in the presence of lower Csf1r transcription. The discordant effects of CSF-1 and IL-34 revealed that cytokine-specific differences in CSF-1R ligation were not simply limited to biochemical affinity. Future investigations into CSF-1R biology within the brain will require an assessment of the cerebral landscape of CSF-1 and IL-34 expression in the context of Csf1r heterozygosity, in addition to the expression of IL-34 binding partners PTP-ζ and syndecan-1. As previously highlighted, CSF-1 has been reported to primarily maintain cerebellar macrophages, resulting in cerebellar and forebrain microglia having distinct gene
expression profiles. Forebrain microglia also tolerate silencing of Csf1, but not Il34, expression. This IL-34 dependency may explain why the cerebellum is conserved in ALSP, and also why there was a strong IL-34 response in the macrophage Csf1r heterozygote. Moreover, isolation of cerebellar microvessels may reveal subsequent changes in gene expression following CSF-1 treatment in these mice. Despite recent research efforts being focused on CSF-1R itself, the differential responses of macrophages, and the BBB, to IL-34 versus CSF-1 may be a critical factor in BBB-macrophage interaction and even more so in the aetiology of ALSP.

Regarding Aβ transport, the Csf1r-deficient cerebrovasculature appeared primed for ligand-induced upregulation of Aβ influx transporter Rage, with concomitant inhibition of efflux transporter expression. An increase in ICAM-1 mediated macrophage association was also seen in the endothelial Csf1r heterozygote in response to CSF-1, and constitutively in the macrophage heterozygote. To discern the functional impacts of these changes in the context of the innate immune response to Aβ, intrahippocampal injections of Aβ were performed on the Csf1r-deficient mice. While the innate immune response was unchanged in the endothelial heterozygotes, Aβ immunopositivity was higher at 3 days post-injection in comparison to controls, indicating a reduced rate of hippocampal Aβ clearance in these mice. This was accompanied by an earlier appearance of Plus Maze special working memory deficits in animals with endothelial Csf1r heterozygosity. This increased Aβ retention may have been due to the decreased baseline Mdr1a expression in the microvessels of these mice. The IB4 and F4/80 positive macrophages recruited to the regions of Aβ deposition may also be secreting CSF-1 which would further inhibit Lrp1 and upregulate Rage expression in the endothelium, increasing net Aβ influx into the brain. In contrast, amyloid retention was unaffected in the macrophage Csf1r heterozygote, although the loss of macrophage Csf1r resulted in poorer T-Maze alternation scores when compared in injected Cre-negative controls. There was also a decrease in the innate immune response in the macrophage heterozygotes, with reduced IB4 and F4/80 immunopositivity cells in the regions of Aβ deposition. This loss of Aβ-induced macrophage localisation and activation was indicative of Csf1r loss producing dysfunctional macrophage populations in this mouse. This loss in macrophage responsivity to the Aβ stimulus, despite the elevated cerebrovascular expression of icam1 and macrophage gene expression, was indicative of a dysregulated macrophage population in this mouse. This was strikingly similar to what was observed when the PBMCs of ALSP patients were examined, with increases in undifferentiated circulating mononuclear cells, dysmorphic macrophages and reduced peripheral macrophage phagocytic capacity. As phagocytosis was not affected in the BV2 microglia following siRNA mediated Csf1r downregulation, nor in primary microglia isolated from the macrophage Csf1r
heterozygous mouse, the changes in the innate immune response observed in intrahippocampally injected mice may be due to depleted recruitment of peripheral macrophages, rather than microglial dysfunction.

This reduction in peripheral and CNS-localising macrophage function would argue in support of the reported therapeutic effect of HSCT in individuals with ALSP\textsuperscript{188,189}. Replenishing the peripheral macrophage population would increase the proportion of circulatory monocytes and macrophages which are homozygous for native \textit{CSF1R}. These cells could then engraft into the niches of tissue-resident macrophages, replacing the endogenous macrophage populations and restoring function. In the context of the brain, macrophage replacement with HSCT donor cells is usually limited by the method of pre-transplantation myeloablative conditioning, as BBB breakdown is required for engraftment of the peripheral monocytes into the microglial niche\textsuperscript{280–282}. As demonstrated in this doctoral work, however, the BBB is compromised in ALSP, with milder leakage detected in the early stages of clinical onset and permeability to high molecular weight macromolecules detected post mortem. This BBB permeability, and deficit in peripheral macrophage function, could explain the success of the HSCT procedures performed thus far in the treatment of ALSP. Restoring macrophage \textit{CSF1R} homozygosity would also be beneficial in ameliorating the dysfunctional impact of microglial-endothelial cross-talk in cells heterozygous for \textit{CSF1R}. In the work presented here, wild type mouse microglia did not induce the same transcriptional changes in \textit{Csf1r}\textsuperscript{+/-} endothelial cells as heterozygous microglia, thereby suggesting microglial replacement may be capable of reducing dysfunctional microglial-endothelial crosstalk. Restoring \textit{CSF1R} expression in human microglia through HSCT-mediated replacement may also help resolve aberrant CSF-1R signalling induced by IL-34 expressing neurons and glial cells. This dual resolution of the peripheral and CNS macrophage compartments may drive the endothelium from a pathogenic pro-CAA state towards one more closely resembling that of the healthy brain.

Moving forward, transitioning away from the use of cell-specific \textit{Csf1r} heterozygotes and towards the global \textit{Csf1r} heterozygote will allow for the therapeutic potential of macrophage replacement to be assessed and characterised. It is still unknown how HSCT drives recovery in ALSP, and indeed why there appears to be an initial worsening of the condition in the months following surgery. A longitudinal study documenting the responses of the circulatory and CNS macrophage compartments to HSCT in mice would provide much needed molecular detail on this therapeutic approach. Using donor cells from GFP-expressing mice would allow for the replacement of each individual macrophage niche to be examined and correlated with recovery of the neuronal lesions and reduced neurocognitive performance documented in the \textit{Csf1r}\textsuperscript{+/-}.
mouse. Furthermore, a characterisation of BBB and vascular Aβ clearance pathways at baseline and post-HSCT in this mouse strain may identify dysfunctional processes which could then be targeted in AD and non-ALSP CAA. Finally, this study could be expanded to include patients with ALSP-associated CSF1R variants. MRI assessment of BBB function and peripheral blood-based analyses of PBMCs and serum components could be included in the current standard of care for ALSP. This would provide a means by which the progression of ALSP may be staged, tracking BBB dysfunction, white matter lesion development and declining peripheral macrophage function in parallel. This approach may provide a much-needed model for the development of ALSP which could be applied during the initial assessment of ALSP patients on receiving their diagnosis, and indeed other family members carrying the same mutation.
Figure 6.1: Contrasting the healthy (left) and ALSP (right) cerebrovasculature.

A: Dysregulated microglial-endothelial crosstalk in the absence of CSF1R homozygosity leading to altered endothelial and tight junction maintenance.

B: BBB breakdown and entry of blood-borne macromolecules into the brain parenchyma.

C: Perivascular astrogliosis and GFAP upregulation.

D: Increase in lineage-negative monocyte populations in ALSP PBMCs.

E: Reduced localisation of peripheral macrophages. ALSP macrophages display an aberrant morphology and have reduced phagocytic activity.

F: A shift from parenchymal to vascular Aβ accumulation, and CAA. Altered Aβ transporter expression drives Aβ influx in the ALSP brain rather than clearance.
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