WHITE MATTER REALLY MATTERS IN CEREBRAL SMALL VESSEL DISEASE

Role of hypoxia signalling in oligodendrocyte precursor cells and its crosstalk with endothelial cells

DISSERTATION

A thesis submitted to the University of Birmingham and Maastricht University for the degree of DOCTOR OF PHILOSOPHY

By

Narek Manukjan

Neuroscience & Ophthalmology
Institute of Inflammation and Ageing
College of Medical and Dental Sciences
University of Birmingham

Department of Pharmacology and Toxicology
CARIM – School for Cardiovascular Diseases
Maastricht University

July 2023

UNIVERSITY OF BIRMINGHAM

University of Birmingham Research Archive
e-theses repository



This unpublished thesis/dissertation is under a Creative Commons Attribution 4.0 International (CC BY 4.0) licence.

You are free to:

Share — copy and redistribute the material in any medium or format

Adapt — remix, transform, and build upon the material for any purpose, even commercially.

The licensor cannot revoke these freedoms as long as you follow the license terms.

Under the following terms:



Attribution — You must give appropriate credit, provide a link to the license, and indicate if changes were made. You may do so in any reasonable manner, but not in any way that suggests the licensor endorses you or your use.

No additional restrictions — You may not apply legal terms or technological measures that legally restrict others from doing anything the license permits.

Notices:

You do not have to comply with the license for elements of the material in the public domain or where your use is permitted by an applicable exception or limitation.

No warranties are given. The license may not give you all of the permissions necessary for your intended use. For example, other rights such as publicity, privacy, or moral rights may limit how you use the material.

Unless otherwise stated, any material in this thesis/dissertation that is cited to a third-party source is not included in the terms of this licence. Please refer to the original source(s) for licencing conditions of any quotes, images or other material cited to a third party.

Promotors:

Prof. Dr. Z. Ahmed, University of Birmingham, UK

Dr. W.M. Blankesteijn, Maastricht University, NL

Co-promotors:

Dr. D. Fulton, University of Birmingham, UK

Dr. S. Foulquier, Maastricht University, NL

Acknowledgements:

The research described in this thesis was supported by a PhD studentship, jointly funded by the University of Birmingham and CARIM, School for Cardiovascular Diseases Maastricht at Maastricht University and performed at:



UNIVERSITY OF BIRMINGHAM

INSTITUTE OF INFLAMMATION AND AGEING

Neuroscience and ophthalmology, Institute of Inflammation and Ageing (IFA), College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom

Maastricht University

School for Cardiovascular Diseases

Department of Pharmacology and Toxicology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands

Abstract

Cerebral small vessel disease (cSVD) is a vascular risk factor associated disorder characterised by white matter lesions. These lesions are associated with cerebral hypoperfusion and increased blood-brain barrier (BBB) permeability. The cellular and molecular mechanisms underlying small vessel dysfunction and loss of white matter integrity remains largely unknown. In that context both endothelial cells (ECs) and oligodendroglia are key players, as ECs have a critical role in cerebral perfusion and BBB permeability and oligodendrocytes and oligodendrocyte precursor cells (OPCs) are responsible for myelination in the brain. Although EC dysfunction could affect oligodendrocytes and OPCs and lead to white matter damage, the role of their interplay is poorly studied in context of cSVD.

In this thesis, we identified Wnt7a as a potential modulator of EC-OPC interaction in a systematic literature search (Chapter 2). We studied the effects of Wnt7a and β-catenin signalling on endothelial barrier integrity in vitro, demonstrating its impact on the expression of tight junction proteins (Chapter 3). Our transcriptomic analysis revealed the role of Wnt7a in regulating a signalling pathway of importance for OPCs. We showed that Wnt7a suppressed *Cxcl12* in ECs but had no influence on Cxcl12 receptor *Cxcr4* in OPCs (Chapter 4). Finally, we demonstrated that cerebral hypoperfusion in mice leads to hypoxia in OPCs and an increased BBB permeability. In vitro experiments with OPCs exposed to hypoxia resulted in upregulation of *Vegfa*, rather than Wnt7a, via *Hif1a* and *Epas1* signalling. We also showed in a retrospective human study that higher VEGFA plasma levels were associated with increased BBB permeability in normal appearing white matter of cSVD patients (Chapter 5).

Taken together, our findings support *(a)* a role for Wnt7a in regulating BBB integrity and OPC migration and *(b)* an early role for OPC-derived VEGFA in hypoxia contributing to BBB impairment and possibly white matter lesion development in cSVD patients.

Table of Contents

Chapter 1 - General introduction **9**

Chapter 2 - A Systematic Review of WNT Signaling in Endothelial Cell Oligodendrocyte Interactions: Potential Relevance to Cerebral Small Vessel Disease **44**

Chapter 3 - Wnt7a decreases brain endothelial barrier function via β-catenin activation **74**

Chapter 4 - Wnt7a decreases Cxcl12 expression in brain endothelial cells **105**

Chapter 5 - Hypoxic oligodendrocyte precursor cell-derived VEGFA is associated with blood-brain barrier impairment **116**

Chapter 6 - Summary and general discussion **174**

Chapter 7 - Impact **196**

Dutch summary - Nederlandse samenvatting **206**

Acknowledgements - Dankwoord **211**

Chapter 1

-

General introduction

This thesis focuses on the molecular mechanisms involved in cerebral small vessel disease (cSVD). CSVD is an umbrella term for various conditions that impact the small blood vessels in the brain including the arterioles, capillaries, and venules, which can lead to brain structural alterations such as lacunae, white matter (WM) hyperintensities, enlarged perivascular spaces, microbleeds, subcortical infarcts and brain atrophy [1]. The global burden of cSVD is widely underestimated, as characteristics of this disease are present in almost every individual over the age of 60 years old and is projected to only increase as the world population ages [2]. Additionally, the underlying cause of about 20% of all ischemic strokes, gait impairments, and mood disturbances is cSVD [1, 3, 4]. Diagnosis relies on MRI brain imaging characteristics while treatments mainly focus on risk and symptoms management [1, 2, 4, 5]. Although advances have been made in recent years, the underlying pathology remains largely unknown. It is thus important to gain a better understanding of the mechanisms leading to cSVD development, especially the early mechanisms to prevent adverse structural brain changes in patients. Thus, my goal in this thesis was to unravel early mechanisms leading to the progressive development of cSVD.

Important structural features of cSVD include blood-brain barrier (BBB) impairments and the presence of white matter lesions (WMLs). However, there is limited evidence about the cellular changes that lead to the development of these features. It is known that endothelial cell (EC) dysfunction leads to BBB impairments and that changes in oligodendrocyte and oligodendrocyte precursor cells (OPCs) results in WM damage, and that these changes might be involved in cSVD pathology [6, 7]. However, there is limited knowledge on the role of their interaction in cSVD. Thus, we conducted a systematic literature investigation on the interaction between ECs and OPCs in context of cSVD to map the current knowledge. To investigate the role of these cells in early cSVD pathology, we conducted several in vitro

and in vivo studies. Additionally, we investigated a potential peripheral blood biomarker associated with vascular and WM damage in a cSVD patient cohort study. In this chapter, I will introduce the relevant topics and research questions related to this project.

Cerebral white matter

The human brain is an unique and complex organ that serves as the central command centre of the entire body, coordinating various cognitive processes, including perception, memory, attention, decision-making and many more vital processes for our daily functioning. The brain communicates through neurons to engage these functions. Neurons are the best-known cells of the brain and reside throughout the whole brain, with their cell bodies and dendrites in the so-called "grey matter" and neuronal fibres in the "white matter" [8]. The WM accounts for more than half the total brain volume and is involved in many key functions including cognition, sensory processing, and motor functions [8–11]. The WM does not only consist of myelinated neuronal fibres, but also glial cells such as oligodendrocytes, OPCs, astrocytes, and microglia and vascular cells [12, 13].

Vascular cells form a physical barrier between the central nervous system (CNS) and the periphery consisting of ECs, astrocytes, pericytes, microglia and oligodendrocytes along with the neuronal cells, referred to as "the neurovascular unit (NVU)", and ensure microenvironmental homeostasis in the CNS and WM integrity [14]. Loss of NVU function and WM integrity can lead to cerebrovascular and glial pathology as seen in diseases such as dementia, Alzheimer's disease (AD), multiple sclerosis (MS), vascular cognitive impairment (VCI), stroke and cSVD [14]. Although understudied in cSVD, oligodendroglial cells are the most important cell types safeguarding WM integrity.

Oligodendroglial lineage cells

Oligodendroglial cells were first described in the 19th century and were known as neuroglia cells that were not able to grow axons or proliferate and only served as connective tissue in the brain [15–17]. Eventually, by using more advanced staining techniques, this group of cells was later characterised into microglia and oligodendroglia [18, 19]. Neurons and macroglia share the common precursor arising in the neural tube during embryonic development, namely the neural progenitor cells (NPCs) [20, 21]. Oligodendroglia originate from multiple sources in the CNS, such as the ventral ventricular zone and dorsal spinal cord, and express DM-20 mRNA, encoding a protein proteolipid protein (PLP) isoform, while microglia originate from progenitor cells in the yolk sack, which migrate to the CNS during development, and astrocytes from NPCs in the neuroepithelium [22–29].

Oligodendroglia can be recognised by a set of phenotypic markers they express depending on their developmental stage (a summary of the most important markers is shown in Figure 1) [30]. The NPCs that are committed to differentiate into cells of the oligodendroglia lineage express markers such as the A2B2 [31]. However, A2B2 expressing cells can also differentiate into astrocyte given specific environmental cues, and are thus called oligodendroctyte-type-2 astrocyte (O-2A) progenitor cells [32, 33]. Olig1/2, SOX10, and Nkx2.2 are expressed by all cells of the oligodendroglia lineage, while the combination of PDGFRα and NG2 is restricted to OPCs in the CNS [23]. However, NG2 expressing cells are also able to differentiate into astrocyte, and potentially neurons, and NG2 is also expressed by pericytes and smooth muscle cells [34–37]. At the next stage, pre-oligodendrocytes start growing their extensions to engage with target axons. At this stage, these pre-oligodendrocytes express CNPase, and the O antigens O4 (a lipid sulfatide) and

O1 (galactocerebroside, GalC), with O4 being expressed first by late OPCs and O1 later by pre-myelinating oligodendrocytes [38–40].

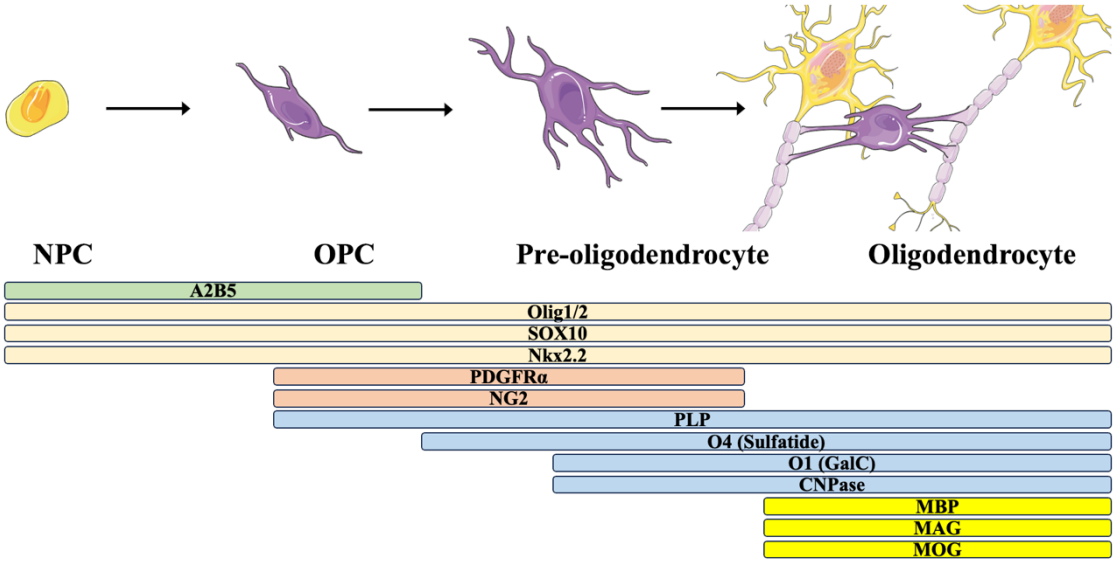


Figure 1: Overview of phenotypic markers for the different stages of development of cells belonging to the oligodendroglial lineage. A2B2 is expressed by neuronal (NPC) and oligodendrocyte progenitor cells (OPC), while Olig1/2, SOX10, and Nkx2.2 is expressed by all cell of the oligodendroglial lineage. Cells committed to the OPCs start to express PDGFRα, NG2, and PLP. Cells start to express the O antigens, O4 (a lipid sulfatide) and O1 (GalC), and CNPase once they start to differentiate into pre-oligodendrocyte. Mature myelinating oligodendrocytes express the specific myelin protein markers MBP, MAG, MOG. Abbreviations: NPC: neuronal progenitor cell; OPC: oligodendrocyte precursor cell; SOX10: SRY-Box transcription Factor 10; Nkx2.2: NK2 Homeobox 2; PDGFRα: platelet-derived growth factor receptor α; NG2: neuron-glial antigen 2; PLP: proteolipid protein; Galc: galactocerebroside; CNPase: 2',3'-cyclic-nucleotide 3'-phosphodiesterase; MBP: myelin basic protein; MAG; myelin associated glycoprotein; MOG: myelin-oligodendrocyte glycoprotein. Adapted from Kuhn et al. (2019) [30].

Differentiated oligodendrocytes are characterised by the production of myelin, and thus express myelin proteins such as myelin basic protein (MBP), myelin associated glycoprotein (MAG), and myelin-oligodendrocyte glycoprotein (MOG) [41–44]. Pre-myelinating oligodendrocytes sense their environment by extending and retracting their processes towards their target axons before connecting and settling into their final position [45, 46]. Once connected, mature oligodendrocytes extend their plasma membrane laterally down the axon to form paranodal loops and wrap the axon with loose layers of plasma membrane that

is subsequently compacted into dense myelin sheaths [47]. This process is tightly regulated by environmental cues, such as the diameter and neuronal activity of the target axon, but also Ca^{2+} activity of the oligodendrocyte [48–52]. This regulation is reciprocal as the neuronal activity can also determine OPC proliferation and differentiation [50, 53]. Myelin sheaths isolate the axons and lead to fast transmission of neuronal action potential by reducing transmembrane capacitance and increasing resistance of the axon and by concentrating high densities of Na^{+} channels at the short unwrapped portions called the nodes of Ranvier [54, 55]. Each oligodendrocyte can form multiple myelin sheaths, each on different axons. In this regard, oligodendrocytes differ from their peripheral nervous system counterpart, the Schwann cells, that form a single myelin sheath around one axon [56]. Additionally, oligodendrocytes provide metabolic support to the axons, such as the production and transfer of lactate to axons through their myelin sheaths when the axons are energy-deprived [57, 58]. Myelin plasticity has been suggested to mediate neural circuit function, as activity dependent myelination can lead to fine tuning action potential conduction, hence complementing synaptic plasticity [59]. Despite these novel myelin functions, the predominant role of the oligodendrocytes is to generate myelin during development and replace damaged myelin (remyelination) during adulthood. To achieve this, OPCs must proliferate and differentiate to generate new oligodendrocyte capable of producing new myelin sheaths [60]. In line with this repair function, OPCs remain one of the most proliferating cells in the adult CNS [61].

The OPCs in the adult brain have functions beyond the generation of new oligodendrocytes. OPCs respond to neuroligands to regulate their fate, form neuron-OPC synapses, modulate neuronal signalling by secreting neuromodulators, modulate immune response, and play a crucial role in the development and maintenance of the BBB [62–72]. The BBB is a

biological barrier between the CNS and is responsible for brain parenchymal homeostasis. The BBB consists of tightly linked ECs that form a physical EC barrier. OPCs and ECs interact in the developing and adult brain to ensure healthy brain functioning [72, 73]. The vascular cells are the first to infiltrate the parenchyma, and the blood vessels they form provide a scaffold that guides OPCs as they migrate through the developing brain. Brain ECs secrete factors to promote the migration and proliferation of these perivascular OPCs, and in turn, the OPCs secrete factors that promote angiogenesis [74]. This "oligovascular niche" persists in the adult brain, where it could play a role in mediating repair by guiding OPCs to demyelinated areas, and in disease since OPCs might induce BBB leakages through secretion of factors in response to injury [71, 75, 76].

The blood-brain barrier

As previously mentioned, the BBB protects the CNS from undesired molecules in the circulating peripheral blood to ensure CNS homeostasis. A schematic overview of the BBB and its cellular elements is provided in Figure 2. Brain ECs present unique characteristics that distinguish them from peripheral vessels, such as the expression of tight junction (TJ) proteins, the absence of fenestrated capillaries, absence of pinocytosis, and the expression of active transporter mechanisms [77]. The TJ proteins seal the paracellular space between adjacent ECs and prevent entrance of molecules from the circulating blood, while oxygen and carbon dioxide can freely cross this barrier [7, 78]. Although small lipophilic molecules can passively cross the BBB, the entrance of essential nutrients and larger molecules such as glucose, amino acids, insulin, and leptin, is facilitated by active transport via transporters and receptor-mediated endocytosis [79–81]. These unique properties tightly regulate the CNS homeostasis by preventing the entrance of nonspecific molecules, while actively taking

up specific nutrients and disposing potential harmful molecules back into the circulating peripheral blood [7]. Even though these mechanisms are crucial for the healthy functioning of the brain, they also present a major obstacle for delivering potential drugs into the CNS. However, there might be a window of opportunity for drug delivery in some conditions such as MS, stroke, and cSVD since these diseases are associated with an increased BBB permeability [82]. The integrity of the BBB is primarily dependent on the proper functioning of the NVU, as previously mentioned, and dysfunction of the brain microvascular ECs is thus considered a significant contributing factor in cSVD pathology [1, 83].

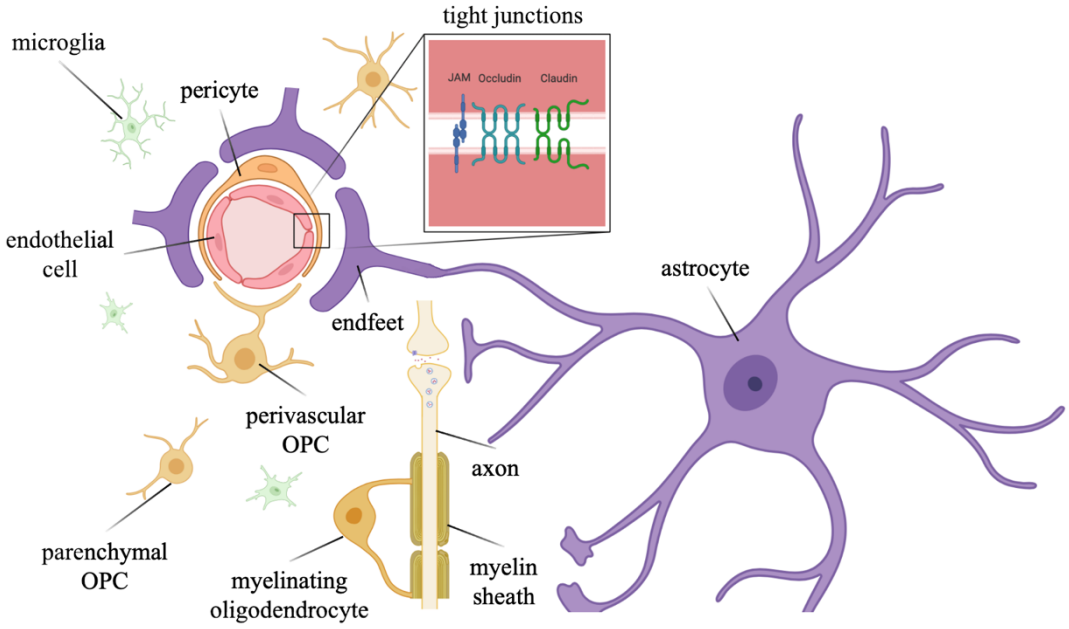


Figure 2: Schematic overview of the blood-brain barrier. The blood-brain barrier (BBB) consists of endothelial cells, astrocytes and their endfeet, microglial cells, and pericytes. The endothelial cells present unique properties distinguishing them from peripheral vessels, such as the absence of fenestrated capillaries, absence of pinocytosis, and the expression of tight junction proteins. Astrocytes have a crucial role in the formation and maintenance of the BBB by secreting factors and ensheath the vessel with their endfeet and provide physical support, regulate blood flow, and contribute to selective permeability. Astrocytes also play a crucial role in modulation of neuronal activity by neurotransmitter uptake and release. Microglia are the immune cells of the brain and their interaction with surrounding cells ensures the proper regulation of the BBB integrity. Pericytes wrap and provide structural support to the endothelial cells and modulate blood flow. Additionally, oligodendrocytes which are responsible for the myelination of axons and oligodendrocyte precursor cells (OPC) secret factors that modulate the BBB integrity.

Brain endothelial cells

Brain ECs line the blood vessel lumen and form the major structural barrier in the BBB. The development and maintenance of these cells and their unique properties depends on the interaction with the other cells of the NVU [84]. In mice, pericytes are the first cells in the developing vasculature to initiate BBB properties at embryonic day 12 (E12), followed by OPCs at E19 and astrocytes directly after birth [84, 85]. The astrocytes are recruited to ensure the postnatal maintenance of the BBB [86]. These interactions persist in the adult brain to ensure proper BBB function and angiogenesis [75, 78, 84, 87]. A major functional and unique property of ECs in the BBB is the expression of TJ proteins [78].

The formation of TJs occurs in the primary stages of brain development and is a critical component of the BBB, indicating its importance in brain health [78]. The TJs consist of three integral membrane proteins, namely claudins, occludins, and junction adhesion molecules (JAMS) in addition to cytoplasmic proteins such as Zonula occludens (ZO), cingulin, and others, which function as links between the membrane proteins and the cytoskeleton of the ECs [7]. The major components forming the BBB in the brain are the TJ proteins claudin-1 and claudin-5 [88, 89]. Loss of function of these proteins increases BBB permeability leading to brain pathology such as ischaemic stroke [89–92]. Occludins seem to have a more regulatory rather than a critical role compared to claudins [93]. They are also only expressed in the adult human brain and not in human foetal or new-born brain [94]. Claudins and occludins can also form heteropolymeric intramembranous strands to allow the selective diffusion of ions and hydrophilic molecules [95]. The role of JAMs in the BBB is less known with studies showing their potential role in EC adhesion and monocyte transmigration into the brain [96, 97].

Blood-brain barrier pathology

BBB dysfunction has been reported in several CNS diseases including AD, MS, stroke, and cSVD [98–101]. Its dysfunction can range from mild changes in permeability and mild leakages, to chronic barrier impairment and ischemic stroke [7, 102, 103]. This can also cause myelin damage and ischemic lesions in the WM [104, 105]. In healthy conditions, the BBB is almost impermeable, however, when dysfunction occurs and BBB is impaired, undesired plasma molecules can infiltrate the CNS and trigger an immune response resulting in disturbance of CNS homeostasis and damage to the brain [7].

Studies have shown that increased BBB permeability and endothelial TJ disruption can be caused by hypoxia in the brain. Focal and global ischemia can lead to an inflammatory response by increasing proinflammatory cytokines and mediators, such as Interleukin 1β (IL-1β), IL-8, tumour necrosis factor α (TNF-α), vascular endothelial growth factor (VEGF), and recruitment of immune cells to hypoxic areas, causing TJ disruption and BBB impairment [7, 106, 107]. In vitro studies revealed that EC barrier disruption might be mediated by factors released by astrocytes in response to ischemia [108, 109]. Disruption of the BBB can also be caused by dysfunctional OPC-EC interaction, leading to WM damage [69, 76]. Clustering of perivascular OPCs due to hypoxia can lead to aberrant Wnt signalling in OPCs, disrupting the astrocyte end-feet and endothelial TJ proteins, resulting in increased vascular permeability, ultimately leading to CNS inflammation and WM damage [69, 70, 76].

The Wnt signalling pathway has a crucial role in both the brain vasculature and OPCs. Wnt signalling molecules bind to their receptor and activate the β-catenin dependent or β-catenin independent intracellular signalling pathways. Activation of the β-catenin dependent pathway leads to the intracellular β-catenin accumulation and nuclear translocation, resulting

in gene modulation [110]. Activation of Wnt signalling in ECs can regulate angiogenesis and barrier function, while in OPCs it is involved in the proliferation, migration, and differentiation [110, 111]. Recent studies have demonstrated the regulation of Wnt pathways in OPCs in response to brain hypoxia, which affected the ECs in a paracrine and the OPCs in an autocrine manner [70, 76, 112]. Thus, making this signalling pathways a promising target for modulating the pathological response to hypoxia leading to BBB impairments and WM damage.

In summary, it is suggested that hypoxia can trigger a cascade of events that lead to endothelial changes, resulting in increased BBB permeability and ultimately disruption of CNS homeostasis and WM damage. Although it is unknown whether BBB impairments are the cause or consequence of diseases such as the sporadic form of cSVD, it is clear that this pathological phenomenon contributes to and exacerbate the development of disease [113–115].

Cerebral small vessel disease

The term cSVD refers to pathology to the small vessels in the brain, including the arteries, arterioles, venules, and capillaries. cSVD is characterised by lacunar infarcts, WM hyperintensities, cerebral microbleeds, enlarged perivascular spaces, and brain atrophy identified using MRI. Diagnosis of cSVD might be accidental, as it is often asymptomatic in its early stages [5]. Clinical manifestations in the acute stage include lacunar stroke or intracerebral haemorrhage, while more chronic stages can affect cognition, motor function (usually gait), balance, and behaviour such as personality changes and depression [116]. The cause of cSVD is difficult to determine as it does not have a single underlying pathologic cause but rather heterogenic pathological processes. The pathology of cSVD differs

depending on the type and subtype of the disease, and there are several suggestions on how to classify the different types of cSVD (Table 1) [83, 117–119]. However, the Standards for Reporting Vascular Changes on Neuroimaging 2 (STRIVE-2) recently revised the recommended standardised neuroimaging features for cSVD to promote consistent and unbiased use of terminology in research and clinical practice [120].

The sporadic form is often characterised by lacunar strokes and parenchymal brain haemorrhages. To have a better understanding of the underlying pathology, this sporadic form can be categorised further into two groups: the amyloidal form and non-amyloidal form for cSVD. The amyloidal form is mostly related to age and is caused by the deposition of amyloid-β in the small arteries, leading to small vessel dysfunction and ultimately vascular occlusions and ruptures [6]. A common form of this subtype is the sporadic cerebral amyloid angiopathy (CAA), which is commonly characterised by spontaneous intracerebral haemorrhages [121]. The non-amyloidal form of cSVD is more difficult to define, as this form is often referred to as the "hypertensive arteriopathy". It is often characterised by atherosclerosis, arteriosclerosis, lipohyalinosis, fibrinoid necrosis or microaneurysms affecting the small vessels, and is related to vascular risk factors such as hypertension, diabetes, or age [116]. Chronic hypertension can lead to lipohyalinosis and thickening and narrowing of the small vessels in the brain that are responsible for the perfusion and nourishment of the deep WM [122]. Hypertension can also cause blood vessel fibrosis and stiffening of the vessel walls, leading to reduction in cerebral blood flow (CBF) [123]. In both instances, hypertension can cause reduced CBF, hypoperfusion, and hypoxia in the brain. Additionally, blood supply to deeper regions of the WM happens in a watershed-like manner, which makes these regions even more vulnerable to CBF impairments [124]. Animal models and human patient data suggest that chronic hypoperfusion in the brain can

lead to major WMLs and cognitive impairments and development of cSVD [125–128]. Hypertension can also lead to impairments in the BBB, which in turn can cause progressive cognitive decline and WM damage in patients with cSVD [129, 130]. The initial mechanism of early development of WM damage and cSVD is largely unknown and future studies are needed to get a better understanding of the underlying pathology in disease progression. Investigations in genetic forms of cSVD might shed light into some of the molecular mechanisms.

Table 1: Classification of cerebral small vessel disease based on sporadic and genetic onset and pathological differences. Adapted from Mustapha et al. (2019) [117].

Sporadic	**Pathology**
Sporadic Cerebral Amyloid Angiopathy	Amyloid-β protein deposition in the wall of small vessels; spontaneous intracerebral haemorrhages.
Hypertensive arteriopathy	Atherosclerosis, arteriosclerosis, lipohyalinosis, fibrinoid necrosis, microaneurysms; lacunar infarcts and white matter damage.
Other	Associated with conditions such as diabetes, smoking, high cholesterol, autoimmune disease, and coagulation disorders.
Genetic	
Genetic Cerebral Amyloid Angiopathy	Amyloid-β associated gene such as *amyloid precursor protein (APP), presenilin 1 (PSEN1),* and *presenilin 2 (PSEN2)* mutations, often autosomal dominant inherited;
Cerebral autosomal-dominant arteriopathy with stroke and ischemic leukoencephalopathy (CADASIL)	*NOTCH3* mutation, autosomal dominant inherited; recurrent strokes, cognitive decline, migraine with aura, and white matter damage.
cerebral autosomal recessive arteriopathy with subcortical infarcts and ischemic leukoencephalopathy (CARASIL)	*HTRA1* mutation; autosomal recessive inherited; early-onset strokes, cognitive impairment, alopecia, and spondylosis.
COL4A1/2 related	*COL4A1/2* mutation; wide range of symptoms, such as strokes, white matter damage, retinal vascular abnormalities, etc.

These genetic forms are often associated with ischemic or haemorrhagic strokes in young people and lead to non-age related cSVD [83]. A less common form of cSVD is the genetic CAA, which can be caused by mutations in genes, such as amyloid precursor protein *(APP)*, presenilin 1 *(PSEN1)*, and presenilin 2 *(PSEN2)* mutations, affecting the production and processing of amyloid-β. The most common form of genetic cSVD is the cerebral autosomal-dominant arteriopathy with stroke and ischemic leukoencephalopathy (CADASIL) which is caused by mutations in the *NOTCH3* gene [83]. NOTCH3 is a transmembrane receptor mainly expressed in vascular smooth muscle cells and vascular pericytes, and accumulation and deposition of the extracellular domain of this receptor within the vessel walls is a key pathological feature in CARDAIL [131]. Similarly, cerebral autosomal recessive arteriopathy with subcortical infarcts and ischemic leukoencephalopathy (CARASIL) is associated with mutation in the high-temperature requirement A serine peptidase 1 (HTRA1) gene, which up-regulates the expression of the transforming growth factor β (TGF-β) and leads to vascular disorders [83, 132]. Understanding these and other genetic forms of cSVD contributes to determining underlying mechanisms leading to disease progression. Genetic studies and animal models can help to unravel biological, cellular, and molecular changes in cSVD development, which can ultimately lead to better diagnostic tools and treatments for patients.

Clinical manifestation

The development of cSVD might progress unnoticed as it remains often asymptomatic in its early stages [133, 134]. Diagnosis can occur accidently when patients undergo MRI of computed tomography (CT) for unrelated conditions [135]. However, in its acute stage, lacunar strokes or intracerebral haemorrhages may also occur. Lacunar strokes manifest as

pure motor strokes leading to motor paresis or paralysis, sensory strokes, sensorimotor stroke, ataxic hemiparesis, or dysarthria-clumsy hand syndrome [136, 137], while the manifestation of intracerebral haemorrhages symptoms depend on the locations of the damage [135]. In chronic cSVD, the main clinical symptoms are associated with progressive cognitive decline due to WM impairment. Posture and gait abnormalities, tremor, pseudobulbar syndrome, and depression are some of signs of chronic cSVD [134, 138, 139]. The presence of these WMLs is associated with risk of cognitive impairments, dementia, stoke, and even death [14, 138, 140, 141]. In the elderly population, cSVD is believed to be the most common cause of cognitive dysfunction and dementia, which can ultimately lead to loss of independence, social withdrawal, and physical injuries [139, 142]. Therefore, it is crucial to underscore the significance of cSVD research and the urgent need for development of novel effective treatment options to enhance quality of life for these patients.

Treatment

Because of the chronic nature of cSVD, prevention strategies should focus on lowering vascular risk factors, with hypertension being the most important one, to protect the brain microvasculature and prevent CNS inflammation and WM damage. The only therapeutic strategy for the sporadic form of cSVD is to limit the exposure to cSVD risk factors by lowering blood pressure and reducing vascular tone with the use of antihypertensive, antiplatelet, cholesterol-lowering, anticoagulant drugs, and glucose-lowering drugs for diabetes [143, 144]. These acute treatments might resemble those for stroke prevention due to pathological similarities of the diseases [83, 143]. Lifestyle interventions, such as diet, sodium restriction, increased physical activity, and smoking cessation might also be implemented to decrease vascular risk factors to prevent vascular events [83]. Some of these

therapeutic strategies could also be implemented for the treatment of genetic forms of cSVD. However, additional genetic screening of young adults with a history of familial cSVD could ensure the most effective prevention strategy [83].

In recent years, there have been promising advancements in the potential treatment of cSVD with cilostazol, a phosphodiesterase inhibitor, and isosorbide mononitrate, a nitric oxide (NO) donor. These medications, already approved for use in other diseases, show potential in cSVD treatment by improving long-term outcomes after lacunar ischaemic stroke [145, 146]. Cilostazol improved cognitive function and protected WM by reducing gliovascular damage, cellular inflammation, and endothelial activation in an in vivo hypoperfusion model, while clinical trials are still ongoing [145–147]. Protecting WM by targeting brain inflammation could be a potential therapeutic strategy in cSVD, as several in vivo studies have demonstrated that the use of anti-inflammatory drugs can lead to reduced WM damage, improved cognitive function, and enhanced overall life expectancy [148, 149]. Hence, investigating the potential of oligodendrocyte and OPC modulation might be a compelling treatment strategy, as studies using stem cells that can differentiate into OPCs and oligodendrocytes have found a positive effect on remyelination and repair of damaged WM [150–153]. These findings suggest that targeting the enhancement of oligodendrocyte function and the regenerative capacity of OPCs may hold great promise for restoring WM integrity in cSVD. Specific targeting of the Wnt7a signalling pathway might hold potential in modulating the OPC-EC crosstalk and normalising BBB function and protecting WM integrity in cerebral hypoperfusion [70, 112, 154, 155].

Thus, it is important to have a better understanding of the disease pathology leading to small vessel damage and associated WMLs to come up with novel treatment options. Having

appropriate models to investigate the biological and molecular mechanisms leading to cSVD pathology is thus crucial for future research.

Experimental models for the study of cerebral small vessel disease

As mentioned before, cSVD can be caused by several pathological mechanisms and no perfect model exists that reflects all aspects of the disease. It is thus important to select and combine the most appropriate models when investigating cSVD. By using different animal models, researchers attempt to mimic parts of cSVD pathophysiology. Some models are also more appropriate for the study of specific pathological features and can thus improve our mechanistic understanding for some cSVD features such as WMLs, arteriopathy, and lacunar infarcts. Here we discuss some of the in vivo models commonly used to investigate cSVD.

Genetic experimental models

One approach is to mimic the human genetic mutations in animal models to investigate associated pathology. Transgenic mice expressing a mutant Notch3 gene in vascular smooth muscle cells show impaired autoregulation and increased resistance of cerebral vessels, indicating vascular dysfunction as an early pathological mechanism in CADASIL [156]. Similarly, transgenic mice for COL4A1, encoding for the basement membrane protein procollagen type IV alpha1, present weak vessels and intracerebral haemorrhages, and can be used to mimic this genetic form seen in cSVD patients [157]. Recently, a genetic model for CARASIL has been proposed, which uses CRISPR/Cas9 to generate the Mut-HTRA1L364P mouse model to study this rare human form [158]. Transgenic mice can also

be used to investigate certain cSVD characteristics without mimicking the human mutations, by for example overexpressing renin and human angiotensinogen which results in hypertension induced vascular remodelling and hypertrophy [159].

Hypertensive animal models

Selective breeding of Wistar-Kyoto rats with high blood pressure rats led to the development of the stroke prone spontaneously hypertensive rats (SHR-SP). These animals develop progressive hypertension from week 8-9 with stroke events occurring at around 10 months of age. Pathology in these animals includes chronic vascular changes and stroke lesions in various regions of the brain [160]. The inducible hypertensive rats (IHR) carry the mouse renin transgene with an inducible promotor. The overexpression of renin, and thus activation of the renin-angiotensin system, can be controlled by administration of indole-3 carbinol in these animals [161]. Although the IHR did not show any histological changes in the brain, stroke lesions could be generated by adding 0.9% saline to their drinking water [162].

Another group proposed to use of deoxy-corticosteroid acetate (DOCA) treated Sabra hypertension prone (SBH/y) rats as a novel model for cSVD. In these animals, the vascular impact of hypertension leading to WMLs and oxidative stress in these animals [163]. In mice, hypertension can be induced by administration of angiotensin II. In this model, animals are continuously injected with angiotensin II for 3 months, and show brain inflammation, BBB impairments, myelin damage, and cognitive impairments [164]. The inbred BHP/2J mice can be used to effects of chronic hypertension as they present spontaneous lifelong hypertension. These animals manifest key features such as microglia activation, BBB impairments, and cognitive decline [165]. Although the list of hypertensive animal models

is more extensive, brain hypoperfusion can also be studied in the absence of peripheral hypertension with the use of other animal models.

Hypoperfusion model

A common model that might reflect the etiology of hypoperfusion seen in cSVD is the bilateral carotid artery stenosis (BCAS) model in mice [166]. Mice undergo narrowing of both carotid arteries via the surgical placement of 0.16-0.20 mm microcoils around these arteries, resulting in a chronic reduction of the CBF, microglial and astroglial activation, cerebral hypoxia, increase in BBB permeability in the WM, and WMLs [125, 167, 168]. The severity and onset of hypoperfusion can be controlled by the diameter and timing of the placed microcoils, and genetic variations can be investigated by using specific strains of mice. The survival rate 1 week after placement of a 0.18 mm microcoils is >85% with CBF reductions of ±30% after 2h after surgery [169]. The strong acute reduction in CBF followed by a gradual recovery is however a limitation of the model, as it does not fully mimic the pathophysiology of chronic gradual increased hypoperfusion seen in cSVD patients [127]. Cerebral WMLs are seen in these animals after 2 weeks of hypoperfusion, while BBB impairments and increases in microglia and astrocytes occur after 1 week [125, 167, 169]. Behaviour and grey matter changes occurs months after, with impairments in motor function being present after 3 months, working memory after 5-6 months, and cortical and hippocampal atrophy at 8 months after BCAS [169, 170]. Additionally, pharmacotherapies can be investigated in BCAS mice, as the procedure is straightforward and inexpensive.

Other hypoperfusion models can be used, such as the rat bilateral common carotid artery occlusion (BCCAO), the mouse asymmetric common carotid artery (ACAS), and non-human primate models, to mimic hypoperfusion. However, these have some additional

limitations such as optic nerve damage in the BCCAO, absence of microvascular pathology in ACAS, and accessibility of non-human primates [169].

Other models

Other animal models for studying cSVD pathological mechanisms could include ischaemic, embolic, and haemorrhagic stroke models, and diabetic and other co-morbidity models. A common model for ischaemic stroke is the middle cerebral artery occlusion (MCAO). In this mouse or rat model, animals present focal ischemic lesions in the cortical, caudate, and subcortical WM and early oligodendrocyte pathology due to an occlusion of the middle cerebral artery [171]. For embolic models, cerebral infarcts in rodents, rabbits and primates were produced by intra-vascular injections of cholesterol crystals, agarose or plastic microspheres, or autologous blood clot emboli [172–175]. Another method of achieving embolic insults in the brain is by the photo-activation of intravascular Rose Bengal dye, which leads to endothelial dysfunction downstream of the photo-activated infarct [176, 177]. A more recent model was proposed which used the combination of ultrasound with microbubble injections to disrupt the BBB through cavitation. This method led to reduced blood flow speed and volume due to vasoconstriction in rats [178]. The direct impact of microhaemorrhages in the small vessels can be studied by inducing these lesions with femtosecond laser irradiation to rupture single arterioles in the rodent brain [179]. This model can be used to investigate the impact of local silent strokes seen in cSVD patients [138]. Experimental animal models of co-morbidies such as diabetes, obesity, and AD, can be used to investigate the interactions of these conditions with cSVD.

This proliferation of models focussing on various aspects of cSVD serves to emphasise the lack of a single model that fully mimics the complex etiopathology of cSVD.

Hypothesis and aims of this thesis

In summary, a vast body of evidence suggests that cSVD is not a simple disease initiated by a single mechanism, but rather a complex interplay between different triggers (peripheral risk factors) and cell types in the brain, that silently and progressively lead to BBB and WML and cognitive decline. Although several risk factors and genetic predispositions are known, the underlying pathology leading to the disease progression remains largely unknown.

Thus, **we hypothesised that cerebral chronic hypoperfusion leads to local hypoxia in the brain, affecting the OPCs, which results in abnormal OPC signalling and an impaired interaction with brain ECs, ultimately causing disruption of the BBB, and unresolved WM damage due to compromised remyelination.**

The overall aims of this thesis are to: **(1)** Identify molecular signalling mediators of the OPC-EC crosstalk that might underlie early mechanisms leading to the development of cSVD (*Chapter 2*). **(2)** Investigate the effects of Wnt7a stimulation on TJ protein and barrier function of brain ECs (*Chapter 3*). **(3)** Understand the involvement of Wnt7a in OPC migration via the vasculature (*Chapter 4*). **(4)** Investigate hypoxic OPC-derived signalling molecules, and their role in the OPC-EC interaction in hypoperfused BCAS mice and **(5)** conduct a retrospective study to support our in vitro and in vivo findings in human blood plasma samples derived from cSVD patients (*Chapter 5*).

Outline of this thesis

Firstly, **Chapter 2** presents a summary of the literature on the signalling for the OPC-EC interaction. Due to findings suggesting the involvement of Wnt signalling in this glial-vascular interplay [70], a summary of this signalling pathway is also provided in chapter 2.

Subsequently, findings via a systematic search on the current knowledge of the role of Wnt7a signalling in OPC-EC interaction and its implications in cSVD pathology are presented.

In **Chapter 3**, the role of Wnt7a stimulation in brain ECs was examined. Literature suggests that hypoxia in the brain can lead to the secretion of Wnt7a by OPCs, which might lead to EC dysfunction. Thus, we hypothesised that Wnt7a stimulation in ECs will lead to the β-catenin mediated signalling, regulating TJ protein expression and barrier function. To test this hypothesis, we used an in vitro mouse EC model to investigate the impact on the endothelial barrier features. Additionally, the transcriptomic signature of Wnt7a stimulation was performed to identify potential modulators of this signalling pathway.

In **Chapter 4**, we further investigated the transcriptomic regulation on brain ECs upon Wnt7a stimulation to identify possible links for OPC recruitment and migration. Thus, we hypothesised that Wnt7a leads to a cellular response in ECs to promoting OPC migration. Chapter 4 presents the transcriptomic data of Wn7a stimulation in brain ECs related to cell migration. Additionally, Wnt7a stimulation in OPCs was also examined to mimic possible autocrine effects. The findings are presented in a short research letter that aims to stimulate future research on Wnt7a mediated Cxcl12-Cxcr4 signalling in context of cSVD.

In **Chapter 5**, we investigated the in vivo OPC-EC interaction in the hypoperfusion BCAS mouse model. We hypothesised that cerebral hypoperfusion causes abnormal OPC-EC interaction, leading to BBB dysfunction and development of WM damage. Myelin content, vascular density, BBB leakages, and hypoxic cell density in the brain was assessed after 1 week of BCAS. Additionally, RNA sequencing was performed on hypoxic OPCs, revealing *Vegfa* regulation. Lastly, we measured VEGFA levels in blood plasma of cSVD patients and correlated them with BBB permeability in normal-appearing WM and WMLs.

Finally, in **Chapter 6**, the implications of our findings are discussed in context of the current knowledge on BBB dysfunction, WMLs, and cSVD pathology. Study gaps and limitations are provided in addition to potential diagnostic and therapeutical implications for the prevention and treatment of cSVD.

Reference

1. Pantoni L (2010) Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. Lancet Neurol 9:689–701. https://doi.org/10.1016/S1474-4422(10)70104-6
2. de Leeuw FE, de Groot JC, Achten E, et al (2001) Prevalence of cerebral white matter lesions in elderly people: a population based magnetic resonance imaging study. The Rotterdam Scan Study. J Neurol Neurosurg Psychiatry 70:9–14. https://doi.org/10.1136/jnnp.70.1.9
3. de Laat KF, van Norden AGW, Gons RAR, et al (2010) Gait in Elderly With Cerebral Small Vessel Disease. Stroke 41:1652–1658. https://doi.org/10.1161/STROKEAHA.110.583229
4. Debette S, Markus HS (2010) The clinical importance of white matter hyperintensities on brain magnetic resonance imaging: systematic review and meta-analysis. BMJ 341:c3666. https://doi.org/10.1136/bmj.c3666
5. Wardlaw JM, Smith EE, Biessels GJ, et al (2013) Neuroimaging standards for research into small vessel disease and its contribution to ageing and neurodegeneration. Lancet Neurol 12:822–838. https://doi.org/10.1016/S1474-4422(13)70124-8
6. Cuadrado-Godia E, Dwivedi P, Sharma S, et al (2018) Cerebral Small Vessel Disease: A Review Focusing on Pathophysiology, Biomarkers, and Machine Learning Strategies. J Stroke 20:302–320. https://doi.org/10.5853/jos.2017.02922
7. Kadry H, Noorani B, Cucullo L (2020) A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity. Fluids and Barriers of the CNS 17:69. https://doi.org/10.1186/s12987-020-00230-3
8. Buyanova IS, Arsalidou M (2021) Cerebral White Matter Myelination and Relations to Age, Gender, and Cognition: A Selective Review. Frontiers in Human Neuroscience 15:
9. Fields RD (2010) Change in the Brain's White Matter. Science 330:768–769. https://doi.org/10.1126/science.1199139
10. Desmond DW (2002) Cognition and White Matter Lesions. Cerebrovascular Diseases 13:53–57. https://doi.org/10.1159/000049151
11. Filley CM (2005) White matter and behavioral neurology. Ann N Y Acad Sci 1064:162–183. https://doi.org/10.1196/annals.1340.028
12. Walhovd KB, Johansen-Berg H, Káradóttir RT (2014) Unraveling the secrets of white matter – Bridging the gap between cellular, animal and human imaging studies. Neuroscience 276:2–13. https://doi.org/10.1016/j.neuroscience.2014.06.058
13. Ohtomo R, Iwata A, Arai K (2018) Molecular Mechanisms of Oligodendrocyte Regeneration in White Matter-Related Diseases. Int J Mol Sci 19:1743. https://doi.org/10.3390/ijms19061743
14. Levit A, Hachinski V, Whitehead SN (2020) Neurovascular unit dysregulation, white matter disease, and executive dysfunction: the shared triad of vascular cognitive impairment and Alzheimer disease. GeroScience 42:445–465. https://doi.org/10.1007/s11357-020-00164-6
15. Virchow R (Rudolf LK, University of Glasgow. Library, University of Glasgow. Library (1856) Gesammelte Abhandlungen zur Wissenschaftlichen Medicin. Frankfurt A. M. : Meidinger Sohn & Comp.
16. Deiters O, Schultze MJS 1825-1874 (1865) Untersuchungen über Gehirn und Rückenmark des Menschen und der Säugethiere. Braunschweig : Vieweg
17. Golgi C, Royal College of Physicians of Edinburgh (1886) Sulla fina anatomia degli organi centrali del sistema nervoso. Milano : U. Hoepli
18. Río Hortega P del (1920) Estudios sobre la neuroglía. La microglía y su transformación en células en bastoncito y cuerpos granuloadiposos. rab Lab Invest Biol Univ Madrid 18:37-82.

19. Río Hortega P del (1933) Arte y artificio de la ciencia histológica.
20. Davis AA, Temple S (1994) A self-renewing multipotential stem cell in embryonic rat cerebral cortex. Nature 372:263–266. https://doi.org/10.1038/372263a0
21. Rogister B, Ben-Hur T, Dubois-Dalcq M (1999) From neural stem cells to myelinating oligodendrocytes. Mol Cell Neurosci 14:287–300. https://doi.org/10.1006/mcne.1999.0790
22. Raff MC, Miller RH, Noble M (1983) A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. Nature 303:390–396. https://doi.org/10.1038/303390a0
23. Pringle NP, Richardson WD (1993) A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. Development 117:525–533. https://doi.org/10.1242/dev.117.2.525
24. Timsit S, Martinez S, Allinquant B, et al (1995) Oligodendrocytes originate in a restricted zone of the embryonic ventral neural tube defined by DM-20 mRNA expression. J Neurosci 15:1012–1024. https://doi.org/10.1523/JNEUROSCI.15-02-01012.1995
25. Warf BC, Fok-Seang J, Miller RH (1991) Evidence for the ventral origin of oligodendrocyte precursors in the rat spinal cord. J Neurosci 11:2477–2488. https://doi.org/10.1523/JNEUROSCI.11-08-02477.1991
26. Vallstedt A, Klos JM, Ericson J (2005) Multiple dorsoventral origins of oligodendrocyte generation in the spinal cord and hindbrain. Neuron 45:55–67. https://doi.org/10.1016/j.neuron.2004.12.026
27. Fogarty M, Richardson WD, Kessaris N (2005) A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. Development 132:1951–1959. https://doi.org/10.1242/dev.01777
28. Ginhoux F, Prinz M (2015) Origin of microglia: current concepts and past controversies. Cold Spring Harb Perspect Biol 7:a020537. https://doi.org/10.1101/cshperspect.a020537
29. Kessaris N, Pringle N, Richardson WD (2008) Specification of CNS glia from neural stem cells in the embryonic neuroepithelium. Philos Trans R Soc Lond B Biol Sci 363:71–85. https://doi.org/10.1098/rstb.2006.2013
30. Kuhn S, Gritti L, Crooks D, Dombrowski Y (2019) Oligodendrocytes in Development, Myelin Generation and Beyond. Cells 8:1424. https://doi.org/10.3390/cells8111424
31. Fok-Seang J, Miller RH (1994) Distribution and differentiation of A2B5+ glial precursors in the developing rat spinal cord. J Neurosci Res 37:219–235. https://doi.org/10.1002/jnr.490370208
32. Raff MC, Abney ER, Miller RH (1984) Two glial cell lineages diverge prenatally in rat optic nerve. Dev Biol 106:53–60. https://doi.org/10.1016/0012-1606(84)90060-5
33. Hughes SM, Lillien LE, Raff MC, et al (1988) Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. Nature 335:70–73. https://doi.org/10.1038/335070a0
34. Otsu M, Ahmed Z, Fulton D (2021) Generation of Multipotential NG2 Progenitors From Mouse Embryonic Stem Cell-Derived Neural Stem Cells. Frontiers in Cell and Developmental Biology 9:
35. Zhu X, Bergles DE, Nishiyama A (2008) NG2 cells generate both oligodendrocytes and gray matter astrocytes. Development 135:145–157. https://doi.org/10.1242/dev.004895
36. Stallcup WB (2018) The NG2 Proteoglycan in Pericyte Biology. Adv Exp Med Biol 1109:5–19. https://doi.org/10.1007/978-3-030-02601-1_2
37. Guo Q, Scheller A, Huang W (2021) Progenies of NG2 glia: what do we learn from transgenic mouse models ? Neural Regeneration Research 16:43. https://doi.org/10.4103/1673-5374.286950

38. Braun P, Sandillon F, Edwards A, et al (1988) Immunocytochemical localization by electron microscopy of 2’3’-cyclic nucleotide 3’-phosphodiesterase in developing oligodendrocytes of normal and mutant brain. J Neurosci 8:3057–3066. https://doi.org/10.1523/JNEUROSCI.08-08-03057.1988
39. Sommer I, Schachner M (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Dev Biol 83:311–327. https://doi.org/10.1016/0012-1606(81)90477-2
40. Jakovcevski I, Filipovic R, Mo Z, et al (2009) Oligodendrocyte Development and the Onset of Myelination in the Human Fetal Brain. Front Neuroanat 3:5. https://doi.org/10.3389/neuro.05.005.2009
41. Barbarese E, Barry C, Chou CH, et al (1988) Expression and localization of myelin basic protein in oligodendrocytes and transfected fibroblasts. J Neurochem 51:1737–1745. https://doi.org/10.1111/j.1471-4159.1988.tb01153.x
42. Trapp BD (1990) Myelin-Associated Glycoprotein Location and Potential Functionsa. Annals of the New York Academy of Sciences 605:29–43. https://doi.org/10.1111/j.1749-6632.1990.tb42378.x
43. Brunner C, Lassmann H, Waehneldt TV, et al (1989) Differential ultrastructural localization of myelin basic protein, myelin/oligodendroglial glycoprotein, and 2’,3’-cyclic nucleotide 3’-phosphodiesterase in the CNS of adult rats. J Neurochem 52:296–304. https://doi.org/10.1111/j.1471-4159.1989.tb10930.x
44. Raff MC, Mirsky R, Fields KL, et al (1978) Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. Nature 274:813–816. https://doi.org/10.1038/274813a0
45. Kirby BB, Takada N, Latimer AJ, et al (2006) In vivo time-lapse imaging shows dynamic oligodendrocyte progenitor behavior during zebrafish development. Nat Neurosci 9:1506–1511. https://doi.org/10.1038/nn1803
46. Simons M, Trotter J (2007) Wrapping it up: the cell biology of myelination. Curr Opin Neurobiol 17:533–540. https://doi.org/10.1016/j.conb.2007.08.003
47. Snaidero N, Möbius W, Czopka T, et al (2014) Myelin membrane wrapping of CNS axons by PI(3,4,5)P3-dependent polarized growth at the inner tongue. Cell 156:277–290. https://doi.org/10.1016/j.cell.2013.11.044
48. Friede RL (1972) Control of myelin formation by axon caliber. (With a model of the control mechanism). Journal of Comparative Neurology 144:233–252. https://doi.org/10.1002/cne.901440207
49. Baraban M, Koudelka S, Lyons DA (2018) Ca 2+ activity signatures of myelin sheath formation and growth in vivo. Nat Neurosci 21:19–23. https://doi.org/10.1038/s41593-017-0040-x
50. Gibson EM, Purger D, Mount CW, et al (2014) Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. Science 344:1252304. https://doi.org/10.1126/science.1252304
51. Fannon J, Tarmier W, Fulton D (2015) Neuronal activity and AMPA-type glutamate receptor activation regulates the morphological development of oligodendrocyte precursor cells. Glia 63:1021–1035. https://doi.org/10.1002/glia.22799
52. Toth E, Rassul SM, Berry M, Fulton D (2021) A morphological analysis of activity-dependent myelination and myelin injury in transitional oligodendrocytes. Sci Rep 11:9588. https://doi.org/10.1038/s41598-021-88887-0
53. Barres BA, Raff MC (1993) Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. Nature 361:258–260. https://doi.org/10.1038/361258a0
54. Waxman SG, Ritchie JM (1993) Molecular dissection of the myelinated axon. Ann Neurol 33:121–136. https://doi.org/10.1002/ana.410330202

55. Seidl AH (2014) Regulation of conduction time along axons. Neuroscience 276:126–134. https://doi.org/10.1016/j.neuroscience.2013.06.047
56. Salzer JL, Zalc B (2016) Myelination. Current Biology 26:R971–R975. https://doi.org/10.1016/j.cub.2016.07.074
57. Fünfschilling U, Supplie LM, Mahad D, et al (2012) Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. Nature 485:517–521. https://doi.org/10.1038/nature11007
58. Lee Y, Morrison BM, Li Y, et al (2012) Oligodendroglia metabolically support axons and contribute to neurodegeneration. Nature 487:443–448. https://doi.org/10.1038/nature11314
59. Xin W, Chan JR (2020) Myelin plasticity: sculpting circuits in learning and memory. Nat Rev Neurosci 21:682–694. https://doi.org/10.1038/s41583-020-00379-8
60. Tepavčević V, Lubetzki C (2022) Oligodendrocyte progenitor cell recruitment and remyelination in multiple sclerosis: the more, the merrier? Brain 145:4178–4192. https://doi.org/10.1093/brain/awac307
61. Dawson MRL, Polito A, Levine JM, Reynolds R (2003) NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Mol Cell Neurosci 24:476–488. https://doi.org/10.1016/s1044-7431(03)00210-0
62. Káradóttir R, Attwell D (2007) Neurotransmitter receptors in the life and death of oligodendrocytes. Neuroscience 145:1426–1438. https://doi.org/10.1016/j.neuroscience.2006.08.070
63. Bergles DE, Roberts JDB, Somogyi P, Jahr CE (2000) Glutamatergic synapses on oligodendrocyte precursor cells in the hippocampus. Nature 405:187–191. https://doi.org/10.1038/35012083
64. Marisca R, Hoche T, Agirre E, et al (2020) Functionally distinct subgroups of oligodendrocyte precursor cells integrate neural activity and execute myelin formation. Nat Neurosci 23:363–374. https://doi.org/10.1038/s41593-019-0581-2
65. Lin S, Bergles DE (2004) Synaptic signaling between GABAergic interneurons and oligodendrocyte precursor cells in the hippocampus. Nat Neurosci 7:24–32. https://doi.org/10.1038/nn1162
66. Sakry D, Yigit H, Dimou L, Trotter J (2015) Oligodendrocyte precursor cells synthesize neuromodulatory factors. PLoS One 10:e0127222. https://doi.org/10.1371/journal.pone.0127222
67. Antel JP, Lin YH, Cui Q-L, et al (2019) Immunology of oligodendrocyte precursor cells in vivo and in vitro. J Neuroimmunol 331:28–35. https://doi.org/10.1016/j.jneuroim.2018.03.006
68. Zeis T, Enz L, Schaeren-Wiemers N (2016) The immunomodulatory oligodendrocyte. Brain Res 1641:139–148. https://doi.org/10.1016/j.brainres.2015.09.021
69. Niu J, Tsai H-H, Hoi KK, et al (2019) Aberrant oligodendroglial–vascular interactions disrupt the blood–brain barrier, triggering CNS inflammation. Nat Neurosci 22:709–718. https://doi.org/10.1038/s41593-019-0369-4
70. Yuen TJ, Silbereis JC, Griveau A, et al (2014) Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell 158:383–396. https://doi.org/10.1016/j.cell.2014.04.052
71. Seo JH, Maki T, Maeda M, et al (2014) Oligodendrocyte Precursor Cells Support Blood-Brain Barrier Integrity via TGF-β Signaling. PLOS ONE 9:e103174. https://doi.org/10.1371/journal.pone.0103174
72. Arai K, Lo EH (2009) An oligovascular niche: cerebral endothelial cells promote the survival and proliferation of oligodendrocyte precursor cells. J Neurosci 29:4351–4355. https://doi.org/10.1523/JNEUROSCI.0035-09.2009
73. Arai K, Lo EH (2009) Oligovascular Signaling in White Matter Stroke. Biol Pharm Bull 32:1639–1644

74. Tsai H-H, Niu J, Munji R, et al (2016) Oligodendrocyte precursors migrate along vasculature in the developing nervous system. Science 351:379–384. https://doi.org/10.1126/science.aad3839
75. Seo JH, Miyamoto N, Hayakawa K, et al (2013) Oligodendrocyte precursors induce early blood-brain barrier opening after white matter injury. J Clin Invest 123:782–786. https://doi.org/10.1172/JCI65863
76. Kishida N, Maki T, Takagi Y, et al (2019) Role of Perivascular Oligodendrocyte Precursor Cells in Angiogenesis After Brain Ischemia. J Am Heart Assoc 8:e011824. https://doi.org/10.1161/JAHA.118.011824
77. Ballabh P, Braun A, Nedergaard M (2004) The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis 16:1–13. https://doi.org/10.1016/j.nbd.2003.12.016
78. Abbott NJ, Patabendige AAK, Dolman DEM, et al (2010) Structure and function of the blood-brain barrier. Neurobiol Dis 37:13–25. https://doi.org/10.1016/j.nbd.2009.07.030
79. Pardridge WM, Eisenberg J, Yang J (1985) Human blood-brain barrier insulin receptor. J Neurochem 44:1771–1778. https://doi.org/10.1111/j.1471-4159.1985.tb07167.x
80. Zhang Y, Pardridge WM (2001) Rapid transferrin efflux from brain to blood across the blood-brain barrier. J Neurochem 76:1597–1600. https://doi.org/10.1046/j.1471-4159.2001.00222.x
81. Pardridge WM (2015) Blood-brain barrier endogenous transporters as therapeutic targets: a new model for small molecule CNS drug discovery. Expert Opin Ther Targets 19:1059–1072. https://doi.org/10.1517/14728222.2015.1042364
82. Profaci CP, Munji RN, Pulido RS, Daneman R (2020) The blood–brain barrier in health and disease: Important unanswered questions. Journal of Experimental Medicine 217:e20190062. https://doi.org/10.1084/jem.20190062
83. Chojdak-Łukasiewicz J, Dziadkowiak E, Zimny A, Paradowski B (2021) Cerebral small vessel disease: A review. Adv Clin Exp Med 30:349–356. https://doi.org/10.17219/acem/131216
84. Maki T (2017) Novel roles of oligodendrocyte precursor cells in the developing and damaged brain. Clinical and Experimental Neuroimmunology 8:33–42. https://doi.org/10.1111/cen3.12358
85. Daneman R, Zhou L, Kebede AA, Barres BA (2010) Pericytes are required for blood–brain barrier integrity during embryogenesis. Nature 468:562–566. https://doi.org/10.1038/nature09513
86. Abbott NJ, Rönnbäck L, Hansson E (2006) Astrocyte–endothelial interactions at the blood–brain barrier. Nat Rev Neurosci 7:41–53. https://doi.org/10.1038/nrn1824
87. Armulik A, Genové G, Mäe M, et al (2010) Pericytes regulate the blood–brain barrier. Nature 468:557–561. https://doi.org/10.1038/nature09522
88. Furuse M, Sasaki H, Tsukita S (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. J Cell Biol 147:891–903. https://doi.org/10.1083/jcb.147.4.891
89. Liebner S, Fischmann A, Rascher G, et al (2000) Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. Acta Neuropathol 100:323–331. https://doi.org/10.1007/s004010000180
90. Liebner S, Kniesel U, Kalbacher H, Wolburg H (2000) Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells. Eur J Cell Biol 79:707–717. https://doi.org/10.1078/0171-9335-00101
91. Lippoldt A, Kniesel U, Liebner S, et al (2000) Structural alterations of tight junctions are associated with loss of polarity in stroke-prone spontaneously hypertensive rat blood-brain barrier endothelial cells. Brain Res 885:251–261. https://doi.org/10.1016/s0006-8993(00)02954-1
92. Nitta T, Hata M, Gotoh S, et al (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161:653–660. https://doi.org/10.1083/jcb.200302070

93. Hirase T, Staddon JM, Saitou M, et al (1997) Occludin as a possible determinant of tight junction permeability in endothelial cells. J Cell Sci 110 (Pt 14):1603–1613. https://doi.org/10.1242/jcs.110.14.1603
94. Papadopoulos MC, Saadoun S, Woodrow CJ, et al (2001) Occludin expression in microvessels of neoplastic and non-neoplastic human brain. Neuropathol Appl Neurobiol 27:384–395. https://doi.org/10.1046/j.0305-1846.2001.00341.x
95. Matter K, Balda MS (2003) Signalling to and from tight junctions. Nat Rev Mol Cell Biol 4:225–236. https://doi.org/10.1038/nrm1055
96. Bazzoni G, Martinez-Estrada OM, Mueller F, et al (2000) Homophilic interaction of junctional adhesion molecule. J Biol Chem 275:30970–30976. https://doi.org/10.1074/jbc.M003946200
97. Martìn-Padura I, Lostaglio S, Schneemann M, et al (1998) Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 142:117–127. https://doi.org/10.1083/jcb.142.1.117
98. Desai BS, Monahan AJ, Carvey PM, Hendey B (2007) Blood-brain barrier pathology in Alzheimer's and Parkinson's disease: implications for drug therapy. Cell Transplant 16:285–299. https://doi.org/10.3727/000000007783464731
99. Correale J, Villa A (2007) The blood-brain-barrier in multiple sclerosis: functional roles and therapeutic targeting. Autoimmunity 40:148–160. https://doi.org/10.1080/08916930601183522
100. Kaur C, Ling EA (2008) Blood brain barrier in hypoxic-ischemic conditions. Curr Neurovasc Res 5:71–81. https://doi.org/10.2174/156720208783565645
101. Thrippleton MJ, Backes WH, Sourbron S, et al (2019) Quantifying blood-brain barrier leakage in small vessel disease: Review and consensus recommendations. Alzheimers Dement 15:840–858. https://doi.org/10.1016/j.jalz.2019.01.013
102. Bernardo-Castro S, Sousa JA, Brás A, et al (2020) Pathophysiology of Blood–Brain Barrier Permeability Throughout the Different Stages of Ischemic Stroke and Its Implication on Hemorrhagic Transformation and Recovery. Frontiers in Neurology 11:
103. van de Haar HJ, Burgmans S, Jansen JFA, et al (2016) Blood-Brain Barrier Leakage in Patients with Early Alzheimer Disease. Radiology 281:527–535. https://doi.org/10.1148/radiol.2016152244
104. Arba F, Leigh R, Inzitari D, et al (2017) Blood–brain barrier leakage increases with small vessel disease in acute ischemic stroke. Neurology 89:2143–2150. https://doi.org/10.1212/WNL.0000000000004677
105. Balasa R, Barcutean L, Mosora O, Manu D (2021) Reviewing the Significance of Blood–Brain Barrier Disruption in Multiple Sclerosis Pathology and Treatment. Int J Mol Sci 22:8370. https://doi.org/10.3390/ijms22168370
106. Tarkowski E, Rosengren L, Blomstrand C, et al (1997) Intrathecal release of pro- and anti-inflammatory cytokines during stroke. Clin Exp Immunol 110:492–499. https://doi.org/10.1046/j.1365-2249.1997.4621483.x
107. Feuerstein GZ, Liu T, Barone FC (1994) Cytokines, inflammation, and brain injury: role of tumor necrosis factor-alpha. Cerebrovasc Brain Metab Rev 6:341–360
108. Zhang W, Smith C, Shapiro A, et al (1999) Increased expression of bioactive chemokines in human cerebromicrovascular endothelial cells and astrocytes subjected to simulated ischemia in vitro. J Neuroimmunol 101:148–160. https://doi.org/10.1016/s0165-5728(99)00137-x
109. Zhang W, Smith C, Howlett C, Stanimirovic D (2000) Inflammatory activation of human brain endothelial cells by hypoxic astrocytes in vitro is mediated by IL-1beta. J Cereb Blood Flow Metab 20:967–978. https://doi.org/10.1097/00004647-200006000-00009

110. Foulquier S, Daskalopoulos EP, Lluri G, et al (2018) WNT Signaling in Cardiac and Vascular Disease. Pharmacol Rev 70:68–141. https://doi.org/10.1124/pr.117.013896
111. Soomro SH, Jie J, Fu H (2018) Oligodendrocytes Development and Wnt Signaling Pathway. IJHA 1:17–35. https://doi.org/10.14302/issn.2577-2279.ijha-18-2407
112. Wang L, Geng J, Qu M, et al (2020) Oligodendrocyte precursor cells transplantation protects blood–brain barrier in a mouse model of brain ischemia via Wnt/β-catenin signaling. Cell Death Dis 11:9. https://doi.org/10.1038/s41419-019-2206-9
113. Persidsky Y, Ramirez SH, Haorah J, Kanmogne GD (2006) Blood-brain barrier: structural components and function under physiologic and pathologic conditions. J Neuroimmune Pharmacol 1:223–236. https://doi.org/10.1007/s11481-006-9025-3
114. Zhang CE, Wong SM, Haar HJ van de, et al (2017) Blood–brain barrier leakage is more widespread in patients with cerebral small vessel disease. Neurology 88:426–432. https://doi.org/10.1212/WNL.0000000000003556
115. Kerkhofs D, Wong SM, Zhang E, et al (2021) Baseline Blood-Brain Barrier Leakage and Longitudinal Microstructural Tissue Damage in the Periphery of White Matter Hyperintensities. Neurology 96:e2192–e2200. https://doi.org/10.1212/WNL.0000000000011783
116. Markus HS, de Leeuw FE (2023) Cerebral small vessel disease: Recent advances and future directions. International Journal of Stroke 18:4–14. https://doi.org/10.1177/17474930221144911
117. Mustapha M, Nassir CMNCM, Aminuddin N, et al (2019) Cerebral Small Vessel Disease (CSVD) – Lessons From the Animal Models. Frontiers in Physiology 10:
118. Litak J, Mazurek M, Kulesza B, et al (2020) Cerebral Small Vessel Disease. Int J Mol Sci 21:9729. https://doi.org/10.3390/ijms21249729
119. Bhagat R, Marini S, Romero JR (2023) Genetic considerations in cerebral small vessel diseases. Frontiers in Neurology 14:
120. Duering M, Biessels GJ, Brodtmann A, et al (2023) Neuroimaging standards for research into small vessel disease—advances since 2013. The Lancet Neurology 22:602–618. https://doi.org/10.1016/S1474-4422(23)00131-X
121. Gatti L, Tinelli F, Scelzo E, et al (2020) Understanding the Pathophysiology of Cerebral Amyloid Angiopathy. Int J Mol Sci 21:3435. https://doi.org/10.3390/ijms21103435
122. Pantoni L, Garcia JH (1995) The significance of cerebral white matter abnormalities 100 years after Binswanger's report. A review. Stroke 26:1293–1301. https://doi.org/10.1161/01.str.26.7.1293
123. Faraco G, Iadecola C (2013) Hypertension: a harbinger of stroke and dementia. Hypertension 62:810–817. https://doi.org/10.1161/HYPERTENSIONAHA.113.01063
124. O'Sullivan M, Lythgoe DJ, Pereira AC, et al (2002) Patterns of cerebral blood flow reduction in patients with ischemic leukoaraiosis. Neurology 59:321–326. https://doi.org/10.1212/wnl.59.3.321
125. Shibata M, Ohtani R, Ihara M, Tomimoto H (2004) White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion. Stroke 35:2598–2603. https://doi.org/10.1161/01.STR.0000143725.19053.60
126. Arba F, Mair G, Carpenter T, et al (2017) Cerebral White Matter Hypoperfusion Increases with Small-Vessel Disease Burden. Data From the Third International Stroke Trial. J Stroke Cerebrovasc Dis 26:1506–1513. https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.03.002
127. Wong SM, Jansen JFA, Zhang CE, et al (2019) Blood-brain barrier impairment and hypoperfusion are linked in cerebral small vessel disease. Neurology 92:e1669–e1677. https://doi.org/10.1212/WNL.0000000000007263

128. Uiterwijk R, Staals J, Huijts M, et al (2017) MRI progression of cerebral small vessel disease and cognitive decline in patients with hypertension. J Hypertens 35:1263–1270. https://doi.org/10.1097/HJH.0000000000001294
129. Kerkhofs D, Wong SM, Zhang E, et al (2021) Blood–brain barrier leakage at baseline and cognitive decline in cerebral small vessel disease: a 2-year follow-up study. GeroScience 43:1643–1652. https://doi.org/10.1007/s11357-021-00399-x
130. Dobrynina LA, Shamtieva KV, Kremneva EI, et al (2022) Daily blood pressure profile and blood–brain barrier permeability in patients with cerebral small vessel disease. Sci Rep 12:7723. https://doi.org/10.1038/s41598-022-11172-1
131. Papakonstantinou E, Bacopoulou F, Brouzas D, et al (2019) NOTCH3 and CADASIL syndrome: a genetic and structural overview. EMBnet J 24:e921. https://doi.org/10.14806/ej.24.0.921
132. Uemura M, Nozaki H, Kato T, et al (2020) HTRA1-Related Cerebral Small Vessel Disease: A Review of the Literature. Frontiers in Neurology 11:
133. Saini M, Ikram K, Hilal S, et al (2012) Silent stroke: not listened to rather than silent. Stroke 43:3102–3104. https://doi.org/10.1161/STROKEAHA.112.666461
134. Das AS, Regenhardt RW, Vernooij MW, et al (2019) Asymptomatic Cerebral Small Vessel Disease: Insights from Population-Based Studies. J Stroke 21:121–138. https://doi.org/10.5853/jos.2018.03608
135. Park Y-S, Chung M-S, Choi B-S (2019) MRI Assessment of Cerebral Small Vessel Disease in Patients with Spontaneous Intracerebral Hemorrhage. Yonsei Med J 60:774–781. https://doi.org/10.3349/ymj.2019.60.8.774
136. Wardlaw JM (2005) What causes lacunar stroke? Journal of Neurology, Neurosurgery & Psychiatry 76:617–619. https://doi.org/10.1136/jnnp.2004.039982
137. Loos CMJ, Staals J, Wardlaw JM, van Oostenbrugge RJ (2012) Cavitation of deep lacunar infarcts in patients with first-ever lacunar stroke: a 2-year follow-up study with MR. Stroke 43:2245–2247. https://doi.org/10.1161/STROKEAHA.112.660076
138. Rensma SP, van Sloten TT, Launer LJ, Stehouwer CDA (2018) Cerebral small vessel disease and risk of incident stroke, dementia and depression, and all-cause mortality: A systematic review and meta-analysis. Neurosci Biobehav Rev 90:164–173. https://doi.org/10.1016/j.neubiorev.2018.04.003
139. Direk N, Perez HS, Akoudad S, et al (2016) Markers of cerebral small vessel disease and severity of depression in the general population. Psychiatry Research: Neuroimaging 253:1–6. https://doi.org/10.1016/j.pscychresns.2016.05.002
140. van der Holst HM, van Uden IWM, Tuladhar AM, et al (2015) Cerebral small vessel disease and incident parkinsonism. Neurology 85:1569–1577. https://doi.org/10.1212/WNL.0000000000002082
141. Hatate J, Miwa K, Matsumoto M, et al (2016) Association between cerebral small vessel diseases and mild parkinsonian signs in the elderly with vascular risk factors. Parkinsonism Relat Disord 26:29–34. https://doi.org/10.1016/j.parkreldis.2016.02.011
142. Aguero-Torres H, Kivipelto M, von Strauss E (2006) Rethinking the dementia diagnoses in a population-based study: what is Alzheimer's disease and what is vascular dementia?. A study from the kungsholmen project. Dement Geriatr Cogn Disord 22:244–249. https://doi.org/10.1159/000094973
143. Clancy U, Appleton JP, Arteaga C, et al (2021) Clinical management of cerebral small vessel disease: a call for a holistic approach. Chin Med J (Engl) 134:127–142. https://doi.org/10.1097/CM9.0000000000001177
144. Cannistraro RJ, Badi M, Eidelman BH, et al (2019) CNS small vessel disease: A clinical review. Neurology 92:1146–1156. https://doi.org/10.1212/WNL.0000000000007654

145. Blair GW, Appleton JP, Flaherty K, et al (2019) Tolerability, safety and intermediary pharmacological effects of cilostazol and isosorbide mononitrate, alone and combined, in patients with lacunar ischaemic stroke: The LACunar Intervention-1 (LACI-1) trial, a randomised clinical trial. EClinicalMedicine 11:34–43. https://doi.org/10.1016/j.eclinm.2019.04.001
146. Wardlaw JM, Woodhouse LJ, Mhlanga II, et al (2023) Isosorbide Mononitrate and Cilostazol Treatment in Patients With Symptomatic Cerebral Small Vessel Disease: The Lacunar Intervention Trial-2 (LACI-2) Randomized Clinical Trial. JAMA Neurol e231526. https://doi.org/10.1001/jamaneurol.2023.1526
147. Kitamura A, Manso Y, Duncombe J, et al (2017) Long-term cilostazol treatment reduces gliovascular damage and memory impairment in a mouse model of chronic cerebral hypoperfusion. Sci Rep 7:4299. https://doi.org/10.1038/s41598-017-04082-0
148. Jalal FY, Yang Y, Thompson JF, et al (2015) Hypoxia-induced neuroinflammatory white-matter injury reduced by minocycline in SHR/SP. J Cereb Blood Flow Metab 35:1145–1153. https://doi.org/10.1038/jcbfm.2015.21
149. Fu Y, Yan Y (2018) Emerging Role of Immunity in Cerebral Small Vessel Disease. Front Immunol 9:67. https://doi.org/10.3389/fimmu.2018.00067
150. Piao J, Major T, Auyeung G, et al (2015) Human embryonic stem cell-derived oligodendrocyte progenitors remyelinate the brain and rescue behavioral deficits following radiation. Cell Stem Cell 16:198–210. https://doi.org/10.1016/j.stem.2015.01.004
151. Li S, Oh BC, Chu C, et al (2019) Induction of immunological tolerance to myelinogenic glial-restricted progenitor allografts. Brain 142:3456–3472. https://doi.org/10.1093/brain/awz275
152. Wang R, Chu C, Wei Z, et al (2021) Traumatic brain injury does not disrupt costimulatory blockade-induced immunological tolerance to glial-restricted progenitor allografts. J Neuroinflammation 18:104. https://doi.org/10.1186/s12974-021-02152-9
153. Li W, He T, Shi R, et al (2021) Oligodendrocyte Precursor Cells Transplantation Improves Stroke Recovery via Oligodendrogenesis, Neurite Growth and Synaptogenesis. Aging Dis 12:2096–2112. https://doi.org/10.14336/AD.2021.0416
154. America M, Bostaille N, Eubelen M, et al (2022) An integrated model for Gpr124 function in Wnt7a/b signaling among vertebrates. Cell Rep 39:110902. https://doi.org/10.1016/j.celrep.2022.110902
155. Martin M, Vermeiren S, Bostaille N, et al (2022) Engineered Wnt ligands enable blood-brain barrier repair in neurological disorders. Science 375:eabm4459. https://doi.org/10.1126/science.abm4459
156. Lacombe P, Oligo C, Domenga V, et al (2005) Impaired cerebral vasoreactivity in a transgenic mouse model of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy arteriopathy. Stroke 36:1053–1058. https://doi.org/10.1161/01.STR.0000163080.82766.eb
157. Gould DB, Phalan FC, van Mil SE, et al (2006) Role of COL4A1 in small-vessel disease and hemorrhagic stroke. N Engl J Med 354:1489–1496. https://doi.org/10.1056/NEJMoa053727
158. Li C, Jin W, Wang X, et al (2019) Establishment and identification of a novel HTRA1 mutation mice model. Rev Cardiovasc Med 20:179–186. https://doi.org/10.31083/j.rcm.2019.03.31813
159. Baumbach GL, Sigmund CD, Faraci FM (2003) Cerebral arteriolar structure in mice overexpressing human renin and angiotensinogen. Hypertension 41:50–55. https://doi.org/10.1161/01.hyp.0000042427.05390.5c
160. Hainsworth AH, Markus HS (2008) Do in vivo experimental models reflect human cerebral small vessel disease? A systematic review. J Cereb Blood Flow Metab 28:1877–1891. https://doi.org/10.1038/jcbfm.2008.91

161. Kantachuvesiri S, Fleming S, Peters J, et al (2001) Controlled hypertension, a transgenic toggle switch reveals differential mechanisms underlying vascular disease. J Biol Chem 276:36727–36733. https://doi.org/10.1074/jbc.M103296200
162. Collidge TA, Lammie GA, Fleming S, Mullins JJ (2004) The role of the renin-angiotensin system in malignant vascular injury affecting the systemic and cerebral circulations. Prog Biophys Mol Biol 84:301–319. https://doi.org/10.1016/j.pbiomolbio.2003.11.003
163. Guy R, Volkman R, Wilczynski E, et al (2022) A Novel Rodent Model of Hypertensive Cerebral Small Vessel Disease with White Matter Hyperintensities and Peripheral Oxidative Stress. International Journal of Molecular Sciences 23:5915. https://doi.org/10.3390/ijms23115915
164. Foulquier S, Namsolleck P, Van Hagen BT, et al (2018) Hypertension-induced cognitive impairment: insights from prolonged angiotensin II infusion in mice. Hypertens Res 41:817–827. https://doi.org/10.1038/s41440-018-0090-9
165. Kerkhofs D, Helgers R, Hermes D, et al (2023) Amlodipine limits microglia activation and cognitive dysfunction in aged hypertensive mice. J Hypertens 41:1159–1167. https://doi.org/10.1097/HJH.0000000000003445
166. Nishio K, Ihara M, Yamasaki N, et al (2010) A Mouse Model Characterizing Features of Vascular Dementia With Hippocampal Atrophy. Stroke 41:1278–1284. https://doi.org/10.1161/STROKEAHA.110.581686
167. Nakaji K, Ihara M, Takahashi C, et al (2006) Matrix Metalloproteinase-2 Plays a Critical Role in the Pathogenesis of White Matter Lesions After Chronic Cerebral Hypoperfusion in Rodents. Stroke 37:2816–2823. https://doi.org/10.1161/01.STR.0000244808.17972.55
168. Dong Y-F, Kataoka K, Toyama K, et al (2011) Attenuation of brain damage and cognitive impairment by direct renin inhibition in mice with chronic cerebral hypoperfusion. Hypertension 58:635–642. https://doi.org/10.1161/HYPERTENSIONAHA.111.173534
169. Washida K, Hattori Y, Ihara M (2019) Animal Models of Chronic Cerebral Hypoperfusion: From Mouse to Primate. Int J Mol Sci 20:6176. https://doi.org/10.3390/ijms20246176
170. Bink DI, Ritz K, Aronica E, et al (2013) Mouse models to study the effect of cardiovascular risk factors on brain structure and cognition. J Cereb Blood Flow Metab 33:1666–1684. https://doi.org/10.1038/jcbfm.2013.140
171. Pantoni L, Garcia JH, Gutierrez JA (1996) Cerebral white matter is highly vulnerable to ischemia. Stroke 27:1641–1646; discussion 1647. https://doi.org/10.1161/01.str.27.9.1641
172. Fukatsu T, Miyake-Takagi K, Nagakura A, et al (2002) Effects of nefiracetam on spatial memory function and acetylcholine and GABA metabolism in microsphere-embolized rats. Eur J Pharmacol 453:59–67. https://doi.org/10.1016/s0014-2999(02)02360-9
173. Roos MW, Ericsson A (1999) N-tert-butyl-alpha-phenylnitrone reduces the number of microinfarctions in the rabbit brain cortex. Exp Brain Res 124:271–272. https://doi.org/10.1007/s002210050622
174. Sato Y, Chin Y, Kato T, et al (2009) White matter activated glial cells produce BDNF in a stroke model of monkeys. Neurosci Res 65:71–78. https://doi.org/10.1016/j.neures.2009.05.010
175. Shen Y, Yao M-J, Su Y-X, et al (2021) Histochemistry of microinfarcts in the mouse brain after injection of fluorescent microspheres into the common carotid artery. Neural Regen Res 17:832–837. https://doi.org/10.4103/1673-5374.322470
176. Dietrich WD, Watson BD, Busto R, et al (1987) Photochemically induced cerebral infarction. I. Early microvascular alterations. Acta Neuropathol 72:315–325. https://doi.org/10.1007/BF00687262
177. Taylor ZJ, Hui ES, Watson AN, et al (2016) Microvascular basis for growth of small infarcts following occlusion of single penetrating arterioles in mouse cortex. J Cereb Blood Flow Metab 36:1357–1373. https://doi.org/10.1177/0271678X15608388

178. Ma H, Yang Y, Gao M, et al (2022) A novel rat model of cerebral small vessel disease and evaluation by super-resolution ultrasound imaging. Journal of Neuroscience Methods 379:109673. https://doi.org/10.1016/j.jneumeth.2022.109673
179. Cianchetti FA, Kim DH, Dimiduk S, et al (2013) Stimulus-Evoked Calcium Transients in Somatosensory Cortex Are Temporarily Inhibited by a Nearby Microhemorrhage. PLOS ONE 8:e65663. https://doi.org/10.1371/journal.pone.0065663

Chapter 2

-

A Systematic Review of WNT Signaling in Endothelial Cell Oligodendrocyte Interactions: Potential Relevance to Cerebral Small Vessel Disease

Narek Manukjan, Zubair Ahmed, Daniel Fulton, W. Matthijs Blankesteijn, Sébastien Foulquier

Received: 2 June 2020

Accepted: 23 June 2020

Published: 25 June 2020

Based on Cells 2020, 9(6), 1545

DOI: 10.3390/cells9061545

Abstract

Key pathological features of cerebral small vessel disease (cSVD) include impairment of the blood brain barrier (BBB) and the progression of white matter lesions (WMLs) amongst other structural lesions, leading to the clinical manifestations of cSVD. The function of endothelial cells (ECs) is of major importance to maintain a proper BBB. ECs interact with several cell types to provide structural and functional support to the brain. Oligodendrocytes (OLs) myelinate axons in the central nervous system and are crucial in sustaining the integrity of white matter. The interplay between ECs and OLs and their precursor cells (OPCs) has received limited attention yet seems of relevance for the study of BBB dysfunction and white matter injury in cSVD. Emerging evidence shows a crosstalk between ECs and OPCs/OLs, mediated by signaling through the Wingless and Int-1 (WNT)/β-catenin pathway. As the latter is involved in EC function (e.g., angiogenesis) and oligodendrogenesis, we reviewed the role of WNT/β-catenin signaling for both cell types and performed a systematic search to identify studies describing a WNT-mediated interplay between ECs and OPCs/OLs. Dysregulation of this interaction may limit remyelination of WMLs and render the BBB leaky, thereby initiating a vicious neuroinflammatory cycle. A better understanding of the role of this signaling pathway in EC–OL crosstalk is essential in understanding cSVD development.

Keywords: cerebral small vessel disease; WNT; β-catenin; oligodendrocytes; oligodendrocyte precursor cells; endothelial cells

1. Introduction

Cerebral small vessel disease (cSVD) is an umbrella term used to describe different pathological processes that affect the small vessels of the brain, including small arteries, arterioles, capillaries, and small veins, and is associated with brain structural lesions and eventual cognitive impairment [1]. With cSVD accounting for up to 25% of all stroke and 45% of dementia, it is seen as a common cause of cognitive impairment worldwide [2]. Beyond cognitive impairment, mood and gait disorders are also often seen in cSVD patients [3]. Although recent advances have led to an improved diagnosis and understanding of cSVD, its exact pathogenesis is still unknown. However, damage to the blood brain barrier (BBB) is recognized as an early pathological step in cSVD, often resulting from the exposure to cardiovascular risk factors, such as hypertension and diabetes [2,4,5].

BBB dysfunction has been evidenced in experimental animal models of cSVD as well as in cSVD patients [6–8]. Endothelial cells (ECs) are essential for BBB function due to their tight junction (TJ) proteins that prevent the passive entrance of cells and macromolecules into the brain. Endothelial dysfunction is therefore a key contributor to BBB dysfunction and is present in several brain pathologies, including stroke, multiple sclerosis (MS), and Alzheimer's disease [9]. In addition to the endothelial barrier, BBB integrity also relies on non-neuronal cells, such as pericytes, astrocytes, and oligodendrocytes (OLs) [10]. Although studies are scarce, the involvement of different cell types, especially OLs, might play a critical role in the pathophysiology of cSVD. Both OLs and oligodendrocyte precursor cells (OPCs) are able to lower the brain endothelial permeability when co-cultured with primary brain ECs [11]. In addition, OL dysfunction can cause white matter loss and BBB disruption, two important features seen in cSVD patients [10,12,13]. A common factor that may facilitate an interaction between ECs and OPCs/OLs is platelet-derived growth factor

(PDGF) as ECs and OPCs secrete PDGF and express its receptor (PDGFRα). The tightening of the BBB by OLs seems, however, not to depend on the PDGF/PDGFRα signaling, as it cannot be prevented by a PDGFRα antagonist [11]. This suggests that other pathways might play a critical role in the interaction between ECs and OPCs/OLs. The Wingless and Int-1 (WNT)/β-catenin pathway, largely described for its function within ECs and OPCs/OLs may be crucial in this crosstalk [14,15].

Although both ECs and OLs (and their precursor cells) are involved in BBB integrity, studies examining these cells and their interaction in the context of cSVD are limited. This review aimed to summarize the published findings on the crosstalk between ECs and OPCs/OLs, and in particular the role played by WNT/β-catenin signaling in this interaction. First, we briefly address the respective roles of WNTs in ECs and OPCs/OLs. Second, we discuss the current knowledge on EC–OL interaction. Third, we present studies found via a systematic search that have identified a role for WNT signaling in this crosstalk. Finally, we discuss the potential implications of these findings for cSVD pathology before we formulate a conclusion.

2. Pathology of Cerebral Small Vessel Disease

cSVD is a heterogeneous disease that includes genetic and sporadic forms. Sporadic cSVD, the most prevalent form of cSVD, can be further subdivided into amyloidal and non-amyloidal subtypes [1,10,16]. Sporadic cSVD is associated with age and the presence of cardiovascular risk factors. Hypertension is considered as the major modifiable risk factor for the development of cSVD and vascular cognitive impairment [16,17]. The thickening, stiffening, and narrowing of extra-cranial and cranial vessels induced by hypertension can lead to brain hypoperfusion, with subsequent hypoxia, BBB impairment,

neuroinflammation, and the appearance of white matter lesions (WMLs), ultimately leading to demyelination and neurodegeneration [18–21]. The pathological cascade induced by hypertension has been summarized by the 'three hit' hypothesis [22]. The initial hit is hypertension itself, which can initiate an ischemic insult leading to local tissue hypoxia. The local hypoxic state, which is considered as the second hit, can promote damage and inflammation, which represents the third and final hit. In the course of this pathological cascade, hypoxia can increase BBB permeability and induce myelin breakdown either directly and/or indirectly via the expression of cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, secreted by microglia and macrophages. Reactive microglia and brain macrophages can alter the BBB integrity itself and also damage the white matter tissue and its supporting cells, such as OLs, leading to myelin breakdown and vasogenic cerebral edema [22]. Altogether, neuroinflammation and myelin breakdown can cause WMLs and the development of cognitive impairment [22,23].

Taken together, our current knowledge on cSVD pathology indicates a role for ECs and OLs in maintaining the integrity of the white matter and the BBB, and suggests that their dysfunction could be critical in the course of cSVD development.

3. Brain Endothelial Cells

Brain ECs harbor specific features compared to peripheral ECs, namely the presence of TJ proteins. This is a key feature in maintaining the central nervous system (CNS) microenvironment and to protect the brain from invading pathogens, immune cells, toxic compounds, or alternating ionic concentrations that can affect normal cerebral function [24]. Pericyte and astrocyte end-feet support this barrier by surrounding the EC [25,26]. TJ link adjacent ECs together by forming homodimer transmembrane proteins, which are important

in maintaining the tightly regulated brain microenvironment. Dysfunction of these proteins can cause leakage of the BBB and disruption of brain functions [27]. The brain requires certain nutrients to maintain its normal function, thus controlled permeability is required for normal brain function. The EC in the BBB are specialized in allowing the transport of certain molecules and cells when needed, such as glucose for energy or immune cells during inflammation [28,29].

ECs also play a key role in regulating local cerebral blood flow in response to neuronal activity and their underlying metabolic needs. This is regulated by the relaxation of smooth muscle cells and pericytes surrounding the larger vessels and capillaries, respectively, mediated by the release of nitric oxide (NO), synthesized by the endothelial NO synthase (eNOS). Upon eNOS deactivation, or impaired vasodilation, the cerebrovascular tone is increased, potentially leading to hypoxia in the corresponding areas [24]. This highlights the importance of tight regulation of the vascular diameter by ECs and the adverse consequences of malfunctioning of this system.

Microvascular dysfunction has been proposed as an early sign of cSVD, preceding the occurrence of structural lesions, such as WMLs. Endothelial dysfunction causes BBB leakage and inflammation, which has been suggested to cause lacunar strokes, white matter hyperintensities, and cerebral microbleeds seen in cSVD [2,24,27,30]. An increased plasma concentration of markers of endothelial activation, such as vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1, has been observed in patients with cSVD compared to control subjects [30]. Furthermore, beyond their own dysfunction, the release of detrimental mediators by diseased ECs may also affect other brain cells, further aggravating cSVD progression [24].

4. Oligodendrocytes

OLs are the cells responsible for the production of myelin, the isolating fatty sheath surrounding axons that provides structural protection, and facilitate fast electrical signal transduction along CNS axons. In addition to this role in action potential conduction, recent work has revealed additional roles in various processes, including trophic and metabolic support of neurons [31–35], information processing in neural circuits [36], and interactions with other CNS cell types, such as ECs [37,38].

Myelinating OLs are generated through a sequence of developmental steps involving four stages: OPCs, late OPCs, immature OLs, and mature myelinating OLs [39]. During this developmental sequence, OPCs migrate to sites requiring myelination, where they proliferate and undergo a morphological differentiation that marks their transition into late OPCs (also known as preoligodendrocytes). Late OPCs exhibit highly branched process arbors whose terminal branches make contact with numerous neuronal compartments, including axons [40,41]. This developmental sequence continues as late OPCs exit the cell cycle and differentiate into immature OLs that upregulate the expression of myelin genes and convert some of their initial axonal contacts into loose membrane wraps. As OL maturation progresses, these early ensheathments are elongated and compacted to form mature myelin sheaths. Other non-myelinating process branches are resorbed so that in the final mature OL, all process branches support myelinating segments. Of note, a significant number of OPCs persist in CNS tissues beyond the period of developmental myelination, where they provide a pool of precursors that can be recruited in the case of injury [42–44]. The rate of OPC differentiation decreases in the adult CNS [43], and while some OPCs differentiate to produce myelinating OLs, the majority remain as OPCs and are not involved in myelin sheath production [42]. Yeung et al. suggest that human OLs have the ability to

remodel the myelin sheaths without the need for the generation of OLs from OPCs [45]. However, other studies show that there is a gradual production of newly differentiated OLs that engage in de novo myelination and myelin remodeling in the adult CNS [46,47]. Factors released by OPCs and OLs, such as insulin-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF), induce neuronal cell survival [31,48], indicating that OLs indeed provide important trophic support for axonal maintenance and survival. Hypertension, hypoxia, and inflammation might disrupt these factors, leading to OL cell death and damage to the integrity of white matter tissues. Loss of white matter due to hypoperfusion-induced OL death and myelin loss in cSVD has been demonstrated in experimental animal models [49–51]. Cognitive impairment associated with the loss of OLs has been observed, for instance, following brain hypoperfusion in rats via the permanent bilateral occlusion of the common carotid arteries [49]. Hypoperfusion led to an initial increase in OPCs, which was followed by an increase in OPC cell death that produced a net decrease in the number of mature OLs in this chronic hypoperfusion rat model [50]. A similar finding was observed in spontaneously hypertensive rat-stroke prone (SHRSP) subjected to brain hypoperfusion in association with increased inflammatory mediators [51]. Overall, the complex multi-step process involved in OL development and function, and its dependence on tightly controlled regulatory mechanisms, renders OLs highly sensitive to pathological conditions in both the developing and mature CNS.

Consequently, the emergence of a local or global brain hypoperfusion can affect both OPC/OL function and the factors secreted by these cells under these conditions. One group of these secreted factors are the WNT proteins and their signaling molecules, an upregulation of which was found after exposure of OPCs to hypoxic conditions, suggesting that they may play major roles in brain hypoxia [52]. In fact, these proteins play a complex role in both

white matter integrity and angiogenesis, with a key role in both OPC fate, OL survival, and EC proliferation [14,15,52]. Taken together, these findings suggest that WNT signaling may be a key regulator of OPC–OL interaction, and an important mediator in cSVD pathology.

5. WNT Signaling in Endothelial Cells and Oligodendrocytes

5.1. WNT Signaling

WNT signaling is typically categorized into two pathways: The β-catenin- and non-β-catenin-mediated pathways, with the latter being further sub-categorized. The non-beta-catenin-mediated pathways involve all WNT pathways that do not lead to the stabilization of β-catenin and plays a role in processes including cell polarization, cell fate, inflammatory response, and cell migration [53]. The β-catenin-mediated WNT signaling pathway leads to the intracellular stabilization of β-catenin, resulting in its translocation to the nucleus and the transcription of numerous genes involved in cell proliferation, differentiation, tissue expansion, cell fate, and many more [54]. In the absence of WNT proteins, β-catenin is phosphorylated by a protein complex formed by glycogen synthase kinase 3β (GSK-3β), Axin, adenomatous polyposis coli (APC), and casein kinase-1 (CK-1). Phosphorylation of β-catenin by this so-called destruction complex leads to its degradation by the ubiquitin proteasome. Secreted WNT proteins require two distinct receptor families for their intracellular signaling, namely the Frizzled (Fzd) and the low-density lipoprotein (LDL) receptor-related protein 5 or 6 (LRP5/6). Binding of WNT to Fzd and its co-receptor LRP5/6 results in the recruitment of the disheveled (Dsh) protein to the plasma membrane. This leads to the recruitment of several components of the β-catenin phosphorylation complex and their

inhibition, which in turn leads to the accumulation of β-catenin in the cytoplasm. Increased cytoplasmic β-catenin promotes its translocation to the nucleus and its binding to transcription factors, leading to transcription of its target genes (Figure 1) [14]. An extensive description of these different WNT signaling pathways is beyond the scope of this review but can be found in a recent publication from our group [14].

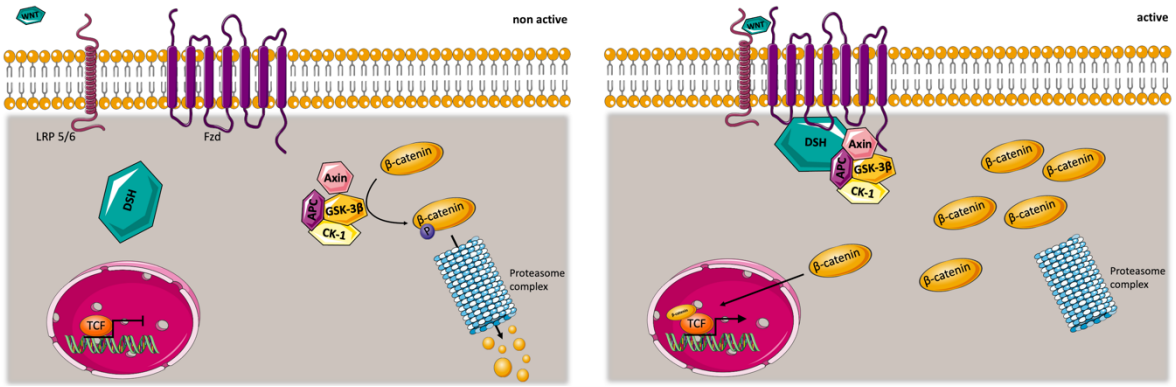


Figure 1. Schematic representation of the Wingless and Int-1 (WNT)/β-catenin signaling pathway. In the non-active state **(left panel)**, β-catenin is phosphorylated by a multi-component complex containing glycogen synthase kinase 3β (GSK-3β), Axin, adenomatous polyposis coli (APC), and casein kinase-1 (CK-1). This causes β-catenin to be phosphorylated for degradation by the proteasome complex. In the active state **(right panel)**, a WNT ligand binds to its receptor and co-receptor, Frizzled (Fzd), and low density lipoprotein (LDL) receptor-related protein 5 or 6 (LRP5/6), respectively, and causes the recruitment of components of the β-catenin phosphorylating complex to the membrane together with disheveled (DSH). This leads to the cytoplasmic accumulation of β-catenin and its translocation to the nucleus, eventually binding members of the T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors and activating transcription of target genes.

5.2. WNT Signaling in Brain Endothelial Cells

β-catenin is essential for the formation and maintenance of vascular integrity and controls BBB TJ formation [55,56]. The first steps of blood vessel formation seem to rely on WNT/β-catenin signaling promoting EC-specific differentiation of pluripotent stem cells, an essential step in vasculogenesis (the initial blood vessel formation) [14]. WNT/β-catenin also induces the expression of TJ and glucose transporter 1 (Glut1) proteins, which are key

features of brain ECs. Besides differentiation, several WNTs have also been reported to induce EC proliferation and migration to promote vessel assembly [57]. WNT signaling is also essential for the formation of new blood vessels from pre-existing ones, called angiogenesis [14]. In a non-angiogenic state, cytoplasmic β-catenin is constantly degraded and thus does not result in the formation of new vasculature. When WNT signaling is activated, stabilization of β-catenin leads to angiogenesis. Angiogenesis is further regulated by the negative feedback mechanism involving the activation of c-Casitas B-lineage lymphoma (c-Cbl). Phosphorylated c-Cbl promotes the degradation of active β-catenin, and thus functions as a negative regulator of angiogenesis [58]. CNS angiogenesis has been shown to be dependent dominantly on the β-catenin-mediated WNT signaling ligands WNT7a and WNT7b [59]. These ligands are dependent on coactivators G protein-coupled receptor 124 (GPR124) and Reck expressed by the EC, which enhance WNT7a/7b signaling pathways [60]. Benz et al. showed that brain areas lacking a BBB have ECs with low levels of β-catenin activation due to the absence of WNT receptors. Increased β-catenin activation resulted in increased endothelial expression of the TJ protein claudin5, and was associated with a reduction of BBB permeability in the corresponding areas [61]. In addition to angiogenesis, the expression of TJ proteins, such as claudin3 and claudin5, which maintain BBB integrity, is also regulated by WNT/β-catenin signaling [56,61]. In vitro stabilization of β-catenin with WNT3a treatment in primary ECs resulted in an increased expression of claudin3, and the formation of TJ and BBB characteristics [56], while in vivo overactivation of β-catenin in transgenic mice led to an increased expression of claudin5 [61].

Taken together, this demonstrates the essential role of WNT/β-catenin signaling in the formation and integrity of the BBB. Interestingly, WNT7a and WNT7b, which drive angiogenesis, are the predominant WNT proteins expressed by OPCs [52,62].

5.3. WNT Signaling in Oligodendrocytes

WNT/β-catenin signaling was initially suggested to exert an inhibitory effect on oligodendrogenesis and differentiation, although it is now clear that this pathway regulates multiple events during the OPC developmental stages [15,63,64]. While some studies have identified a WNT/β-catenin-mediated repressive function for the OPC specification from neuronal stem cells (NSCs) during prenatal development [63,65], other studies showed that WNT signaling was dispensable or could even enhance OPC differentiation [66,67]. It has become clear that WNT signaling plays a complex role in the fate of OPCs in a context-dependent manner, depending on the developmental stage, location in the CNS, cell type, exposure level, and possible interactions with other pathways affecting the cell fate [15,64]. Guo et al. proposed a working model on the multimodal role of WNT/β-catenin signaling in OPC development [15]. Low levels of β-catenin signaling promote OPC differentiation to immature OLs during development, whereas high levels inhibit OPC density and differentiation. Similar findings were observed during OL maturation, where the exact role of WNT/β-catenin signaling on OL maturation remains unclear [15]. Olig2Cre; Da-Cat mice, which have dominant-active β-catenin in OPCs and OLs, had decreased numbers of myelin proteolipid protein (PLP) expressing OLs, which was associated with hypomyelination, while OPC numbers were not affected [66]. On the contrary, Tawk et al. reported that WNT/β-catenin signaling plays a role in activating this myelin gene in OLs. They showed that inhibition of WNT signaling components by small interfering RNA (siRNA) resulted in a decrease in *plp* expression levels, while a three-fold increase in the expression of this myelin gene protein was observed following WNT/β-catenin activation by WNT1 [67]. Myelin is a major component within white matter, and damage to myelin proteins is widely seen in cSVD as discussed previously. It is therefore important to clarify

the effects of WNT signaling on OPC differentiation and maturation in the context of demyelination and remyelination.

After demyelination, activation of WNT/β-catenin seems to inhibit OPC differentiation and to decrease myelin density. Fancy et al. reported a decrease in PLP-expressing OLs, 14 days after a lysolecithin (LPC)-induced lesion in Olig2Cre;Da-Cat mice. They also found a decrease in remyelination after LPC-induced demyelination in mice lacking one *Apc* allele or containing a complete deletion of the *Axin2* gene, which both caused an increase in β-catenin levels [66]. In line with this finding, the inhibition of WNT/β-catenin by aspirin was associated with increased OL differentiation [68]. However, in another study, neither *Apc* single-allele conditional knockout nor one-allele nonsense truncated mutation differed in the levels of WNT/β-catenin signal when compared to wild type (WT) mice [69]. This suggests that the previous reported delay in OPC differentiation might have resulted from β-catenin-independent effects of *Apc* deletion. On the contrary, an increase in the WNT transcription mediator gene, transcription factor-4 (TCF4 (also known as TCF7L2)), was reported following white matter demyelination in rodents and in active areas of MS lesions, indicating a beneficial role for WNT signaling in remyelination [66]. It was, however, demonstrated that TCF4 was expressed only early in remyelination in both mice and MS patients, and not in later stages or in chronic lesions [70,71]. This might indicate an early OPC recruitment via WNT/β-catenin signaling, which has been reported to play a role in OPC migration and the attachment to vessels [72], as a rescue mechanism in response to demyelination.

Although sometimes contradictory at first sight, these results highlight a crucial role for WNT/β-catenin in OL function that may largely depend on the timing, context, and cellular environment. Taken together, activation of WNT/β-catenin signaling in demyelination

appears to inhibit OL development and decreases myelin production, resulting in a remyelination failure that could contribute to the development of the WML seen in cSVD.

6. Oligodendrocyte–Endothelial Cell Crosstalk

Although both ECs and OPCs/OLs are of importance for the BBB integrity, the interaction between ECs and OPCs/OLs and their role in cSVD remain largely unclear. It has been suggested that EC dysfunction might alter OL function, and vice versa [10]. There is no direct evidence linking EC–OL interaction and cSVD. However, early investigations on the SHRSP, a hypertensive rat model that recapitulates many brain structural abnormalities characteristic of cSVD pathology, including WMLs, suggest that endothelial dysfunction precedes and leads to alterations in myelin and white matter [73–75]. A reciprocal interaction between ECs and OLs was also demonstrated in an animal model of neurofibromatosis. OL-specific overactivation of Ras signaling in these animals led to an aberrant production of NO in addition to dysregulation of TJ proteins in both ECs and myelin sheaths. Ras overexpression also led to enlarged perivascular spaces and BBB leakages, which correlated with myelin breakdown. This indicates that OL-specific changes might have an effect on ECs and lead to features of cSVD pathology [76]. In addition, mice with endothelial dysfunction presented increased remnants of capillaries in the form of string vessels and decreased numbers of OPCs in the white matter [77]. These abnormalities predate other cSVD pathology in both animal models and human post-mortem tissue, suggesting an interplay between the endothelium and myelinating cells [77,78].

Aria and Lo were the first to describe a clear interaction between ECs and OLs in an in vitro model by culturing rat OPCs in human brain EC-conditioned medium, which induced an increased OPC proliferation rate. They suggested that EC-secreted factors, such as fibroblast

growth factor (FGF) and BDNF, mediated these effects [79]. In turn, OPCs seem to affect ECs in culture as well. Culturing ECs in conditioned medium from OPCs led to EC proliferation and angiogenesis by secreted factors, such as transforming growth factor (TGF)-β and matrix metalloproteases (MMPs). OPCs support BBB integrity by releasing either TGF-β1 in normal conditions, or disrupt it and induce angiogenesis by releasing MMP-9 when stressed by inflammatory cytokines [80,81]. Taken together, these results indicate that factors released by ECs and OPCs play an important role in promoting their interactions and influencing their functions.

More recently, abnormal WNT signaling was suggested to alter the EC–OPC interaction, leading to abnormal OPC migration and dysfunction of both ECs and OLs [82,83]. Hence, we performed a systematic literature search to investigate the exact role of WNT signaling in the interaction between ECs and OPCs/OLs and its involvement in cSVD.

7. Literature Search Method

Publications on ECs and OPCs/OLs and interaction related to cSVD were exclusively identified through PubMed search engine and reported following the Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) [84]. The search was conducted on 9 April 2020 and the following search terms and combinations were used to identify articles: Cerebral small vessel disease AND endothelial cells; Cerebral small vessel disease AND endothelium; Cerebral small vessel disease AND oligodendrocyte; Cerebral small vessel disease AND OPC; Cerebral small vessel disease AND WNT; Oligodendrocyte AND endothelial; OPC AND endothelial; Endothelial AND interaction AND (oligodendrocyte OR OPC OR OPC); Endothelial AND oligodendrocyte AND WNT; Endothelial AND OPC AND WNT; WNT AND angiogenesis; WNT AND BBB AND

oligodendrocyte; WNT AND hypoperfusion; WNT AND hypertension; WNT7 AND endothelial; WNT7 AND oligodendrocyte. Two additional relevant papers were later added by the authors.

Duplicate papers were removed, and studies underwent an initial screen based on their title, followed by a screening of their abstract. Studies using the following terms were included during title screening: Endothelial cells; oligodendrocytes and/or oligodendrocyte precursor cells; and cerebral small vessel disease. Papers using the following terms were included after screening the abstract: Interaction EC–OPC/OL; inflammation; vasculature; cerebrovascular; animal and human studies. We excluded publications matching the following topics: Review, methodology, periphery, oncology, and cancer. Furthermore, studies were excluded if they referred to EC interaction with OPCs or OLs in the context that was not related to the brain or irrelevant for cSVD (e.g., WNT signaling in brain tumor environment) after full text reviewing. There were no commentaries, editorials, rectifications, or non-English publications in our search results. Screening and extraction of articles was performed by the first author (NM) under the guidance of the other authors. For each study, the following variables were recorded: Language, year of publication, type of disease, clinical design, experimental design, animal model, and results. The results are described in a flow diagram (Figure 2).

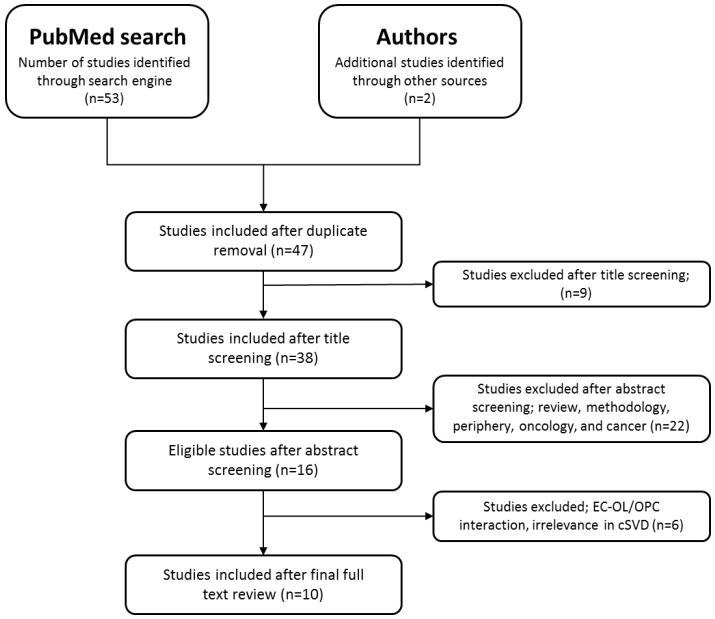


Figure 2. Flow diagram showing the systematic Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) search protocol and identification, screening, and extraction of the corresponding studies. ECs, endothelial cells; OLs, oligodendrocytes; OPCs, oligodendrocyte precursor cells; cSVD, cerebral small vessel disease.

8. WNT Signaling in Endothelial Cell–Oligodendrocyte Crosstalk

The key role of WNT/β-catenin signaling in various brain cells is subject to complex regulation. Miyamoto et al. reviewed recent findings indicating a WNT-dependent single cell migration of adult OPCs from the subventricular zone to damaged areas under the guidance of ECs, which may contribute to white matter recovery [83]. Other findings supporting a key role for WNT signaling in white matter recovery via OPC recruitment include the initial upregulation of WNT expression in OPCs during hypoxia, WNT-mediated migration of OPCs, and an early increase of OPCs after injury [50,52,85,86]. Evidence for a reciprocal interaction came from Iijima et al. by the induction of remyelination due to EC dysfunction via endothelin (ET)-1 injection, indicated by a decrease in immunohistochemical neuron-glial antigen 2 (NG2) and myelin basic protein (MBP) levels,

which was rescued by transplantation of healthy ECs. In addition, the results suggested a potential intrinsic rescue mechanism via an increase in OPC density, 7 days after ET-1 injection [87]. EC dysfunction, indicated by an impaired dilatation response to acetylcholine (ACh) and calcitonin gene-related peptide (CGRP), and hypoperfusion, marked by an increased number of string vessels, due to TGF-β1 overexpression in ECs in a transgenic mouse model led to a decreased OPC density in white matter areas and cognitive impairment [77]. Treatment of EC dysfunction with simvastatin, a drug that improves overall endothelial function [88], counteracted these negative effects, possibly via mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling [77]. This might indicate a cross-talk with the WNT/β-catenin signaling pathway in the interaction of EC–OL, as MAPK can increase β-catenin expression through direct regulation of GSK3β by p38 [89]. These indirect evidence suggest that EC dysfunction precedes OL dysfunction and white matter abnormalities, possibly via WNT signaling. We thus suggest that WNT/β-catenin signaling modulates both EC and OL function and their interaction with potential pleiotropic roles in cSVD.

In cSVD animal models and patients, increased OPC proliferation and migration has been observed following EC dysfunction [78,90]. Rajani et al. showed that SHRSP rats and human post-mortem tissue displayed EC abnormalities before any classical signs of cSVD pathology or stroke. Direct evidence for the ECs and OPCs/OLs interaction can be found in in vitro assays with OPCs cultured with conditioned media from SHRSP rat primary brain microvascular EC cultures, which exhibit decreased OPC differentiation and increased proliferation [78]. In vitro and in vivo increased OPC density and proliferation together with an initial increase in OPCs, as observed in patients with leukoencephalopathy, suggests an attempt by the brain to recruit myelinating cells for white matter repair [78,87,90]. In fact,

WNT signaling is involved in the migration of OPCs using the vascular tree [72,85,86]. OPCs respond to demyelination by becoming activated, proliferating, migrating, and ultimately differentiating into myelinating OLs [72]. Under physiological conditions, demyelination results in the activation of WNT signaling in OPCs, which in turn leads to the upregulation of C-X-C chemokine receptor type 4 (Cxcr4). This protein binds to its ligand stromal cell-derived factor 1 (Sdf1, also known as C-X-C motif chemokine 12 [Cxcl12]) expressed by ECs and mediates the single cell migration of OPCs to the recruitment site [85,86]. Tsai et al. showed that overexpressing Cxcr4 led to increased attachment of OPCs to the vasculature and OPC clustering, which could be reversed by inhibiting the Cxc4–Sdf1 interaction. Once these OPCs are at their intended destination, OPCs can detach from the vessel and differentiate into mature myelinating OLs due to the downregulation of WNT and Cxcr4. However, in disease states, abnormal EC function was shown to result in disruption of OPC migration, OPC clustering, delayed OPC differentiation, and a decrease in myelination. These effects might involve WNT signaling pathways as upregulation of Cxcr4 was detected in WNT-activated OPC clusters [85]. OPC clusters were shown to be present in lesions in both animal models and human white matter injury, where aberrant OPC migration and remyelination seem to be associated with overactive WNT signaling [86]. These effects are both autocrine and paracrine, thus also affecting surrounding ECs. OPCs are indeed able to activate WNT-mediated angiogenesis [52,72], and regulate it via the expression of c-Cbl, acting as a negative feedback mechanism for WNT/β-catenin signaling [58]. These results indicate an important role for OPC clustering in the dysregulation of EC and OPC function.

OPC clustering, and the dysfunctional overexpression of WNT signaling seen in Olig2-cre: APC floxed/floxed mice, in turn lead to EC dysfunction, BBB disruption, and inflammation

in non-injury settings [86]. A key component in this process is the WNT-mediated expression of WNT inhibitory factor 1 (Wif1), which is expressed following β-catenin activation. Wif1 is highly upregulated in OPCs, activated due to WNT/β-catenin signaling, and functions as a negative feedback control mechanism to decrease WNT activation in OPCs. However, overexpression of Wif1 also has a paracrine effect on ECs by downregulating the TJ protein claudin5, leading to BBB dysfunction and subsequent neuroinflammation [86]. Altogether, WNT/β-catenin signaling and its target genes are involved in the tight regulation of the BBB and angiogenesis to adjust the blood supply according to demand. However, this is a complex synergistic interaction since oxygen levels also influence WNT expression [52].

Hypoxic conditions lead to the upregulation of WNT7a and WNT7b, mediated by the stabilization of hypoxia-inducible factor (HIF)1/2α, which resulted in decreased myelination in mouse white matter [52,91]. These effects were normalized by XAV939, an inhibitor of WNT/β-catenin-mediated transcription, via the stabilization of Axin [92]. Cell culture experiments also demonstrated an arrest of OPC maturation when WNT signaling was overactive. In vitro, OPC-conditioned media promoted β-catenin-mediated endothelial tip sprouting and tube formation in mouse brain ECs, which was inhibited by XAV939 [52]. The secretion and release of WNT7a and WNT7b proteins from OPCs was shown to directly induce angiogenesis and this was prevented by XAV939, demonstrating that this effect was WNT/β-catenin dependent [52]. Wang et al. suggested that OPCs might have a positive effect in reducing the infarct volume and brain edema and improving cognitive function after middle cerebral artery occlusion in mice [82]. Transplantation of OPCs into the infarct area reduced protein leakage into the brain parenchyma, and rescued claudin5 expression. This was associated with an upregulation of endothelial β-catenin and activation of WNT/β-

catenin signaling, as using XAV939 increased BBB leakages and decreased claudin5 expression. These results are in agreement with in vitro findings showing a decreased permeability of brain ECs treated with either WNT7a or OPC-conditioned medium. In addition, WNT7a was upregulated in mice transplanted with OPCs, suggesting a key role in this interaction. OPC-conditioned media or WNT7a treatment increased β-catenin and claudin5 expressions in brain ECs, an effect that was reversed by WNT7a knockdown in cultured OPCs [82]. Interestingly, recent evidence suggests that M2c anti-inflammatory microglia secrete WNT7a and promote oligodendrogenesis, indicating that pleiotropic actions are also involved in microglia and OL crosstalk [93].

9. Discussion

In summary, direct and indirect evidence suggests an interplay between ECs and OPCs/OLs driven by WNT/β-catenin signaling. The mechanistic/molecular hypothesis of their interaction may differ between physiological and pathological conditions as proposed below (Figure 3). We suggest that the activation of WNT7a and WNT7b signaling in healthy ECs and OPCs/OLs in response to injury may help the recruitment of new OPCs at sites of demyelination via the structural support of cerebral vessels. In the meantime, this endothelial signaling promotes angiogenesis to supply oxygen and necessary nutrients for recovery. Once OPCs have reached their destination, feedback mechanisms are activated via Wif1, leading to the downregulation of WNT/β-catenin signaling. Subsequently, angiogenesis stops and OPCs detach from the vessels to differentiate into mature myelinating OLs. In cSVD however, local hypoxic injuries can alter the brain endothelium [52], causing activation of HIF1/2α, which in turn excessively upregulates WNT7a and WNT7b signaling. Dysfunctional ECs exhibit an overexpression of WNT proteins, leading to dysfunctional

OPC migration and clustering, and local angiogenesis. The expression of negative feedback proteins can have paracrine effects, resulting in inflammation, abnormal angiogenesis, and a leaky BBB due to a low expression of the TJ protein claudin5 in inflamed or newly formed vessels. EC dysfunction and WNT signaling may also lead to decreased OPC detachment, leading to decreased OPC differentiation and decreased myelination. Ultimately, these effects may stimulate further damage to the brain by decreasing the rate of myelin repair, potentiating the development of WML and ensuing cognitive impairment.

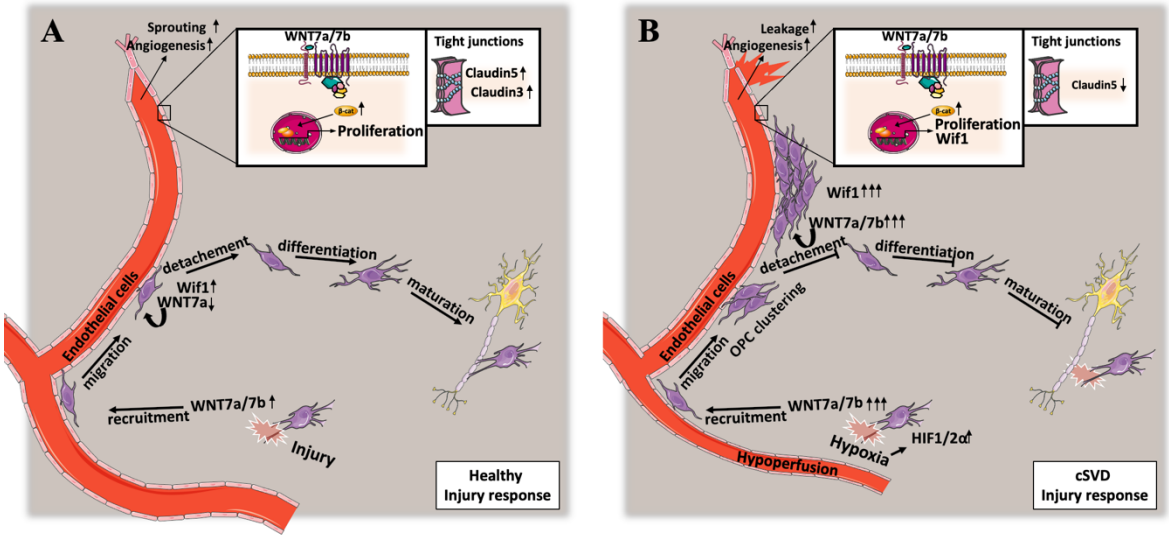


Figure 3. Schematic overview of the proposed contribution of WNT/β-catenin signaling in cSVD. (**A**) In a healthy situation, hypoxic injury induces WNT7a and WNT7b signaling in OPCs/OLs to promote the recruitment of new OPCs at sites of demyelination. OPCs migrate to the site of injury partly by using the physical support offered by cerebral vessels. WNT/β-catenin signaling in ECs promotes angiogenesis to supply oxygen and necessary nutrients for recovery. Once OPCs have reached their destination, feedback mechanisms are activated via Wif1, leading to the downregulation of WNT/β-catenin signaling and detachment from cerebral vessels. Subsequently, angiogenesis stops and OPCs differentiate into mature OLs, capable of initiating remyelination. (**B**) In cSVD, activation of HIF1/2α due to local hypoxia results in an exaggerated upregulation of WNT7a and WNT7b, which is aggravated by EC dysfunction. This leads to dysfunctional OPC migration and clustering. Local angiogenesis induced by WNT/β-catenin signaling results in a leaky and inflamed BBB partly due to the expression of negative feedback proteins that alter the expression of the tight junction protein claudin5. In addition, decreased detachment and differentiation of OPCs in turn leads to attenuated remyelination and white matter repair.

10. Conclusion

It this review, we identified an important role for WNT signaling in the EC–OPC/OL interplay, whose contribution (both autocrine and paracrine) should be further investigated. The use of animal and post-mortem human material will be determinative to decipher the pleiotropic role of WNT in this interaction and its relevance for cSVD pathobiology and other cerebrovascular disorders. Beyond a greater pathological understanding, it may bring therapeutic opportunities to seal an impaired BBB, reverse OPC clustering, and repair white matter injuries in cSVD.

Author Contributions: Conceptualization, N.M., Z.A., D.F., W.M.D., and S.F.; methodology, N.M., W.M.B., and S.F.; writing—original draft preparation, N.M. and, S.F.; writing—review and editing, N.M., Z.A., D.F., W.M.B., and S.F.; illustration, N.M.; and supervision, Z.A., D.F., W.M.B., and S.F. All authors have read and agreed to the published version of the manuscript.

Funding: N.M. is the recipient of a Ph.D. studentship from University of Birmingham and Maastricht University.

Acknowledgments: Servier Medical Art was used to generate Figures 1 and 3 (Creative Common Attribution 3.0 Generic License). http://smart.servier.com/.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Pantoni, L. Cerebral small vessel disease: From pathogenesis and clinical characteristics to therapeutic challenges. Lancet Neurol. 2010, 9, 689–701.
2. Shi, Y.; Wardlaw, J.M. Update on cerebral small vessel disease: A dynamic whole-brain disease. Stroke Vasc. Neurol. 2016, 1, 83–92.
3. Pinter, D.; Ritchie, S.J.; Doubal, F.; Gattringer, T.; Morris, Z.; Bastin, M.E.; Hernández, M.D.C.V.; Royle, N.A.; Corley, J.; Muñoz Maniega, S.; et al. Impact of small vessel disease in the brain on gait and balance. Sci. Rep. 2017, 7, 41637.
4. Farrall, A.J.; Wardlaw, J.M. Blood-brain barrier: Ageing and microvascular disease-systematic review and meta-analysis. Neurobiol. Aging 2009, 30, 337–352.
5. Liu, Y.; Dong, Y.H.; Lyu, P.Y.; Chen, W.H.; Li, R. Hypertension-Induced Cerebral Small Vessel Disease Leading to Cognitive Impairment. Chin. Med. J. (England) 2018, 131, 615–619.
6. Zhang, C.E.; Wong, S.M.; Van De Haar, H.J.; Staals, J.; Jansen, J.F.A.; Jeukens, C.R.L.P.N.; Hofman, P.A.M.; Van Oostenbrugge, R.J.; Backes, W.H. Blood-brain barrier leakage is more widespread in patients with cerebral small vessel disease. Neurology 2017, 88, 426–432.
7. Foulquier, S.; Namsolleck, P.; Van Hagen, B.T.; Milanova, I.; Post, M.J.; Blankesteijn, W.M.; Rutten, B.P.; Prickaerts, J.; Van Oostenbrugge, R.J.; Unger, T. Hypertension-induced cognitive impairment: Insights from prolonged angiotensin II infusion in mice. Hypertens. Res. 2018, 41, 817–827.
8. Wong, S.M.; Jansen, J.F.A.; Zhang, C.E.; Hoff, E.I.; Staals, J.; van Oostenbrugge, R.J.; Backes, W.H. Blood-brain barrier impairment and hypoperfusion are linked in cerebral small vessel disease. Neurology 2019, 92, 1669–1677.
9. Hawkins, B.T. The Blood-Brain Barrier/Neurovascular Unit in Health and Disease. Pharmacol. Rev. 2005, 57, 173–185.
10. Cuadrado-Godia, E.; Dwivedi, P.; Sharma, S.; Ois Santiago, A.; Roquer Gonzalez, J.; Balcells, M.; Laird, J.; Turk, M.; Suri, H.S.; Nicolaides, A.; et al. Cerebral Small Vessel Disease: A Review Focusing on Pathophysiology, Biomarkers, and Machine Learning Strategies. J. Stroke 2018, 20, 302–320.
11. Kimura, I.; Dohgu, S.; Takata, F.; Matsumoto, J.; Watanabe, T.; Iwao, T.; Yamauchi, A.; Kataoka, Y. Oligodendrocytes upregulate blood-brain barrier function through mechanisms other than the PDGF-BB/PDGFRα pathway in the barrier-tightening effect of oligodendrocyte progenitor cells. Neurosci. Lett. 2020, 715, 134594.
12. Liu, C.; Zou, L.; Tang, X.; Zhu, W.; Zhang, G.; Qin, Y.; Zhu, W. Changes of white matter integrity and structural network connectivity in nondemented cerebral small-vessel disease. J. Magn. Reson. Imaging 2019, 51, 1162–1169.
13. Hamanaka, G.; Ohtomo, R.; Takase, H.; Lok, J.; Arai, K. Role of oligodendrocyte-neurovascular unit in white matter repair. Neurosci. Lett. 2018, 684, 175–180.
14. Foulquier, S.; Daskalopoulos, E.P.; Lluri, G.; Hermans, K.C.M.; Deb, A.; Blankesteijn, W.M. WNT Signaling in Cardiac and Vascular Disease. Pharmacol. Rev. 2018, 70, 68–141.
15. Guo, F.; Lang, J.; Sohn, J.; Hammond, E.; Chang, M.; Pleasure, D. Canonical Wnt signaling in the oligodendroglial lineage-puzzles remain. Glia 2015, 63, 1671–1693.
16. Mustapha, M.; Nassir, C.M.N.C.M.; Aminuddin, N.; Safri, A.A.; Ghazali, M.M. Cerebral Small Vessel Disease (CSVD)–Lessons from the Animal Models. Front Physiol. 2019, 10, 1317.
17. Dichgans, M.; Leys, D. Vascular Cognitive Impairment. Circ. Res. 2017, 120, 573–591.
18. Mok, V.; Kim, J.S. Prevention and Management of Cerebral Small Vessel Disease. J. Stroke 2015, 17, 111–122.

19. Pantoni, L.; Garcia, J.H. The significance of cerebral white matter abnormalities 100 years after binswanger's report: A review. Stroke 1995, 26, 1293–1301.
20. Faraco, G.; Iadecola, C. Hypertension: A harbinger of stroke and dementia. Hypertension 2013, 62, 810–817.
21. Hooghiemstra, A.M.; Bertens, A.S.; Leeuwis, A.E.; Bron, E.E.; Bots, M.L.; Brunner-La Rocca, H.P.; De Craen, A.J.M.; Van Der Geest, R.J.; Greving, J.P.; Kappelle, L.J.; et al. The Missing Link in the Pathophysiology of Vascular Cognitive Impairment: Design of the Heart-Brain Study. Cerebrovasc. Dis. Extra 2017, 7, 140–152.
22. Rosenberg, G.A. Extracellular matrix inflammation in vascular cognitive impairment and dementia. Clin. Sci. 2017, 131, 425–437.
23. Ivanova, N.; Liu, Q.; Agca, C.; Agca, Y.; Noble, E.G.; Whitehead, S.N.; Cechetto, D.F. White matter inflammation and cognitive function in a co-morbid metabolic syndrome and prodromal Alzheimer's disease rat model. J. Neuroinflammation 2020, 17, 29.
24. Rajani, R.M.; Williams, A. Endothelial cell–oligodendrocyte interactions in small vessel disease and aging. Clin. Sci. 2017, 131, 369–379.
25. Nedergaard, M.; Ransom, B.; Goldman, S.A. New roles for astrocytes: Redefining the functional architecture of the brain. Trends Neurosci. 2003, 26, 523–530.
26. Dermietzel, R.; Spray, D.C.; Nedergaard, M. Blood-Brain Barriers: From Ontogeny to Artificial Interfaces; Wiley-Blackwell: Hoboken, NJ, USA, 2007; ISBN 3527310886.
27. Liu, W.Y.; Wang, Z. Bin; Zhang, L.C.; Wei, X.; Li, L. Tight junction in blood-brain barrier: An overview of structure, regulation, and regulator substances. CNS Neurosci. Ther. 2012, 18, 609–615.
28. Mergenthaler, P.; Lindauer, U.; Dienel, G.A.; Meisel, A. Sugar for the brain: The role of glucose in physiological and pathological brain function. Trends Neurosci. 2013, 36, 587–597.
29. Takeshita, Y.; Ransohoff, R.M. Inflammatory cell trafficking across the blood-brain barrier: Chemokine regulation and in vitro models. Immunol. Rev. 2012, 248, 228–239.
30. Rouhl, R.P.W.; Damoiseaux, J.G.M.C.; Lodder, J.; Theunissen, R.O.M.F.I.H.; Knottnerus, I.L.H.; Staals, J.; Henskens, L.H.G.; Kroon, A.A.; de Leeuw, P.W.; Tervaert, J.W.C.; et al. Vascular inflammation in cerebral small vessel disease. Neurobiol. Aging 2012, 33, 1800–1806.
31. Wilkins, A.; Chandran, S.; Compston, A. A role for oligodendrocyte-derived IGF-1 in trophic support of cortical neurons. Glia 2001, 36, 48–57.
32. Nave, K.A. Myelination and support of axonal integrity by glia. Nature 2010, 468, 244–252.
33. Nave, K.A. Myelination and the trophic support of long axons. Nat. Rev. Neurosci. 2010, 11, 275–283.
34. Fünfschilling, U.; Supplie, L.M.; Mahad, D.; Boretius, S.; Saab, A.S.; Edgar, J.; Brinkmann, B.G.; Kassmann, C.M.; Tzvetanova, I.D.; Möbius, W.; et al. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. Nature 2012, 485, 517–521.
35. Lee, Y.; Morrison, B.M.; Li, Y.; Lengacher, S.; Farah, M.H.; Hoffman, P.N.; Liu, Y.; Tsingalia, A.; Jin, L.; Zhang, P.W.; et al. Oligodendroglia metabolically support axons and contribute to neurodegeneration. Nature 2012, 487, 443–448.
36. Steadman, P.E.; Xia, F.; Ahmed, M.; Mocle, A.J.; Penning, A.R.A.; Geraghty, A.C.; Steenland, H.W.; Monje, M.; Josselyn, S.A.; Frankland, P.W. Disruption of Oligodendrogenesis Impairs Memory Consolidation in Adult Mice. Neuron 2020, 105, 150–164.
37. Gibson, E.M.; Purger, D.; Mount, C.W.; Goldstein, A.K.; Lin, G.L.; Wood, L.S.; Inema, I.; Miller, S.E.; Bieri, G.; Zuchero, J.B.; et al. Neuronal activity promotes oligodendrogenesis and adaptive myelination in the mammalian brain. Science 2014, 344, 1252304.

38. Domingues, H.S.; Portugal, C.C.; Socodato, R.; Relvas, J.B. Oligodendrocyte, Astrocyte, and Microglia Crosstalk in Myelin Development, Damage, and Repair. Front. Cell Dev. Biol. 2016, 4, 71.
39. Ceprian, M.; Fulton, D. Glial cell AMPA Receptors in nervous system health, injury and disease. Int. J. Mol. Sci. 2019, 20, 2450.
40. Fannon, J.; Tarmier, W.; Fulton, D. Neuronal activity and AMPA-type glutamate receptor activation regulates the morphological development of oligodendrocyte precursor cells. Glia 2015, 63, 1021–1035.
41. Butt, A.M.; Hamilton, N.; Hubbard, P.; Pugh, M.; Ibrahim, M. Synantocytes: The fifth element. J. Anat. 2005, 207, 695–706.
42. Rivers, L.E.; Young, K.M.; Rizzi, M.; Jamen, F.; Psachoulia, K.; Wade, A.; Kessaris, N.; Richardson, W.D. PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. Nat. Neurosci. 2008, 11, 1392–1401.
43. Psachoulia, K.; Jamen, F.; Young, K.M.; Richardson, W.D. Cell cycle dynamics of NG2 cells in the postnatal and ageing brain. Neuron Glia Biol. 2009, 5, 57–67.
44. Michalski, J.-P.; Kothary, R. Oligodendrocytes in a Nutshell. Front. Cell. Neurosci. 2015, 9, 340.
45. Yeung, M.S.Y.; Zdunek, S.; Bergmann, O.; Bernard, S.; Salehpour, M.; Alkass, K.; Perl, S.; Tisdale, J.; Possnert, G.; Brundin, L.; et al. Dynamics of Oligodendrocyte Generation and Myelination in the Human Brain. Cell 2014, 159, 766–774.
46. Young, K.M.; Psachoulia, K.; Tripathi, R.B.; Dunn, S.J.; Cossell, L.; Attwell, D.; Tohyama, K.; Richardson, W.D. Oligodendrocyte dynamics in the healthy adult CNS: Evidence for myelin remodeling. Neuron 2013, 77, 873–885.
47. Hughes, E.G.; Orthmann-Murphy, J.L.; Langseth, A.J.; Bergles, D.E. Myelin remodeling through experience-dependent oligodendrogenesis in the adult somatosensory cortex. Nat. Neurosci. 2018, 21, 696–706.
48. Linker, R.A.; Lee, D.H.; Demir, S.; Wiese, S.; Kruse, N.; Siglienti, I.; Gerhardt, E.; Neumann, H.; Sendtner, M.; Lühder, F.; et al. Functional role of brain-derived neurotrophic factor in neuroprotective autoimmunity: Therapeutic implications in a model of multiple sclerosis. Brain 2010, 133, 22–48–2263.
49. Choi, B.R.; Kim, D.H.; Back, D. Bin; Kang, C.H.; Moon, W.J.; Han, J.S.; Choi, D.H.; Kwon, K.J.; Shin, C.Y.; Kim, B.R.; et al. Characterization of White Matter Injury in a Rat Model of Chronic Cerebral Hypoperfusion. Stroke 2016, 47, 542–547.
50. Miyamoto, N.; Tanaka, R.; Shimura, H.; Watanabe, T.; Mori, H.; Onodera, M.; Mochizuki, H.; Hattori, N.; Urabe, T. Phosphodiesterase III inhibition promotes differentiation and survival of oligodendrocyte progenitors and enhances regeneration of ischemic white matter lesions in the adult mammalian brain. J. Cereb. Blood Flow Metab. 2010, 30, 299–310.
51. Jalal, F.Y.; Yang, Y.; Thompson, J.; Lopez, A.C.; Rosenberg, G.A. Myelin loss associated with neuroinflammation in hypertensive rats. Stroke 2012, 43, 1115–1122.
52. Yuen, T.J.; Silbereis, J.C.; Griveau, A.; Chang, S.M.; Daneman, R.; Fancy, S.P.J.; Zahed, H.; Maltepe, E.; Rowitch, D.H. Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell 2014, 158, 383–396.
53. De, A. Wnt/Ca2+ signaling pathway: A brief overview. Acta Biochim. Biophys. Sin. (Shanghai) 2011, 43, 745–756.
54. Clevers, H. Wnt/β-Catenin Signaling in Development and Disease. Cell 2006, 127, 469–480.
55. Cattelino, A.; Liebner, S.; Gallini, R.; Zanetti, A.; Balconi, G.; Corsi, A.; Blanco, P.; Wolburg, H.; Moore, R.; Oreda, B.; et al. The conditional inactivation of the β-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility. J. Cell Biol. 2003, 162, 1111–1122.

56. Liebner, S.; Corada, M.; Bangsow, T.; Babbage, J.; Taddei, A.; Czupalla, C.J.; Reis, M.; Felici, A.; Wolburg, H.; Fruttiger, M.; et al. Wnt/β-catenin signaling controls development of the blood-brain barrier. J. Cell Biol. 2008, 183, 409–417.
57. Franco, C.A.; Liebner, S.; Gerhardt, H. Vascular morphogenesis: A Wnt for every vessel? Curr. Opin. Genet. Dev. 2009, 19, 476–483.
58. Shivanna, S.; Harrold, I.; Shashar, M.; Meyer, R.; Kiang, C.; Francis, J.; Zhao, Q.; Feng, H.; Edelman, E.R.; Rahimi, N.; et al. The C-Cbl ubiquitin ligase regulates nuclear β-catenin and angiogenesis by its tyrosine phosphorylation mediated through the Wnt signaling pathway. J. Biol. Chem. 2015, 290, 12537–12546.
59. Cho, C.; Smallwood, P.M.; Nathans, J. Reck and Gpr124 Are Essential Receptor Cofactors for Wnt7a/Wnt7b-Specific Signaling in Mammalian CNS Angiogenesis and Blood-Brain Barrier Regulation. Neuron 2017, 95, 1056-1073.e5.
60. Vanhollebeke, B.; Stone, O.A.; Bostaille, N.; Cho, C.; Zhou, Y.; Maquet, E.; Gauquier, A.; Cabochette, P.; Fukuhara, S.; Mochizuki, N.; et al. Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/β-catenin pathway during brain angiogenesis. Elife 2015, 8, e06489.
61. Benz, F.; Wichitnaowarat, V.; Lehmann, M.; Germano, R.F.; Mihova, D.; Macas, J.; Adams, R.H.; Mark Taketo, M.; Plate, K.H.; Guérit, S.; et al. Low wnt/β-catenin signaling determines leaky vessels in the subfornical organ and affects water homeostasis in mice. Elife 2019, 8, e43818.
62. Cahoy, J.D.; Emery, B.; Kaushal, A.; Foo, L.C.; Zamanian, J.L.; Christopherson, K.S.; Xing, Y.; Lubischer, J.L.; Krieg, P.A.; Krupenko, S.A.; et al. A transcriptome database for astrocytes, neurons, and oligodendrocytes: A new resource for understanding brain development and function. J. Neurosci. 2008, 28, 264–278.
63. Shimizu, T.; Kagawa, T.; Wada, T.; Muroyama, Y.; Takada, S.; Ikenaka, K. Wnt signaling controls the timing of oligodendrocyte development in the spinal cord. Dev. Biol. 2005, 282, 397–410.
64. Soomro, S.H.; Jie, J.; Fu, H. Oligodendrocytes Development and Wnt Signaling Pathway. Int. J. Hum. Anat. 2018, 1, 17–35.
65. Langseth, A.J.; Munji, R.N.; Choe, Y.; Huynh, T.; Pozniak, C.D.; Pleasure, S.J. Wnts Influence the Timing and Efficiency of Oligodendrocyte Precursor Cell Generation in the Telencephalon. J. Neurosci. 2010, 30, 13367–13372.
66. Fancy, S.P.J.; Baranzini, S.E.; Zhao, C.; Yuk, D.I.; Irvine, K.A.; Kaing, S.; Sanai, N.; Franklin, R.J.M.; Rowitch, D.H. Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. Genes Dev. 2009, 23, 1571–1585.
67. Tawk, M.; Makoukji, J.; Belle, M.; Fonte, C.; Trousson, A.; Hawkins, T.; Li, H.; Ghandour, S.; Schumacher, M.; Massaad, C. Wnt/β-Catenin Signaling Is an Essential and Direct Driver of Myelin Gene Expression and Myelinogenesis. J. Neurosci. 2011, 31, 3729–3742.
68. Huang, N.; Chen, D.; Wu, X.; Chen, X.; Zhang, X.; Niu, J.; Shen, H.Y.; Xiao, L. Aspirin Promotes Oligodendroglial Differentiation Through Inhibition of Wnt Signaling Pathway. Mol. Neurobiol. 2016, 53, 3258–3266.
69. Lang, J.; Maeda, Y.; Bannerman, P.; Xu, J.; Horiuchi, M.; Pleasure, D.; Guo, F. Adenomatous Polyposis Coli Regulates Oligodendroglial Development. J. Neurosci. 2013, 33, 3113–3130.
70. Fu, H.; Kesari, S.; Cai, J. Tcf7l2 is tightly controlled during myelin formation. Cell. Mol. Neurobiol. 2012, 32, 345–352.
71. Lürbke, A.; Hagemeier, K.; Cui, Q.L.; Metz, I.; Brück, W.; Antel, J.; Kuhlmann, T. Limited TCF7L2 Expression in MS Lesions. PLoS ONE 2013, 8, e72822.

72. Ulanska-Poutanen, J.; Mieczkowski, J.; Zhao, C.; Konarzewska, K.; Kaza, B.; Pohl, H.B.F.; Bugajski, L.; Kaminska, B.; Franklin, R.J.M.; Zawadzka, M. Injury-induced perivascular niche supports alternative differentiation of adult rodent CNS progenitor cells. Elife 2018, 7, e30325.
73. Yamori, Y.; Horie, R. Developmental course of hypertension and regional cerebral blood flow in stroke-prone spontaneously hypertensive rats. Stroke 1977, 8, 456–461.
74. Lin, J.X.; Tomimoto, H.; Akiguchi, I.; Wakita, H.; Shibasaki, H.; Horie, R. White matter lesions and alteration of vascular cell composition in the brain of spontaneously hypertensive rats. Neuroreport 2001, 12, 1835–1839.
75. Bailey, E.L.; Smith, C.; Sudlow, C.L.M.; Wardlaw, J.M. Is the spontaneously hypertensive stroke prone rat a pertinent model of sub cortical ischemic stroke? A systematic review. Int. J. Stroke 2011, 6, 434–444.
76. Mayes, D.A.; Rizvi, T.A.; Titus-Mitchell, H.; Oberst, R.; Ciraolo, G.M.; Vorhees, C.V.; Robinson, A.P.; Miller, S.D.; Cancelas, J.A.; Stemmer-Rachamimov, A.O.; et al. Nf1 Loss and Ras Hyperactivation in Oligodendrocytes Induce NOS-Driven Defects in Myelin and Vasculature. Cell Rep. 2013, 4, 1197–1212.
77. Tong, X.K.; Trigiani, L.J.; Hamel, E. High cholesterol triggers white matter alterations and cognitive deficits in a mouse model of cerebrovascular disease: Benefits of simvastatin. Cell Death Dis. 2019, 10, 89.
78. Rajani, R.M.; Quick, S.; Ruigrok, S.R.; Graham, D.; Harris, S.E.; Verhaaren, B.F.J.; Fornage, M.; Seshadri, S.; Atanur, S.S.; Dominiczak, A.F.; et al. Reversal of endothelial dysfunction reduces white matter vulnerability in cerebral small vessel disease in rats. Sci. Transl. Med. 2018, 10, eaam9507.
79. Arai, K.; Lo, E.H. An Oligovascular Niche: Cerebral Endothelial Cells Promote the Survival and Proliferation of Oligodendrocyte Precursor Cells. J. Neurosci. 2009, 29, 4351–4355.
80. Seo, J.H.; Miyamoto, N.; Hayakawa, K.; Pham, L.D.D.; Maki, T.; Ayata, C.; Kim, K.W.; Lo, E.H.; Arai, K. Oligodendrocyte precursors induce early blood-brain barrier opening after white matter injury. J. Clin. Investig. 2013, 123, 782–786.
81. Seo, J.H.; Maki, T.; Maeda, M.; Miyamoto, N.; Liang, A.C.; Hayakawa, K.; Pham, L.D.D.; Suwa, F.; Taguchi, A.; Matsuyama, T.; et al. Oligodendrocyte precursor cells support blood-brain barrier integrity via TGF-β signaling. PLoS ONE 2014, 9, e103174.
82. Wang, L.; Geng, J.; Qu, M.; Yuan, F.; Wang, Y.; Pan, J.; Li, Y.; Ma, Y.; Zhou, P.; Zhang, Z.; et al. Oligodendrocyte precursor cells transplantation protects blood–brain barrier in a mouse model of brain ischemia via Wnt/β-catenin signaling. Cell Death Dis. 2020, 11, 9.
83. Miyamoto, N.; Pham, L.D.D.; Seo, J.H.; Kim, K.W.; Lo, E.H.; Arai, K. Crosstalk between cerebral endothelium and oligodendrocyte. Cell. Mol. Life Sci. 2014, 71, 1055–1066.
84. Shamseer, L.; Moher, D.; Clarke, M.; Ghersi, D.; Liberati, A.; Petticrew, M.; Shekelle, P.; Stewart, L.A.; Altman, D.G.; Booth, A.; et al. Preferred reporting items for systematic review and meta-analysis protocols (prisma-p) 2015: Elaboration and explanation. BMJ 2015, 349, g7647.
85. Tsai, H.H.; Niu, J.; Munji, R.; Davalos, D.; Chang, J.; Zhang, H.; Tien, A.C.; Kuo, C.J.; Chan, J.R.; Daneman, R.; et al. Oligodendrocyte precursors migrate along vasculature in the developing nervous system. Science 2016, 351, 379–384.
86. Niu, J.; Tsai, H.-H.; Hoi, K.K.; Huang, N.; Yu, G.; Kim, K.; Baranzini, S.E.; Xiao, L.; Chan, J.R.; Fancy, S.P.J. Aberrant oligodendroglial–vascular interactions disrupt the blood–brain barrier, triggering CNS inflammation. Nat. Neurosci. 2019, 22, 709–718.
87. Iijima, K.; Kurachi, M.; Shibasaki, K.; Naruse, M.; Puentes, S.; Imai, H.; Yoshimoto, Y.; Mikuni, M.; Ishizaki, Y. Transplanted microvascular endothelial cells promote oligodendrocyte precursor cell survival in ischemic demyelinating lesions. J. Neurochem. 2015, 135, 539–550.

88. Altun Effect of Statins on Endothelial Function in Patients with Acute Coronary Syndrome: A Prospective Study Using Adhesion Molecules and Flow-Mediated Dilatation. J. Clin. Med. Res. 2014, 6, 354.
89. Zhang, Y.; Pizzute, T.; Pei, M. A review of crosstalk between MAPK and Wnt signals and its impact on cartilage regeneration. Cell Tissue Res. 2014, 358, 633–649.
90. Bugiani, M.; Kevelam, S.H.; Bakels, H.S.; Waisfisz, Q.; Ceuterick-De Groote, C.; Niessen, H.W.M.; Abbink, T.E.M.; Lesnik Oberstein, S.A.M.J.; Van Der Knaap, M.S. Cathepsin A-related arteriopathy with strokes and leukoencephalopathy (CARASAL). Neurology 2016, 87, 1777–1786.
91. Zhang, Y.; Zhang, M.; Li, L.; Wei, B.; He, A.; Lu, L.; Li, X.; Zhang, L.; Xu, Z.; Sun, M. Methylation-reprogrammed Wnt/β-catenin signalling mediated prenatal hypoxia-induced brain injury in foetal and offspring rats. J. Cell. Mol. Med. 2018, 22, 3866–3874.
92. Li, C.; Zheng, X.; Han, Y.; Lv, Y.; Lan, F.; Zhao, J. XAV939 inhibits the proliferation and migration of lung adenocarcinoma A549 cells through the WNT pathway. Oncol. Lett. 2018, 15, 8973–8982.
93. Mecha, M.; Yanguas-Casás, N.; Feliú, A.; Mestre, L.; Carrillo-Salinas, F.J.; Riecken, K.; Gomez-Nicola, D.; Guaza, C. Involvement of Wnt7a in the role of M2c microglia in neural stem cell oligodendrogenesis. J. Neuroinflammation 2020, 17, 88.

Chapter 3

-

Wnt7a decreases brain endothelial barrier function via β-catenin activation

Narek Manukjan, Steven Chau, Florian Caiment, Marcel van Herwijnen, Hubert J. Smeets, Daniel Fulton, Zubair Ahmed, W. Matthijs Blankesteijn, Sébastien Foulquier

Submitted for publication

Abstract

The blood-brain barrier consists of tightly connected endothelial cells protecting the brain's microenvironment from the periphery. These endothelial cells are characterised by specific tight junction proteins such as Claudin-5 and Occludin, forming the endothelial barrier. Disrupting these cells might lead to blood-brain barrier dysfunction. The Wnt/β-catenin signalling pathway can regulate the expression of these tight junction proteins and subsequent barrier permeability. The aim of this study was to investigate the in vitro effects of Wnt7a mediated β-catenin signalling on endothelial barrier integrity. Mouse brain endothelial cells, bEnd.3, were treated with recombinant Wnt7a protein or XAV939, a selective inhibitor of Wnt/β-catenin mediated transcription to modulate the Wnt signalling pathway. The involvement of Wnt/HIF1α signalling was investigated by inhibiting Hif1α signalling with *Hif1a* siRNA. Wnt7a stimulation led to activation and nuclear translocation of β-catenin, which was inhibited by XAV939. Wnt7a stimulation decreased Claudin-5 expression mediated by β-catenin and decreased endothelial barrier formation. Wnt7a increased *Hif1a* and *Vegfa* expression mediated by β-catenin. However, *Hif1a* signalling pathway did not regulate tight junction proteins Claudin-5 and Occludin. Our data suggest that Wnt7a stimulation leads to a decrease in tight junction proteins mediated by the nuclear translocation of β-catenin, which hampers proper endothelial barrier formation. This process might be crucial in initiating endothelial cell proliferation and angiogenesis. Although HIF1α did not modulate the expression of tight junction proteins, it might play a role in brain angiogenesis and underlie pathogenic mechanisms in Wnt/HIF1α signalling in diseases such as cerebral small vessel disease.

Key words: beta-catenin; TEER; BBB; hypoxia; vascular dementia; cSVD

Introduction

The blood-brain barrier (BBB) constitutes a highly specialised vascular structure, which separates the blood circulation from the central nervous system (CNS) and functions to control the passage of molecules and ions to the brain in a protective manner. The BBB prevents the entry of harmful toxins, inflammatory cells, and pathogens, while still providing oxygen and nutrients necessary for the normal functioning of the brain [1]. An important feature of the BBB is the specific characteristics of the endothelial cells (ECs) that line the blood vessels in the CNS, such as tight junctions (TJs) and reduced pinocytosis [2]. Other cells, such as astrocytes, pericytes and oligodendrocytes, are also involved in the tight regulation of the brain microenvironment, forming the neurovascular unit (NVU) [3].

Brain ECs possess TJ proteins and maintain a homeostatic environment by tightly holding cells together and thus form a protective structural barrier [4]. These TJ proteins also link adjacent brain ECs together by forming homodimer transmembrane proteins, and normal function of TJ proteins ensures the correct regulation of intercellular communication and paracellular transport [4, 5]. Claudins, one class of TJ proteins, exhibit homophilic binding to other claudins, and heterophilic binding to other TJ-associated proteins to form multiprotein junctional complexes between adjacent cells [6]. Claudin-5 (Cldn5) is the most abundant isoform in the BBB and is crucial for the regulation of its properties [5–8]. Dysfunction of ECs can disrupt TJ proteins and BBB function, ultimately leading to neurodegenerative disorders such as Alzheimer's disease, multiple sclerosis, stroke, and cerebral small vessel disease (cSVD) [4, 8–10]. Dysregulation of secreted factors, such as Wingless-related integration site (Wnt), by cells of the NVU might play a key role in this EC dysfunction [11, 12]. A recent study demonstrated the contribution of Wnt/β-catenin

signalling to the regulation of the BBB permeability by affecting TJ proteins such as Cldn5 and occludin (Ocln) in adult mice [13].

The β-catenin mediated Wnt signalling pathway leads to the recruitment of the β-catenin destruction complex upon binding of Wnt molecules to receptor protein Frizzled (Fzd) 4 and co-receptor low density lipoprotein receptor-related protein (Lrp) 5 or 6. This leads to the intracellular stabilisation of β-catenin, resulting in its translocation to the nucleus. Once in the nucleus, β-catenin mediates the transcription of numerous genes involved in processes such as EC proliferation and differentiation, and TJ protein expression, implying an important role in the BBB [7, 14–16]. The Wnt/β-catenin signalling pathway is the most important pathway regulating the BBB in development, but seems to also play a role in adulthood [7, 17–20]. Thus, understanding the involvement of Wnt signalling in regulating TJ proteins in adult ECs might give insight into BBB pathology in diseases such as stroke and cSVD.

Of the 19 Wnt ligands, the regulation of BBB maturation is controlled by one of the most investigated Wnt ligands, namely Wnt7a. The interaction of Wnt7a with receptor Fzd4 and Lrp5/Lrp6 co-receptor controls brain angiogenesis and vessel formation by regulating endothelial tip cell formation [21]. In addition, Wnt7a mediated β-catenin activation regulates neural progenitor cell proliferation and differentiation [22]. Deletion of Wnt7a leads to major defects in CNS angiogenesis, while knocking out β-catenin resulted in similar angiogenic abnormalities [17]. Treatment with Wnt7a protein increases the expression of *Cldn5* in an immortalised brain endothelioma cell line (bEnd.3), leading to increased trans-endothelial electrical resistance (TEER) and decreased permeability of the endothelial monolayer following β-catenin activation [23–25]. The expression of CLDN5 was increased in both passage 1 and 3 ECs derived from human pluripotent stem cell (hPSC) in response

to β-catenin activation, but the effects were less prominent in the later passage cells [25]. In passage 4 cells, β-catenin activation did not increase the number of ECs or CLDN5 levels, indicating a stage-dependent response to Wnt7a stimulation [25]. On the contrary, activation and nuclear accumulation of β-catenin resulted in the inhibition of *Cldn5* expression and promotion of angiogenesis [26, 27]. Thus, the molecular effects of Wnt7a mediated β-catenin activation on mature ECs and BBB integrity remains unclear.

The aim of this study was to investigate the underlying pathways determining in vitro effects of Wnt7a mediated β-catenin signalling on mature brain endothelial barrier integrity. The impact of Wnt7a stimulation on TJ protein expression mediated by β-catenin activation was investigated in bEnd.3 mouse brain ECs along with TEER measurements to investigate the effects on barrier function. Additionally, analysis of the transcriptomic signature was performed to identify modulators of this signalling pathway. We hypothesised that Wnt7a activates β-catenin-mediated Wnt signalling leading to regulation of TJ protein expression, affecting the barrier function of ECs. A clear understanding of this pathway might present a potential therapeutic target in repairing BBB damage in disease.

Materials and Methods

Cell culture

The immortalised murine brain ECs, bEnd.3 cells (Cat no. CRL-2299, American Type Culture Collection, Manassas, VA, USA [28]) were cultured in Dulbecco's Modified Eagle's Medium high glucose (DMEM, Cat no. D6429, Merck Millipore, Burlington, MA, USA) supplemented with 10% foetal bovine serum (FBS) and 100 µg/mL penicillin/streptomycin (P/S) at 37°C and in an atmosphere of 5% CO_2. BEnd.3 cells between passage 26 and 28 were seeded onto multi-well plates, membrane inserts, or glass coverslips at a density of

$1.5x10^4$ to $1.0x10^5$ cells/cm^2 for expression and functional studies and treated at confluency (1-3 days after seeding).

Cells were treated with recombinant Wnt7a (Cat no. SRP3296, Sigma-Aldrich, St. Louis, MO, USA), dissolved in PBS containing 0.1% bovine serum albumin (BSA-PBS) to study the effects of paracrine mediated Wnt signalling on ECs. Briefly, cells were grown to confluency and treated with control (0.1% BSA-PBS) or Wnt7a (50 or 100 ng/ml in 0.1% BSA-PBS) for 24h [29–31]. Wnt7a activation of β-catenin was investigated by co-administration with XAV939 (XAV, Cat no. X3004, Sigma-Aldrich), a Tankyrase inhibitor known to selectively inhibit Wnt/β-catenin mediated transcription [32, 33]. Briefly, cells were grown to confluency and treated with control, Wnt7a (100 ng/ml), XAV (10 μM) or a co-administrated of Wnt7a with XAV (100 ng/ml and 10 μM, respectively) for 24h.

Hypoxia-inducible factor 1α (Hif1a) activation mediated by Wnt7a was investigated by silencing the *Hif1a* gene with siRNA. Briefly, cells were grown to confluency and transfected with 4 μg/ml Lipofectamine™ 2000 Transfection Reagent (Cat no. 11668, Invitrogen, Waltham, MA, USA) and 20 μM siRNA in DMEM high glucose containing 10% FBS and 10% Opti-MEM (Cat no. 31985070, ThermoFisher Scientific, Waltham, MA, USA) for 24h. Negative control siRNA (Silencer™ Cy™3-labeled Negative Control No. 1 siRNA, Cat no. AM4621, Invitrogen), or a validated *Hif1a* siRNA (Silencer™ Select Pre-Designed mouse *Hif1a* siRNA, sequence 5'→3': Sense CCUUUACCUUCAUCGGGAAAtt; Antisense UUUCCGAUGAAGGUAAAGGag, Cat no. 4390771, Invitrogen) were used. After 24h, cells were washed with PBS and treated with either control or Wnt7a (100 ng/ml) for 24h. Cells were washed and fixed with 4% paraformaldehyde (PFA) for immunocytochemistry or lysed for RNA isolation, after

respective treatment. Immunocytochemistry samples were kept at 4°C and RNA samples at -20°C until further use.

Immunocytochemistry

Immunocytochemistry was performed after seeding bEnd.3 cells at a density of 1.5-5.0x10^4 cells/cm^2 on glass coverslips or on a 96-well plate. Cells were washed in PBS and fixed with 4% PFA for 10 min. at room temperature. Cells were then blocked in PBS blocking buffer containing 1% normal donkey serum and 0.3% Triton X-100 for 1h. Then, cells were incubated with appropriate primary antibody (Supplementary Table 1) overnight at 4°C followed by incubation with appropriate secondary antibodies in blocking buffer (Supplementary Table 1) for 2h. Lastly, a counterstaining of the nuclei with NucBlue (Cat no. R37605, Invitrogen) was performed and cells were mounted using antifading Mounting Medium (Prolong gold, Agilent Technologies, Santa Clara, CA, USA) before imaging.

Image acquisition and analysis

Images were captured by an investigator masked to the treatment conditions using a confocal microscope (DMI 4000, Leica, Freiburg, Germany) or a fluorescent live cell imager (ImageXpress Pico Automated Cell Imaging System, Molecular Devices, San Jose, CA, USA). For confocal imaging, six image volumes (175 x 175 µm) were acquired with a 1 µm step size at a magnification of 63x. Images captured with the fluorescent live cell imager (690 x 690 µm) were acquired at a magnification of 40x. Subsequently, image stacks underwent maximal intensity projections and mean grey values and integrated density values of the resulting images were obtained using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Three fields of view were acquired from every replicate to compare

the different treatment conditions. In each field of view, the overall mean grey value and integrated density values were normalised to the number of cells. Additionally, for active β-catenin the average nuclear, cytoplasmic, and membrane concentrations were measured by averaging values of the mean intensity of three randomly selected cells per field of view. All quantifications were performed in ImageJ and expressed as intensity levels corrected versus control.

Quantitative PCR (qPCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the TRIzol method and stored at -80°C before use. Quality and quantity were checked using NanoDrop 1000 spectrophotometer and the RNA was reverse transcribed into cDNA using the high-capacity RNA-to-cDNA kit (Cat no. 1708891, Bio-rad laboratories, Inc., Hercules, CA, USA) according to manufacturer's manual. CDNA samples were stored at -20°C before use. QPCR was performed using Sensimix^{TM} SYBER® & Fluorescein kit (Cat no. QT615-05, Meridian Bioscience Inc., Cincinnati, OH, USA) on the Light Cycler 480 (Roche Applied Science, Penzberg, Germany) with the following qPCR program: 10 min. at 95°C followed by 55 cycles a 10s at 95°C and 20s at 60°C. Temperature was increased from 60 to 95°C for melting curve analyses. Primers were designed to cover exon-exon junctions and all possible splice variants using NCBI Primer-BLAST tool. Primers were synthesised by Eurofins Genomics (Ebersberg, Germany) and quality was ensured by testing on cell cultures, as well as by calculation of primer efficiency. At least two stable reference housekeeping genes were selected from a selection of three genes by using the GeNorm Software (Primerdesign, Southampton, NY, USA). Primers are listed in Supplementary Table 2. Gene expression

analysis was performed using LinReg PCR (Ver. 2014.0) and the Light Cycler 480 data converter (Ver. 2014.1) and shown as fold change (FC) compared to control.

RNA sequencing

Total isolated RNA quantity was checked using Qubit 2.0 Fluorometer (Invitrogen) and RNA quality was assessed using Bioanalyzer (Cat no. RNA 6000 Nano kit; 2100 Bioanalyzer, Agilent Technologies). Purification of mRNA from total RNA (NEXTFLEX Poly(A) Beads 2.0, Cat no. NOVA-512992, PerkinElmer, Waltham, MA, USA) and directional, strand-specific RNA library preparation (NEXTFLEX Rapid Directional RNA-Seq Kit 2.0, Cat no. NOVA-5198, PerkinElmer) was performed according to manufacturer's protocol. Sequencing was performed using NovaSeq 6000 Sequencing system (NovaSeq S Prime flow cell 200 cycles; NovaSeq 6000, Illumina, Inc, San Diego, CA, USA) according to manufacturer's protocol. The raw sequencing data was trimmed using *fastp*, remaining reads were then mapped against the Ensembl mouse genome (release 100) using STAR (version 2.7.3a) and quantified using RSEM (v.1.3.1). The resulting raw read counts were processed using the R package DESeq2. Genes with were not sequenced (0 reads) in more than 75% of the samples of any given condition were removed. Genes were considered differentially expressed with an adjusted p-value (false discovery rate; FDR) below 0.01. Gene ontology (GO) enrichment analysis was performed using g:Profiler [34] and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 [35, 36]. The modified Fisher exact p-value (EASE score) <0.05 and FDR <0.05 were considered enriched. Gene interaction network analysis was performed using Cytoscape version 3.9.1 [37].

Trans endothelial electrical resistance (TEER) assay

TEER was monitored every 2-3 days using an epithelial volt ohm meter (EVOM-2, WPI, UK) connected to an electrode (STX4 EVOM, Cat no. EVM-EL-03-03-01, WPI). Experiments were performed using PET membrane inserts (Cat no. 353095, Falcon) with an insert diameter of 6.4 mm and pore diameter of 0.4 μm in combination with a 24-well companion plate (Cat no. 353504, Falcon). Inserts were coated with rat tail collagen I (100 μg/ml, Cat no. 3440-005-01, R&D Systems, Minneapolis, MN, USA) for 1h at 37°C. Before seeding cells, resistance measurements were performed on all coated wells to determine the blank resistance. Measurements were performed on a heating surface to maintain a stable temperature of 37°C during measurements. BEnd.3 cells were plated on the membrane inserts at a density of $4.5x10^4$ cells/cm^2 and left to attach and reach full confluency for 3 days. Treatment with either control or 100 ng/ml Wnt7a was initiated at day 3 and refreshed every 2-3 days before measurements. The TEER was calculated using the following formula:

$$TEER\,(\Omega * cm^2) = (R_{total} - R_{blank}) * Insert\ membrane\ area$$

Statistical analysis

Data was analysed using GraphPad Prism 9 (Dotmatics). Data distribution was tested using Shapiro-Wilk test for normality. Unpaired Student t-tests were used to compare Wnt7a vs control. One-way ANOVA or Kruskal-Wallis test (for non-parametric data) with post-hoc Tukey's multiple comparisons test was used to assess multiple comparisons. $P<0.05$ was considered statistically significant and data are expressed as mean±SEM.

Results

Wnt7a leads to increased levels of nuclear active β-catenin

To investigate whether Wnt7a stimulation causes β-catenin mediated signalling in a mouse brain EC line, we performed immunocytochemistry on bEnd.3 cells treated with recombinant Wnt7a protein for 24h (Figure 1A). The overall signal intensity of the active form of β-catenin was quantified in addition to the signal intensity in the membrane, cytoplasm, and nucleus. A significant increase in overall active β-catenin intensity was observed when cells were treated with 100 ng/ml Wnt7a compared to control (1.46±0.17 FC, p=0.025). Similar increases in active β-catenin were observed in the membrane (1.24±0.05 FC, p=0.036), cytoplasm (1.48±0.11 FC, p=0.001), and nucleus (1.52±0.06 FC, p=0.002). Treatment with 50 ng/ml Wnt7a, however, did not lead to a significant increase in overall active β-catenin intensity (1.24±0.04 FC, p=0.305), as well as in membrane (1.12±0.06 FC, p=0.370), cytoplasmic (1.23±0.07, p=0.116), or nuclear intensity (1.40±0.15 increase, p=0.092) compared to control. These results indicate a dose dependent effect of Wnt7a protein on β-catenin activity.

Next, we investigated whether XAV, a known Tankyrase inhibitor, was able to inhibit the Wnt7a-mediated increase in active β-catenin. XAV inhibits the PARylation of Axin, which is one of the proteins in the β-catenin destruction complex. Tankyrase mediated PARylation of Axin leads to the ubiquitination and degradation of this protein. Thus, by inhibiting Tankyrase, the β-catenin destruction complex can assemble and lead to the ubiquitination and degradation of β-catenin [38].

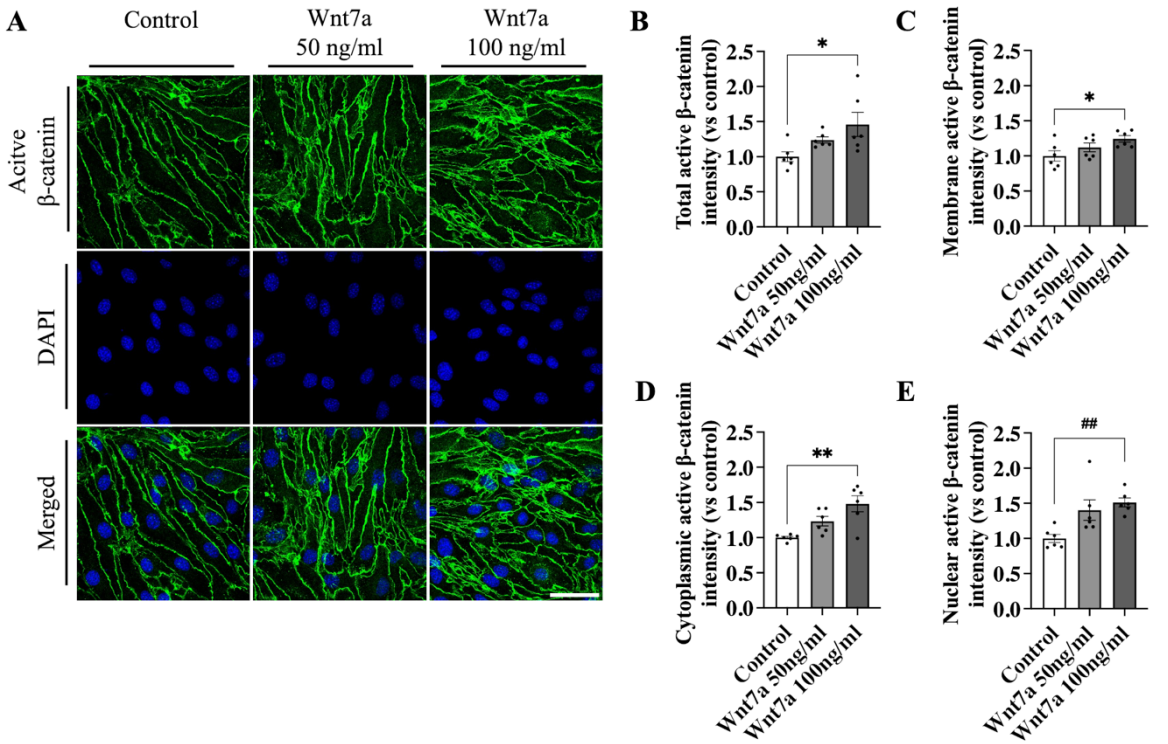


Figure 1: Wnt7a led to increase in active β-catenin levels in bEnd.3 cells in vitro. (A) Immunocytochemistry of active β-catenin after treatment with either 50 or 100 ng/ml recombinant Wnt7a. Scale bar, 50 µm. **(B)** Quantification of relative active β-catenin intensity revealed an increase in total active β-catenin in bEnd.3 cells when treated with 100 ng/ml Wnt7a. **(C)** Membrane bound active β-catenin was increased in cells treated with 100 ng/ml Wnt7a and in the **(D)** cytoplasm and **(E)** nucleus. Treatment with 50 ng/ml did not lead to significant changes in active β-catenin. Graph represents mean±SEM; n=6; Data points (dots) indicate technical replicates; *p<0.05, **p<0.01, One-way ANOVA with post-hoc Tukey's multiple comparisons; ##p<0.01, Kruskal-Wallis with post-hoc Dunn's multiple comparisons. Statistically non-significant comparisons are not shown.

Treatment with 100 ng/ml Wnt7a did not lead to a significant increase in the transcription of *Axin2* (0.98±0.29 FC, p>0.99; Figure 2A), a direct target of the Tcf/LEF factor mediated Wnt pathway [39]. Inhibition of the active β-catenin with XAV also did not lead to any significant changes in *Axin2* mRNA expression (1.66±0.33 FC, p=0.26; Figure 2A). However, overall β-catenin activity, assessed by immunocytochemistry (Figure 2B), showed a significant increase in active β-catenin in cells treated with 100 ng/ml Wnt7a compared to controls (1.20±0.02 FC, p<0.0001; Figure 2C). The increase in active β-catenin was not observed when cells were treated with XAV (0.90±0.03 FC compared to control, p=0.07; Figure 2C) or when cells were co-treated with Wnt7a and XAV (1.02±0.03 FC compared to

control, p=0.96; Figure 2C). Total active β-catenin levels in Wnt7a treated cells were significantly increased compared to co-treatment with Wnt7a and XAV (p=0.0002; Figure 2C), indicating the ability of XAV to inhibit the Wnt7a mediated activation of β-catenin.

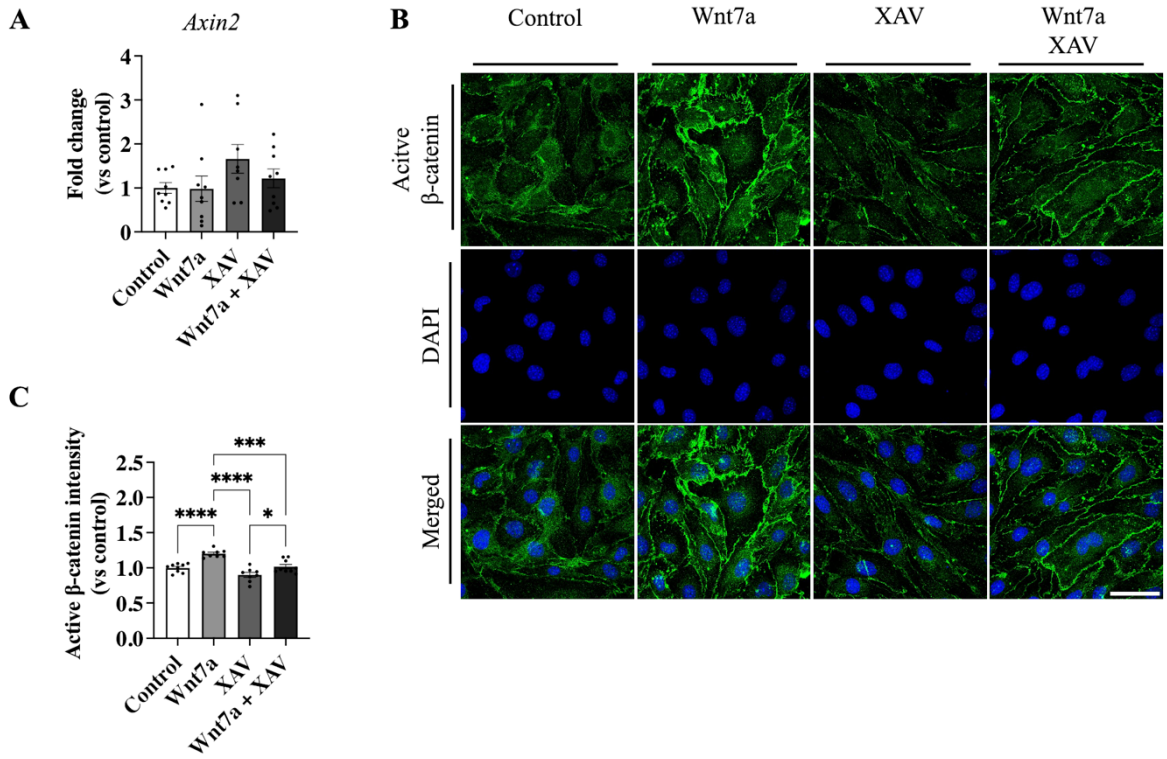


Figure 2: **XAV inhibited the activation of β-catenin in response to Wnt7a treatment. (A)** In vitro Wnt7a treatment did not lead to any changes in the β-catenin target gene *Axin2* mRNA expression **(B)** Immunocytochemistry of active β-catenin in bEnd.3 cells treated with either control, 100 ng/ml Wnt7a, 10 µM XAV, or co-treatment with Wnt7a and XAV. Scale bar, 50 µm. **(C)** The overall total β-catenin activation mediated by Wnt7a was significantly inhibited by XAV. XAV led to decreased active β-catenin levels compared to control. Graph represents mean±SEM; n=8-9; Data points (dots) indicate technical replicates; *p<0.05, ***p<0.001, ****p<0.0001; One-way ANOVA with post-hoc Tukey's multiple comparisons. Statistically non-significant comparisons are not shown.

Wnt7a stimulation decreases Claudin-5 expression via β-catenin mediated signalling and reduces endothelial barrier function

The effects of Wnt7a signalling on the TJ proteins *Cldn5* and *Ocln* mRNA expression and protein levels mediated by β-catenin activation were investigated with qPCR and immunocytochemistry, respectively (Figure 3). Wnt7a stimulation led to a significant

decrease in both *Cldn5* (p=0.0002; Figure 3A) and *Ocln* (p=0.007; Figure 3B) mRNA expression levels compared to cells in which β-catenin was inhibited with XAV. Similar results were observed for protein levels of Cldn5, which was significantly decreased by Wnt7a stimulation compared to control (0.80±0.04 FC, p=0.022; Figure 3D). No difference in protein levels of Cldn5 was observed when Wnt7a and XAV were co-administered compared to control (0.92±0.05 FC, p=0.730; Figure 3D). Despite reducing *Ocln* mRNA levels, Wnt7a treatment did not lead to changes in Ocln protein levels compared to control (1.10±0.06 FC, p=0.483; Figure 3E). However, treatment with XAV, and co-treatment with Wnt7a and XAV, significantly increased protein levels of Ocln compared to control (1.21±0.05 FC and 1.27±0.06 FC, p=0.031 and p=0.005, respectively; Figure 3E). These results indicate that β-catenin mediated the effects of Wnt7a stimulation on TJ proteins, which are responsible for EC barrier function.

The EC barrier integrity was studied by measuring the electrical resistance of the endothelial monolayer in culture by TEER. The establishment of the BBB is characterised by the endothelial barrier formation comprised of TJ proteins tightly connecting the ECs, resulting in an increased electrical resistance over time [40]. No overall differences were observed in electrical resistance between cultures treated with Wnt7a protein and control over a period of 14 days (Figure 4A). However, a significant difference in change in resistance from day 3 to day 5 was observed, with Wnt7a treatment leading to a reduced increase in resistance change compared to control (10.17±0.39 vs 16.58±1.15 Ω, p<0.0001; Figure 4B). These results suggest that Wnt7a stimulation inhibits endothelial barrier formation.

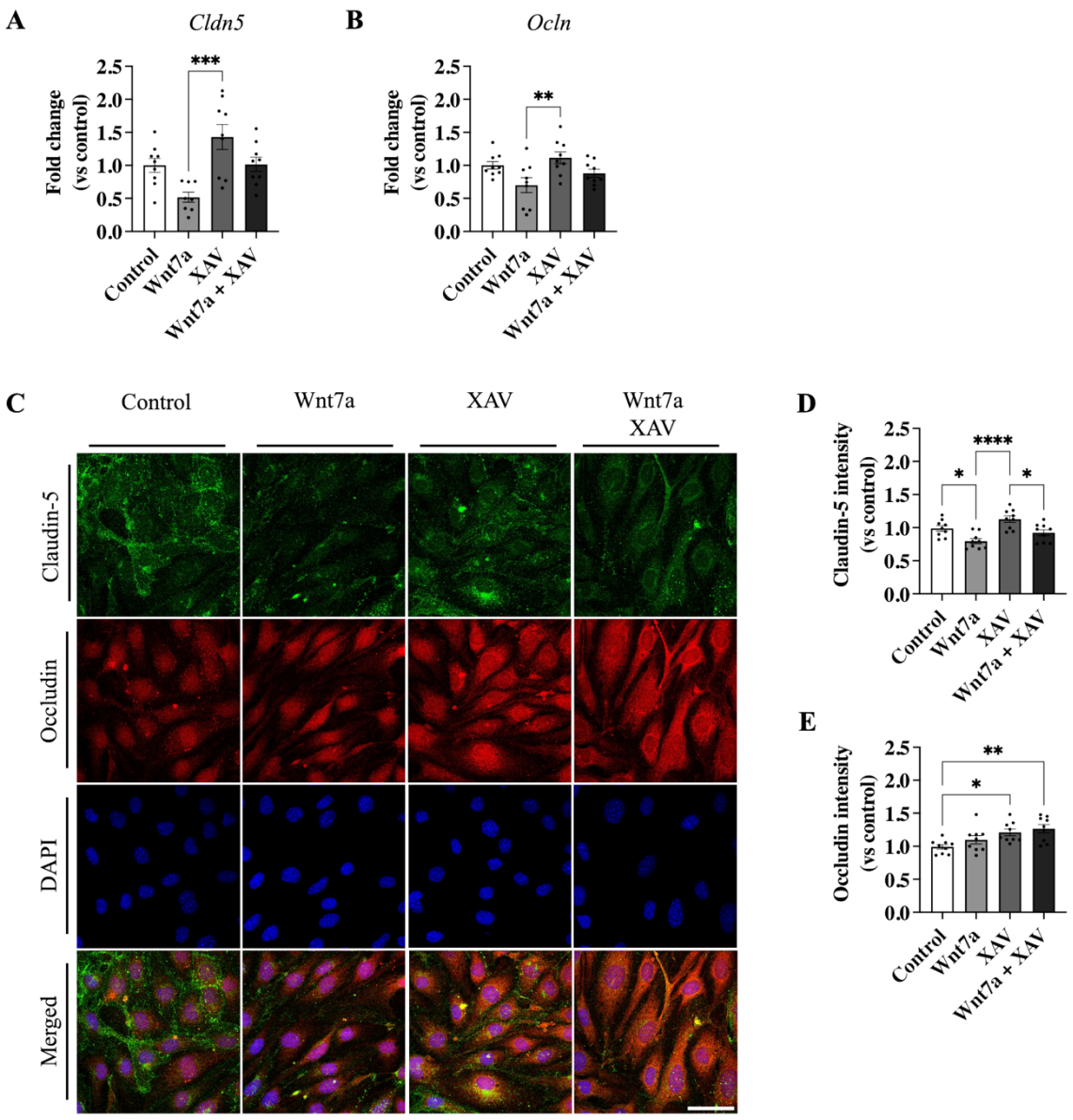


Figure 3: **Modulation of β-catenin led to changes in TJ proteins Claudin-5 and Occludin. (A)** In vitro activation of β-catenin mediated by Wnt7a led to downregulation of *claudin-5* and **(B)** *occludin* mRNA. **(C)** Immunocytochemistry of Claudin-5 or Occludin in bEnd.3 cells treated with either control, 100 ng/ml Wnt7a, XAV, or co-treatment with Wnt7a and XAV. Scale bar, 50 µm. **(D)** Decreased levels of Claudin-5 protein were mediated by wnt7a activation of β-catenin. (E) inhibition β-catenin levels by XAV led to a significant increase in Occludin compared to control. However, Wnt7a did not decrease levels of Occludin. Abbreviations: Cldn5 = claudin-5; Ocln = Occludin. Graph represents mean±SEM; n=8-9, Data points (dots) indicate technical replicates; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA with post-hoc Tukey's multiple comparisons. Statistically non-significant comparisons are not shown.

Hif1a activation in response to β-catenin mediated Wnt7a signalling is not involved in the decrease of tight junction proteins Claudin-5 and Occludin

To decipher the molecular mechanisms involved in the response of brain ECs to Wnt7a stimulation, RNA sequencing was performed to assess their transcriptomic regulation. This revealed 2,107 differently expressed genes (DEG) when comparing Wnt7a to control treatment (Figure 5A). GO enrichment analysis of the top 100 DEG revealed enriched pathways such as vasculature development (6.2-fold enriched), blood vessel development (6.3-fold enriched) and angiogenesis (6.8-fold enriched, Figure 5B). Pathway analysis of the genes mediated by β-catenin in the angiogenesis pathway showed upregulation of *Hif1a* and vascular endothelial growth factor A (*Vegfa)* (0.28 and 0.26 log2FC, respectively), genes typically involved in response to hypoxia (Figure 5C) [41]. Quantification of mRNA levels in cells treated with Wnt7a showed an increase in both *Hif1a* and *Vegfa* compared to control (1.76±0.20 and 1.42±0.07 FC, p=0.010 and p=0.047; Figure 5D and 5E respectively).

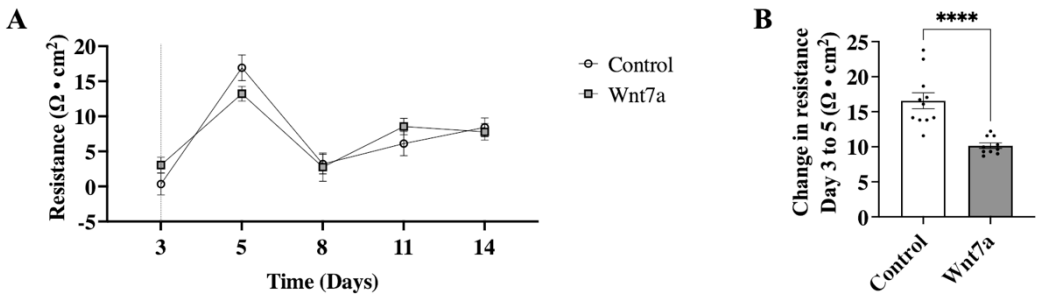


Figure 4: **Wnt7a led to a reduction in formation of the endothelial barrier. (A)** Trans endothelial electrical resistance (TEER) measurements of the bEnd.3 monolayer in vitro. No differences in electrical resistance were observed after 14 days of Wnt7a treatment compared to control. Dotted vertical line indicates start of Wnt7a treatment. **(B)** Wnt7a blunted the increase in the electrical resistance change between day 3 to day 5 compared to control. Graph represents mean±SEM; n=10-11, Data points (dots) indicate technical replicates; ****p<0.0001; Unpaired students t-test. Statistically non-significant comparisons are not shown.

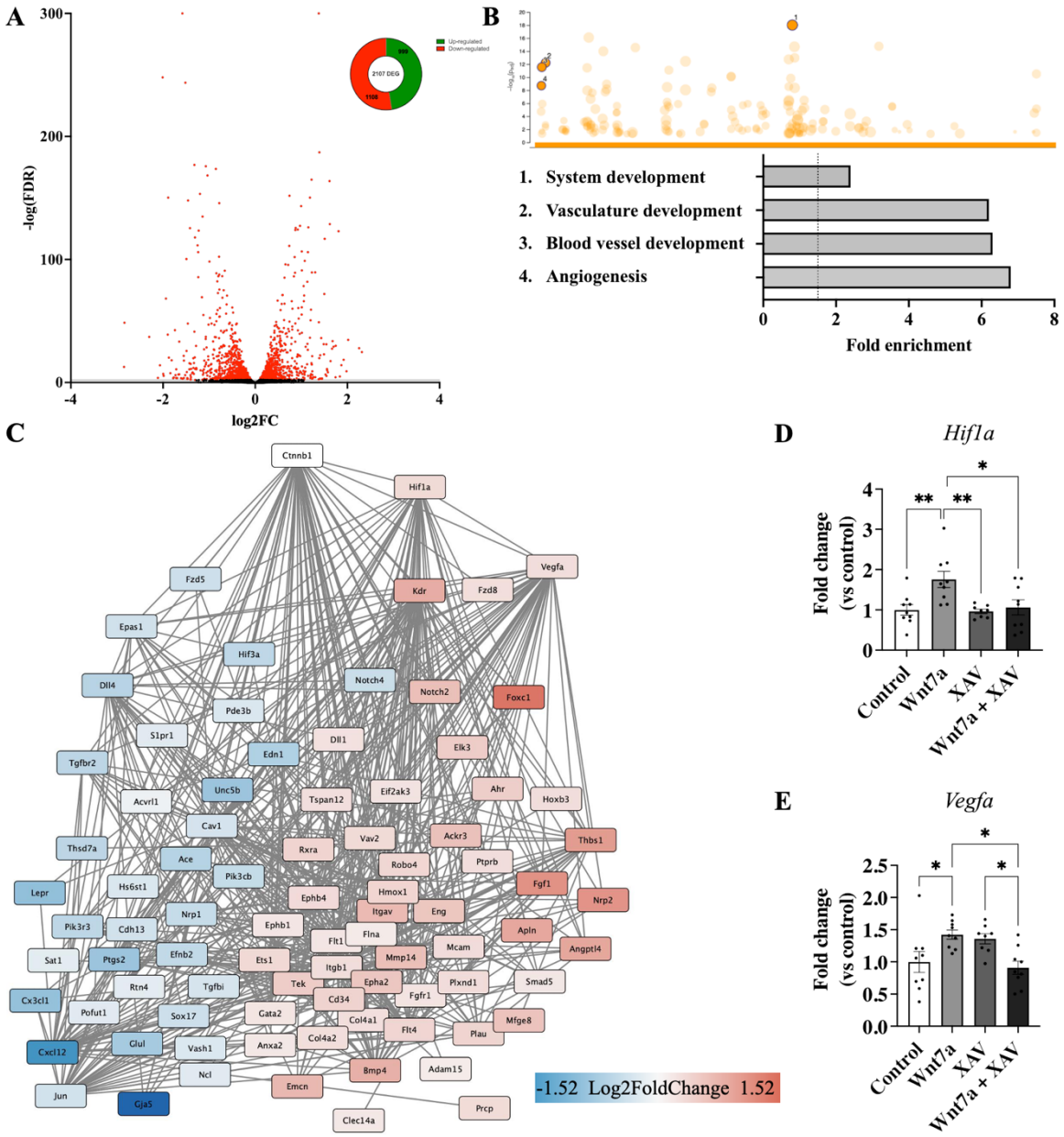


Figure 5: **Wnt7a led to regulation of 2,017 differentially expressed genes involved in e.g. angiogenesis. (A)** Volcano plot showing 2,017 differentially expressed genes (DEG) in bEnd.3 cells treated with Wnt7a for 24h (n=3) **(B)** Gene Ontology (GO) enrichment analysis revealed the enrichment of biological processes such as vascular development, blood vessel development, and angiogenesis. **(C)** Analysis of Wnt7a regulated genes mediated by β-catenin in the angiogenesis pathway revealed the upregulation of *Hif1a and Vegfa*. **(D)** The upregulation of *Hif1a* and **(E)** *Vegfa* by Wnt7a was mediated by β-catenin (n=8-9). Graph represents mean±SEM; Data points (dots) indicate technical replicates; *p<0.05, **p<0.01; One-way ANOVA with post-hoc Tukey's multiple comparisons. Statistically non-significant comparisons are not shown.

The contribution of *Hif1a* activation in regulating TJ proteins was investigated by silencing the *Hif1a* gene with a validated commercially available siRNA. *Hif1a* was significantly downregulated when treated with Wnt7a and *Hif1a* siRNA compared to Wnt7a and negative control siRNA (0.14±0.02 FC, p<0.0001; Figure 6A). No differences were found in *Vegfa* expression when treated with either Wnt7a or co-treated with Wnt7a and *Hif1a* siRNA (Figure 6B). The expression of *Axin2* was significantly downregulated by Wnt7a (0.42±0.08 FC, *p*=0.019; Figure 6C), with no difference compared to when treated with Wnt7a and *Hif1a* siRNA (*p*=0.42). *Cldn5* expression was significantly downregulated by Wnt7a only when inhibiting *Hif1a* mRNA (0.70±0.08 FC, *p*=0.01; Figure 6D), while *Ocln* expression was downregulated by Wnt7a treatment alone (0.59±0.02 FC, *p*=0.009; Figure 6E).

When *Hif1a* was silenced in cells treated with Wnt7a, *Ocln* mRNA levels normalised compared to control (0.86±0.12 FC, *p*=0.48; Figure 6E), suggesting that HIF1α signalling might play a role in regulating the effects of Wnt7a on endothelial barrier function. Protein levels of Cldn5 and Ocln were investigated after *Hif1a* knockdown by immunocytochemistry (Figure 6F). Consistent with our previous data (Figure 3D), treatment with Wnt7a reduced levels of Cldn5 protein compared to control (0.73±0.06 FC, *p*=0.002; Figure 6G). However, no differences in Cldn5 levels were observed between Wnt7a treated cells treated with either negative control or *Hif1a* siRNA (*p*=0.68). Similar effects on Ocln levels were observed, with Wnt7a treatment inducing a reduction compared to control (0.71±0.05 FC, *p*=0.0003; Figure 6H), and with no differences observed between the negative control and *Hif1a* siRNA treated cells treated with Wnt7a (*p*=0.59). These results show that HIF1α signalling does not directly interact with Wnt7a signalling in regulating the expression of TJ proteins Cldn5 and Ocln but might modulate gene expression (Figure 6D-E).

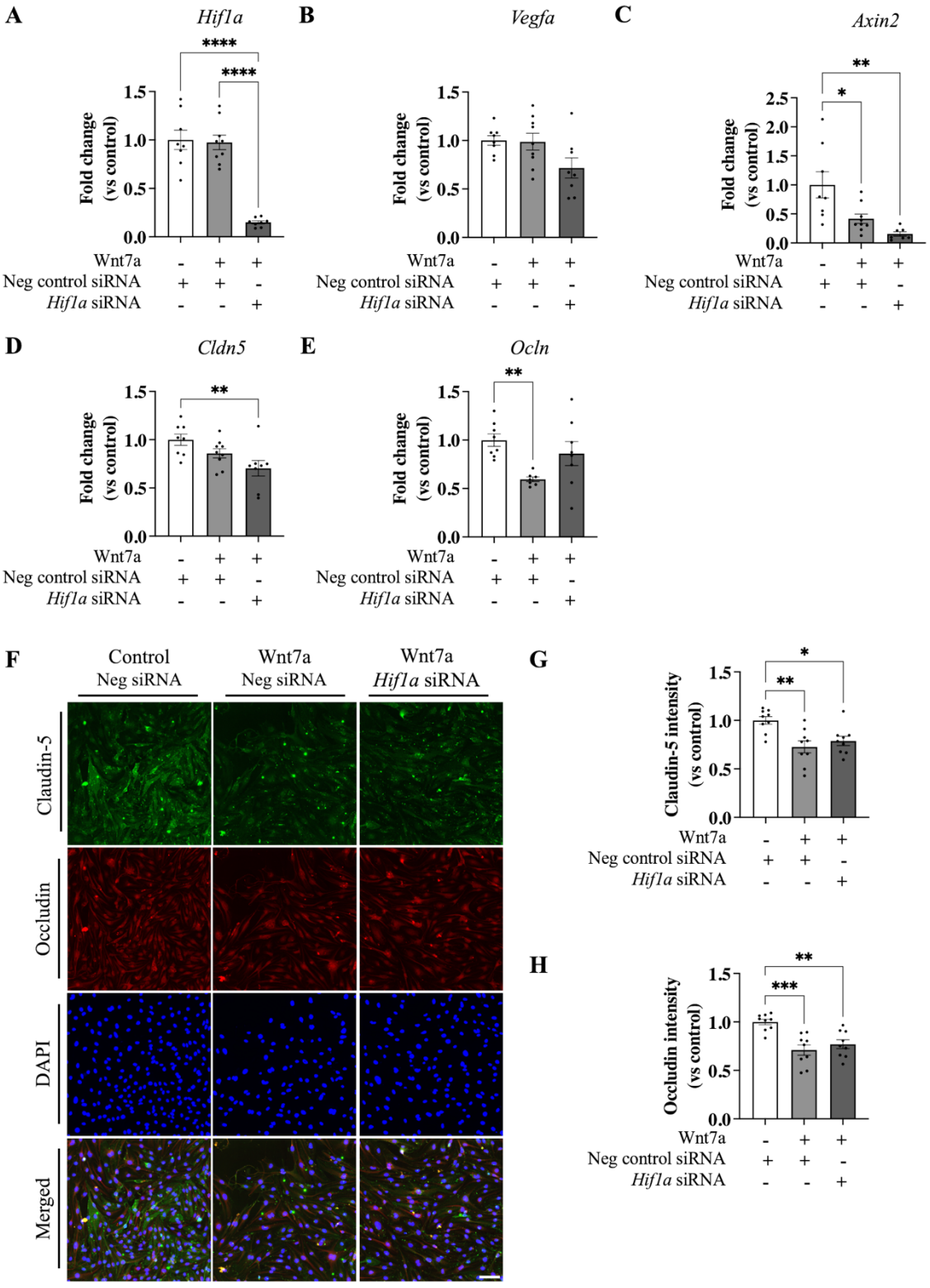


Figure 6: **Wnt7a regulation of tight junction proteins Claudin-5 and Occludin was not mediated by *Hif1a*. (A)** Transfection with *Hif1a* siRNA led to the downregulation of *Hif1a*. **(B)** Wnt7a did not change *Vegfa* mRNA expression, with no effect of *Hif1a.* **(C)** Wnt7a led to the significant downregulation of the β-catenin target gene *Axin2*, which was not mediated by *Hif1a*. **(D)** *Claudin-5* was downregulated by Wnt7a only when *Hif1a* was silenced, **(E)** while *Occludin* was normalised by silencing *Hif1a* in the present of Wnt7a. **(F)** Immunocytochemistry of Claudin-5 or Occludin in bEnd.3 cells treated with either

control and negative control siRNA, 100 ng/ml Wnt7a and negative control siRNA, or 100 ng/ml Wnt7a and *Hif1a* siRNA. Scale bar, 100 µm. **(G)** Decreased Claudin-5 protein level by Wnt7a was not mediated by *Hif1a*. **(H)** Similar results were observed for Occludin. Abbreviations: Neg = negative; Cldn5 = claudin-5; Ocln = Occludin. Graph represents mean±SEM; n=7-9, Data points (dots) indicate technical replicates; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; One-way ANOVA with post-hoc Tukey's multiple comparisons. Statistically non-significant comparisons are not shown.

Discussion

The aim of this study was to investigate the effects of the Wnt7a/β-catenin signalling pathway on EC barrier function. We showed that 100 ng/ml Wnt7a increased protein levels of active β-catenin in the cytoplasm, membrane, and nucleus of the bEnd.3 EC line. This increase was reversed when degradation of the destruction complex was prevented by the Tankyrase inhibitor XAV, validating Wnt7a mediated activation of β-catenin signalling. Of note, increased β-catenin activation did not translate to an increase in expression of its target gene *Axin2*, implying that other factors might influence the transcription of the downstream β-catenin target genes, in response to Wnt7a stimulation. This Wnt7a/β-catenin signalling had functional significance since Wnt7a stimulation impaired EC barrier formation and reduced expression of the TJ protein, Cldn5. Endothelial genes commonly activated in response to hypoxia, *Hif1a* and *Vegfa*, were upregulated by Wnt7a activation of β-catenin. Interestingly, other studies have described the expression of Wnt7a following hypoxic conditions, indicating that the interplay of these signalling molecules might contribute to hypoxia-induced events, during pathological conditions such as stroke or cSVD [42]. However, decreases in TJ proteins in Cldn5 and Ocln were not mediated by *Hif1a* in our study. Overall, our analysis of TJ protein expression and EC barrier function suggest the involvement of Wnt7a in increasing the permeability of ECs, an effect that could have consequences for the endothelial barrier function.

Activation of β-catenin signalling by Wnt7a has been shown to depend on the cell type and developmental stage within the CNS. Activation of Wnt7a in the developing hippocampus triggers β-catenin mediated signalling, leading to positive influences on synaptogenesis [43]. Here, β-catenin mediated signalling by Wnt7a is enhanced by the presence of receptor co-factors Gpr124 and Reck [21]. However, β-catenin is not involved in all aspects of Wnt7a function at the synapse. For example, localisation of Wnt7a in mouse cerebellar synapses increased the size and spreading of axonal growth cones and was essential for neurotransmitter release via actions on the cytoskeleton that do not involve β-catenin [44]. These findings suggest that Wnt7a might activate both β-catenin dependent and independent signalling, or one or the other, depending on environmental factors. Based on our findings that Wnt7a increases levels of active β-catenin, the ability of XAV to reverse Wnt7a effects, and evidence from previous work, we suggest that the effects of Wnt7a on brain ECs are predominantly mediated via β-catenin signalling. However, β-catenin independent signalling effects might also play a role [21, 43, 45]. In addition to confirming the role of the Wnt7a/β-catenin pathway, our findings highlight XAV as a potent molecule to modulate Wnt7a induced β-catenin signalling in bEnd.3 brain ECs.

In our study, Wnt7a activation of β-catenin in vitro led to a decrease in the TJ proteins Cldn5 and Ocln in mouse brain ECs (bEnd.3 cells). However, previous studies have shown β-catenin mediated increases in TJ proteins. Stabilisation of β-catenin via glycogen synthase kinase 3β (GSK-3β) inhibitor CHIR99021 in hPSC, or LiCl in an immortalised human brain microvascular EC line (hCMEC/D3), led to the upregulation of Cldn5 [13, 23]. Similarly, in vivo deletion of β-catenin in the brain endothelium led to decreased protein levels of both Cldn5 and Ocln in the cerebral cortex of mice [25]. Wnt7a derived from oligodendrocyte precursor cell (OPC) conditioned medium showed increased β-catenin and *Cldn5* expression

in bEnd.3 cells, while siRNA knock down of Wnt7a in OPCs blocked these effects [46]. Conversely, stabilisation of β-catenin with LiCl in ECs derived from murine embryonic stem cells showed a significant reduction in *Cldn5* mRNA and protein levels [27]. Here, translocation of β-catenin to the nucleus led to its binding to Foxo1, forming a Foxo1–β-catenin–Tcf complex at the *Cldn5* gene promotor site that inhibited its expression [27]. A correlation was observed between increased β-catenin and decreased *CLDN5* expression in both patient glioma tissue and malignant glioma cells lines [47]. The expression of β-catenin gradually increased in higher glioma tumour grades, while the expression of TJ proteins CLDN1 and CLDN5 were both decreased [47].

There is some discrepancy in the literature regarding the Wnt7a/β-catenin regulation of TJ proteins. For example, an in vitro study in bEnd.3 cells detected significant increases in levels of BBB-specific influx transporters but did not observe changes in the expression of TJ proteins such as Ocln [17]. Functional effects of Wnt7a/β-catenin have also been examined. For example, an in vitro bEnd.3 permeability assay using Evans blue dye, decreased endothelial permeability in cells treated with Wnt7a compared to control treated cells [46]. Similarly, CHIR99021 treated hPSC showed increased TEER resistance and decreased permeability to a small molecule tracer, sodium fluorescein [25]. However, primary mouse brain ECs treated with Wnt3a, another activator of the β-catenin dependent Wnt pathway, did not produce an effect on EC permeability [48]. These findings contrast with the decrease in TEER values we observed after a 2-day stimulation with Wnt7a. Of note, results similar to ours were observed in a study of human aortic ECs where similar β-catenin mediated increase in permeability were detected [49].

The observed decrease in TEER values in both Wnt7a and control treated cells after 5 days may be explained by a model of EC function in which β-catenin levels must be maintained

above a certain threshold level of β-catenin to ensure barrier function in adult CNS vasculature [50]. In this context, the β-catenin signalling response of bEnd.3 cells treated with 100 ng/ml Wnt7a might decrease after repeated stimulations due to desensitisation, leading to disabled tight barrier maintenance and a decrease in electrical resistance. In support of this idea, previous studies suggest that activation of β-catenin due to phosphorylation and internalisation of LRP6, leads to desensitisation [51, 52].

Modulation of EC barrier permeability might be a crucial step towards EC proliferation and the initiation of brain angiogenesis through EC sprouting, both of which are processes known to require Wnt signalling [17, 21, 53]. An interesting factor that might modulate the angiogenic response of ECs is hypoxia induced transcription factor, HIF1α. Our data suggest that β-catenin activation through Wnt7a can increase the expression of *Hif1a* in bEnd.3 ECs. HIF1α can regulate Wnt signalling and be the target of Wnt induced regulation [54–56], whilst hypoxia and HIF1α signalling regulates Wnt/β-catenin signalling in a cell- and developmental stage-specific manner [57, 58]. It is also suggested that these signalling pathways might have an indirect interaction rather than exerting direct regulation [56]. On the other hand, nuclear β-catenin/Tcf complex formation has been shown to induce *Hif1a* expression [55]. These results indicate that Wnt/β-catenin signalling can affect the expression of *Hif1a* to modulate the cellular response to events such as hypoxia. Our data support this notion of Wnt7a/β-catenin mediating the expression of *Hif1a*. However, in our study, the silencing of *Hif1a* with siRNA did not reverse the Wnt7a/β-catenin mediated downregulation of TJ proteins Cldn5 and Ocln. Interestingly, mRNA levels of *Ocln* were normalised by inhibiting *Hif1a*, while Ocln proteins levels were increased independently from β-catenin, suggesting that these signalling pathways might interact and indirectly regulate the endothelial barrier function. Thus, Wnt/HIF1α signalling in mature ECs may

not be critical for barrier formation but may play an indirect role in a context-dependent manner, for example via HIF1α/VEGFA signalling during hypoxic conditions in diseases such as stroke and cSVD. There was some discrepancy regarding the regulation of Ocln by Wnt7a in two of our data sets (Figure 3E vs 6H). On one hand (Figure 3E), Ocln was not changed by Wnt7a stimulation while blocking of β-catenin with XAV had a positive effect. On the other hand (Figure 6H), Ocln was downregulated by Wnt7a stimulation and unchanged by *Hif1a* silencing. While these data are contradicting, it also indicates that the Wnt7a/β-catenin signalling can lead to HIF1α independent regulation of Ocln.

In conclusion, we suggest that Wnt7a activates the β-catenin mediated Wnt signalling pathway, causing nuclear translocation of β-catenin, suppression of TJ protein expression, and ultimately a decrease in EC barrier function. Furthermore, we propose that these changes in EC properties are associated with events such as endothelial proliferation and angiogenesis, which are stimulated by Wnt7a signalling. Wnt7a/β-catenin mediated regulation of TJ proteins occurs independently of the Wnt/HIF1α signalling pathway. This pathway may play other roles in inducing angiogenesis in response to environmental factors such as hypoxia. However, future studies are needed to determine the specific role of HIF1α in modulating the Wnt/β-catenin signalling pathway. Understanding the role of Wnt/β-catenin signalling in hypoxia might lead to a better understanding of the cellular mechanisms involved in diseases such as cSVD.

Statements & Declarations

Funding

This work was supported by a PhD studentship, jointly funded by the University of Birmingham and CARIM, School for Cardiovascular Diseases Maastricht at Maastricht University.

Competing Interests

The authors have no financial or non-financial competing interests to declare that are relevant to the content of this article.

Author Contributions

NM designed and performed research with input from SF, ZA, WMB, and DF. SC supported NM in performing TEER measurements. MW performed the RNA sequencing and FC data processing. NM wrote manuscript with input from all authors. All authors read and approved the final version of the manuscript. Corresponding authors are ZA, WMB, and SF.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Raw data are available from the corresponding author, upon request. Supplementary information is available at *Molecular Neurobiology* online.

References

1. Haddad-Tóvolli R, Dragano NRV, Ramalho AFS, Velloso LA (2017) Development and Function of the Blood-Brain Barrier in the Context of Metabolic Control. Frontiers in Neuroscience 11:
2. Kadry H, Noorani B, Cucullo L (2020) A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity. Fluids and Barriers of the CNS 17:69. https://doi.org/10.1186/s12987-020-00230-3
3. Quick S, Moss J, Rajani RM, Williams A (2021) A Vessel for Change: Endothelial Dysfunction in Cerebral Small Vessel Disease. Trends in Neurosciences 44:289–305. https://doi.org/10.1016/j.tins.2020.11.003
4. Liu W-Y, Wang Z-B, Zhang L-C, et al (2012) Tight Junction in Blood-Brain Barrier: An Overview of Structure, Regulation, and Regulator Substances. CNS Neuroscience & Therapeutics 18:609–615. https://doi.org/10.1111/j.1755-5949.2012.00340.x
5. Bhat AA, Uppada S, Achkar IW, et al (2019) Tight Junction Proteins and Signaling Pathways in Cancer and Inflammation: A Functional Crosstalk. Frontiers in Physiology 9:
6. Anderson JM, Itallie CMV (2009) Physiology and Function of the Tight Junction. Cold Spring Harb Perspect Biol 1:a002584. https://doi.org/10.1101/cshperspect.a002584
7. Liebner S, Corada M, Bangsow T, et al (2008) Wnt/β-catenin signaling controls development of the blood–brain barrier. Journal of Cell Biology 183:409–417. https://doi.org/10.1083/jcb.200806024
8. Greene C, Hanley N, Campbell M (2019) Claudin-5: gatekeeper of neurological function. Fluids and Barriers of the CNS 16:3. https://doi.org/10.1186/s12987-019-0123-z
9. Rouhl RPW, Damoiseaux JGMC, Lodder J, et al (2012) Vascular inflammation in cerebral small vessel disease. Neurobiology of Aging 33:1800–1806. https://doi.org/10.1016/j.neurobiolaging.2011.04.008
10. Hawkins BT, Davis TP (2005) The Blood-Brain Barrier/Neurovascular Unit in Health and Disease. Pharmacol Rev 57:173–185. https://doi.org/10.1124/pr.57.2.4
11. Manukjan N, Ahmed Z, Fulton D, et al (2020) A Systematic Review of WNT Signaling in Endothelial Cell Oligodendrocyte Interactions: Potential Relevance to Cerebral Small Vessel Disease. Cells 9:1545. https://doi.org/10.3390/cells9061545
12. Galatius S, Wroblewski H, Sørensen VB, et al (1999) Endothelin and von Willebrand factor as parameters of endothelial function in idiopathic dilated cardiomyopathy: Different stimuli for release before and after heart transplantation? American Heart Journal 137:549–554. https://doi.org/10.1016/S0002-8703(99)70505-3
13. Hussain B, Fang C, Huang X, et al (2022) Endothelial β-Catenin Deficiency Causes Blood-Brain Barrier Breakdown via Enhancing the Paracellular and Transcellular Permeability. Front Mol Neurosci 15:895429. https://doi.org/10.3389/fnmol.2022.895429
14. Foulquier S, Daskalopoulos EP, Lluri G, et al (2018) WNT Signaling in Cardiac and Vascular Disease. Pharmacol Rev 70:68–141. https://doi.org/10.1124/pr.117.013896
15. Clevers H (2006) Wnt/β-Catenin Signaling in Development and Disease. Cell 127:469–480. https://doi.org/10.1016/j.cell.2006.10.018
16. Cattelino A, Liebner S, Gallini R, et al (2003) The conditional inactivation of the β-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility. Journal of Cell Biology 162:1111–1122. https://doi.org/10.1083/jcb.200212157
17. Daneman R, Agalliu D, Zhou L, et al (2009) Wnt/β-catenin signaling is required for CNS, but not non-CNS, angiogenesis. PNAS 106:641–646. https://doi.org/10.1073/pnas.0805165106

18. Stenman JM, Rajagopal J, Carroll TJ, et al (2008) Canonical Wnt Signaling Regulates Organ-Specific Assembly and Differentiation of CNS Vasculature. Science 322:1247–1250. https://doi.org/10.1126/science.1164594
19. Wang Y, Rattner A, Zhou Y, et al (2012) Norrin/Frizzled4 Signaling in Retinal Vascular Development and Blood Brain Barrier Plasticity. Cell 151:1332–1344. https://doi.org/10.1016/j.cell.2012.10.042
20. Zhou Y, Wang Y, Tischfield M, et al (2014) Canonical WNT signaling components in vascular development and barrier formation. J Clin Invest 124:3825–3846. https://doi.org/10.1172/JCI76431
21. Vanhollebeke B, Stone OA, Bostaille N, et al Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/β-catenin pathway during brain angiogenesis. eLife 4:e06489. https://doi.org/10.7554/eLife.06489
22. Qu Q, Sun G, Murai K, et al (2013) Wnt7a Regulates Multiple Steps of Neurogenesis. Mol Cell Biol 33:2551–2559. https://doi.org/10.1128/MCB.00325-13
23. Paolinelli R, Corada M, Ferrarini L, et al (2013) Wnt Activation of Immortalized Brain Endothelial Cells as a Tool for Generating a Standardized Model of the Blood Brain Barrier In Vitro. PLOS ONE 8:e70233. https://doi.org/10.1371/journal.pone.0070233
24. Watanabe T, Dohgu S, Takata F, et al (2013) Paracellular Barrier and Tight Junction Protein Expression in the Immortalized Brain Endothelial Cell Lines bEND.3, bEND.5 and Mouse Brain Endothelial Cell 4. Biological and Pharmaceutical Bulletin 36:492–495. https://doi.org/10.1248/bpb.b12-00915
25. Gastfriend BD, Nishihara H, Canfield SG, et al (2021) Wnt signaling mediates acquisition of blood–brain barrier properties in naïve endothelium derived from human pluripotent stem cells. eLife 10:e70992. https://doi.org/10.7554/eLife.70992
26. Morini MF, Giampietro C, Corada M, et al (2018) VE-Cadherin–Mediated Epigenetic Regulation of Endothelial Gene Expression. Circulation Research 122:231–245. https://doi.org/10.1161/CIRCRESAHA.117.312392
27. Taddei A, Giampietro C, Conti A, et al (2008) Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat Cell Biol 10:923–934. https://doi.org/10.1038/ncb1752
28. Montesano R, Pepper MS, Möhle-Steinlein U, et al (1990) Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. Cell 62:435–445. https://doi.org/10.1016/0092-8674(90)90009-4
29. Bentzinger CF, von Maltzahn J, Dumont NA, et al (2014) Wnt7a stimulates myogenic stem cell motility and engraftment resulting in improved muscle strength. J Cell Biol 205:97–111. https://doi.org/10.1083/jcb.201310035
30. Bikkavilli RK, Avasarala S, Van Scoyk M, et al (2015) Wnt7a is a novel inducer of β-catenin-independent tumor-suppressive cellular senescence in lung cancer. Oncogene 34:5317–5328. https://doi.org/10.1038/onc.2015.2
31. Huang X, Zhu H, Gao Z, et al (2018) Wnt7a activates canonical Wnt signaling, promotes bladder cancer cell invasion, and is suppressed by miR-370-3p. J Biol Chem 293:6693–6706. https://doi.org/10.1074/jbc.RA118.001689
32. Huang S-MA, Mishina YM, Liu S, et al (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461:614–620. https://doi.org/10.1038/nature08356
33. Laksitorini MD, Yathindranath V, Xiong W, et al (2019) Modulation of Wnt/β-catenin signaling promotes blood-brain barrier phenotype in cultured brain endothelial cells. Sci Rep 9:1–13. https://doi.org/10.1038/s41598-019-56075-w

34. Raudvere U, Kolberg L, Kuzmin I, et al (2019) g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Research 47:W191–W198. https://doi.org/10.1093/nar/gkz369
35. Huang DW, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37:1–13. https://doi.org/10.1093/nar/gkn923
36. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57. https://doi.org/10.1038/nprot.2008.211
37. Shannon P, Markiel A, Ozier O, et al (2003) Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res 13:2498–2504. https://doi.org/10.1101/gr.1239303
38. Palazzo L, Ahel I (2018) PARPs in genome stability and signal transduction: implications for cancer therapy. Biochemical Society Transactions 46:1681–1695. https://doi.org/10.1042/BST20180418
39. Jho E, Zhang T, Domon C, et al (2002) Wnt/β-Catenin/Tcf Signaling Induces the Transcription of Axin2, a Negative Regulator of the Signaling Pathway. Mol Cell Biol 22:1172–1183. https://doi.org/10.1128/MCB.22.4.1172-1183.2002
40. Vigh JP, Kincses A, Ozgür B, et al (2021) Transendothelial Electrical Resistance Measurement across the Blood–Brain Barrier: A Critical Review of Methods. Micromachines (Basel) 12:685. https://doi.org/10.3390/mi12060685
41. Liang Y, Li X-Y, Rebar EJ, et al (2002) Activation of Vascular Endothelial Growth Factor A Transcription in Tumorigenic Glioblastoma Cell Lines by an Enhancer with Cell Type-specific DNase I Accessibility. J Biol Chem 277:20087–20094. https://doi.org/10.1074/jbc.M201766200
42. Yuen TJ, Silbereis JC, Griveau A, et al (2014) Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell 158:383–396. https://doi.org/10.1016/j.cell.2014.04.052
43. Davis EK, Zou Y, Ghosh A (2008) Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation. Neural Development 3:32. https://doi.org/10.1186/1749-8104-3-32
44. Acebron SP, Niehrs C (2016) β-Catenin-Independent Roles of Wnt/LRP6 Signaling. Trends in Cell Biology 26:956–967. https://doi.org/10.1016/j.tcb.2016.07.009
45. Cerpa W, Godoy JA, Alfaro I, et al (2008) Wnt-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. J Biol Chem 283:5918–5927. https://doi.org/10.1074/jbc.M705943200
46. Wang L, Geng J, Qu M, et al (2020) Oligodendrocyte precursor cells transplantation protects blood–brain barrier in a mouse model of brain ischemia via Wnt/β-catenin signaling. Cell Death Dis 11:9. https://doi.org/10.1038/s41419-019-2206-9
47. Karnati HK, Panigrahi M, Shaik NA, et al (2014) Down Regulated Expression of Claudin-1 and Claudin-5 and Up Regulation of B-Catenin: Association with Human Glioma Progression. CNS Neurol Disord Drug Targets 13:1413–1426
48. Cottarelli A, Corada M, Beznoussenko GV, et al (2020) Fgfbp1 promotes blood-brain barrier development by regulating collagen IV deposition and maintaining Wnt/β-catenin signaling. Development 147:dev185140. https://doi.org/10.1242/dev.185140
49. Rickman M, Ghim M, Pang K, et al (2023) Disturbed flow increases endothelial inflammation and permeability via a Frizzled-4-β-catenin-dependent pathway. Journal of Cell Science 136:jcs260449. https://doi.org/10.1242/jcs.260449

50. Wang Y, Cho C, Williams J, et al (2018) Interplay of the Norrin and Wnt7a/Wnt7b signaling systems in blood–brain barrier and blood–retina barrier development and maintenance. Proceedings of the National Academy of Sciences 115:E11827–E11836. https://doi.org/10.1073/pnas.1813217115
51. Yamamoto H, Komekado H, Kikuchi A (2006) Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. Dev Cell 11:213–223. https://doi.org/10.1016/j.devcel.2006.07.003
52. Liu C-C, Kanekiyo T, Roth B, Bu G (2014) Tyrosine-based Signal Mediates LRP6 Receptor Endocytosis and Desensitization of Wnt/β-Catenin Pathway Signaling. J Biol Chem 289:27562–27570. https://doi.org/10.1074/jbc.M113.533927
53. Okumura N, Nakamura T, Kay EP, et al (2014) R-spondin1 Regulates Cell Proliferation of Corneal Endothelial Cells via the Wnt3a/β-Catenin Pathway. Investigative Ophthalmology & Visual Science 55:6861–6869. https://doi.org/10.1167/iovs.14-14091
54. Krock BL, Skuli N, Simon MC (2011) Hypoxia-Induced Angiogenesis. Genes Cancer 2:1117–1133. https://doi.org/10.1177/1947601911423654
55. Boso D, Rampazzo E, Zanon C, et al (2019) HIF-1α/Wnt signaling-dependent control of gene transcription regulates neuronal differentiation of glioblastoma stem cells. Theranostics 9:4860–4877. https://doi.org/10.7150/thno.35882
56. Shen X, Li M, Wang C, et al (2022) Hypoxia is fine-tuned by Hif-1α and regulates mesendoderm differentiation through the Wnt/β-Catenin pathway. BMC Biology 20:219. https://doi.org/10.1186/s12915-022-01423-y
57. Mazumdar J, O'Brien WT, Johnson RS, et al (2010) O2 regulates stem cells through Wnt/β-catenin signalling. Nat Cell Biol 12:1007–1013. https://doi.org/10.1038/ncb2102
58. Kaidi A, Williams AC, Paraskeva C (2007) Interaction between β-catenin and HIF-1 promotes cellular adaptation to hypoxia. Nat Cell Biol 9:210–217. https://doi.org/10.1038/ncb1534

Supplementary Material

Supplementary Table 1: Primary and secondary antibodies used for immunocytochemistry.

Primary antibody	**Host**	**Dilution**	**Cat no.**	**Supplier**
Anti-Active β-catenin	Mouse	1:300	05-665	Millipore
Anti-Claudin-5	Mouse	1:300	35-2500	Invitrogen
Anti-Occludin	Rabbit	1:300	40-6100	Invitrogen
Secondary antibody				
Anti-Mouse IgG Alexa Fluor 488	Donkey	1:200	A-21202	Invitrogen
Anti-Rabbit IgG Alexa Fluor 594	Donkey	1:200	A-21207	Invitrogen

Supplementary Table 2: Primers used in quantitative PCR.

Primer name	**Primer sequence**	
Rpl13a	Forward	AGCCTACCAGAAAGTTTGCTTAC
	Reverse	GCTTCTTCTTCCGATAGTGCATC
Ywhaz	Forward	GAAAAGTTCTTGATCCCCAATGC
	Reverse	TGTGACTGGTCCACAATTCCTT
Gusb	Forward	CCGACCTCTCGAACAACCG
	Reverse	GCTTCCCGTTCATACCACACC
Axin2	Forward	AACCTATGCCCGTTTCCTCTA
	Reverse	GAGTGTAAAGACTTGGTCCACC
Cldn5	Forward	CCACGGCCAATGGCGATTAC
	Reverse	TCGTCATCCACACACGGCTT
Ocln	Forward	CCTCGGTACAGCAGCAATGG
	Reverse	TAGTGGTCAGGGTCCGTCCT
Hif1a	Forward	AATGAAGTGCACCCTAACAAGCCG
	Reverse	TGGCCCGTGCAGTGAAGC
Vegfa	Forward	GCACATAGAGAGAATGAGCTTCC
	Reverse	CTCCGCTCTGAACAAGGCT

Chapter 4

-

Wnt7a decreases Cxcl12 expression in brain endothelial cells

Narek Manukjan, Florian Caiment, Marcel van Herwijnen, Hubert J. Smeets, Daniel Fulton, Zubair Ahmed, W. Matthijs Blankesteijn, Sébastien Foulquier

In preparation for publication

Introduction

A recent genome-wide association study (GWAS) in more than 40,000 participants identified polymorphisms near the *WNT7A* gene as one of the white matter perivascular space burden risk loci [1]. The perivascular space burden is a marker for cerebral small vessel disease (cSVD), which is a major cause of stroke and vascular dementia [1]. Following damage to myelin in the white matter, pro-repair oligodendrocyte precursor cells (OPCs) migrate to sites of damage utilising the vasculature [2, 3]. Increased perivascular OPC densities in stroke-related hypoxic areas, and OPC clusters in active myelin lesions, suggest abnormal vascular-OPC interaction in diseases such as cSVD and stroke [4]. Wnt7a is involved in regulating angiogenesis and blood-brain barrier integrity [5], but its contribution to vascular-OPC interactions remains poorly understood. Based on these recent findings, we investigated the impact of Wnt7a on brain endothelial cells (ECs) with a focus on EC-OPC crosstalk pathways using a transcriptomic approach. We hypothesised that Wnt7a mediates cellular signals involved in vascular OPC migration. This study aims to stimulate research on Wnt7a in context of cSVD.

Methods

Immortalised murine brain ECs (bEnd.3) and OPCs (Oli-neu) were treated with recombinant Wnt7a (100 ng/ml), or a selective β-catenin inhibitor, XAV939 (XAV, 10 μM), as well as co-treatment of Wnt7a and XAV for 24h followed by RNA sequencing and quantitative PCR. Additional methodological details are described in the Supplemental Materials.

Results

Gene ontology analysis identified two cell migration related pathways (GO:0016477 and GO:0030334) among the top 5 most enriched biological processes in bEnd.3 cells stimulated with Wnt7a (Figure 1A). Pathway analysis of genes regulated by Wnt/β-catenin signalling revealed *Cxcl12* as the gene showing the greatest degree of regulation (Figure 1B). Wnt7a stimulation in bEnd.3 cells decreased *Cxcl12* mRNA compared to control (Figure 1C) or XAV treated cells (Figure 1D). A similar decrease in *Cxcl12* was observed when cells were co-treated with Wnt7a and XAV (Figure 1D), whilst Wnt7a stimulation in Oli-neu cells had no effect on the β-catenin target gene, *Axin2* (Figure 1E) nor the *Cxcl12* receptor gene, *Cxcr4* (Figure 1F). These results reveal that Wnt7a stimulation downregulates *Cxcl12* in bEnd.3 cells independent of β-catenin but does not affect its receptor in Oli-neu cells.

Discussion

Cxcl12 is a chemokine protein expressed by the ECs which mediates OPC migration along the vasculature via its interaction with Cxcr4 [3, 5]. Here, we report that Wnt7a stimulation suppresses *Cxcl12* expression in bEnd.3 ECs independent of β-catenin but does not influence the *Cxcl12* receptor *Cxcr4* in the OPC Oli-neu cell line. Therefore, our finding of reduced *Cxcl12* levels after Wnt7a stimulation might suggest a link between Wnt7a polymorphisms and OPC migration dysfunction in cSVD and stroke [1, 3].

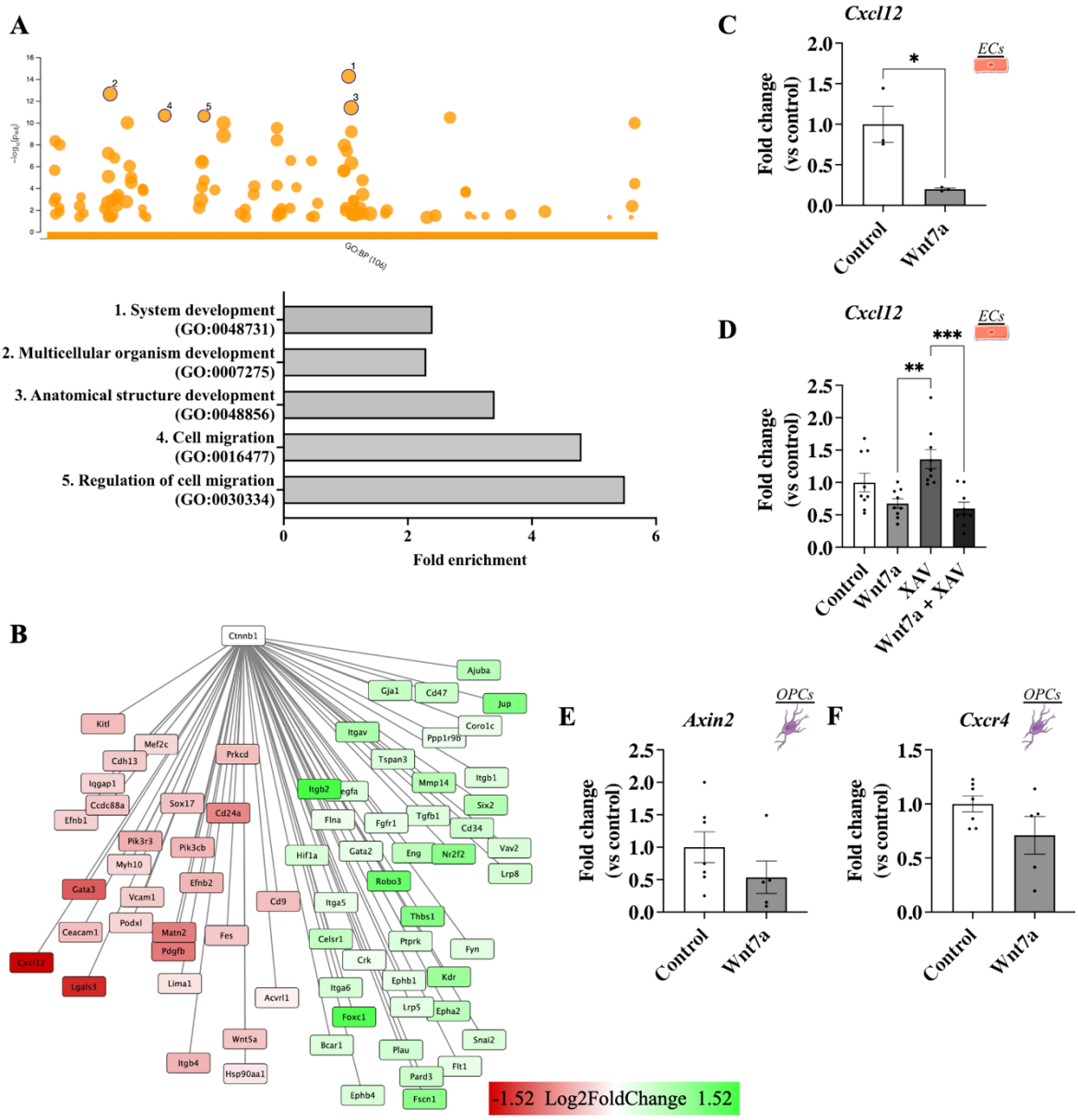


Figure 1: Wnt7a decreased *Cxcl12* expression in endothelial cells independent of β-catenin signalling. (A) Gene ontology enrichment analysis of the top 100 regulated genes following Wnt7a stimulation. Cell migration related pathways represent the top two biological processes. **(B)** Cell migration pathway analysis revealed the downregulation of *Cxcl12*. **(C)** *Cxcl12* was downregulated upon Wnt7a stimulation compared to control in brain endothelial cells (ECs); (n=3). **(D)** Downregulation of *Cxcl12* by Wnt7a was not mediated by β-catenin (n=9). **(E)** Wnt7a stimulation of oligodendrocyte precursor cells (OPCs) did not lead to changes in the β-catenin target gene *Axin2* or **(F)** the *Cxcl12* receptor gene, *Cxcr4;* (n=5-7). Graphs represent Mean±SEM; Data points (dots) indicate technical replicates; *p<0.05, **p<0.01, ***p<0.001; student t-test or One-way ANOVA with post-hoc Tukey's test. Statistically non-significant comparisons are not shown.

References

1. Duperron M-G, Knol MJ, Le Grand Q, et al (2023) Genomics of perivascular space burden unravels early mechanisms of cerebral small vessel disease. Nat Med 29:950–962. https://doi.org/10.1038/s41591-023-02268-w
2. Russo MV, McGavern DB (2015) Immune Surveillance of the CNS following Infection and Injury. Trends Immunol 36:637–650. https://doi.org/10.1016/j.it.2015.08.002
3. Tsai H-H, Niu J, Munji R, et al (2016) Oligodendrocyte precursors migrate along vasculature in the developing nervous system. Science 351:379–384. https://doi.org/10.1126/science.aad3839
4. Kishida N, Maki T, Takagi Y, et al (2019) Role of Perivascular Oligodendrocyte Precursor Cells in Angiogenesis After Brain Ischemia. J Am Heart Assoc 8:e011824. https://doi.org/10.1161/JAHA.118.011824
5. Niu J, Tsai H-H, Hoi KK, et al (2019) Aberrant oligodendroglial–vascular interactions disrupt the blood–brain barrier, triggering CNS inflammation. Nat Neurosci 22:709–718. https://doi.org/10.1038/s41593-019-0369-4

Supplemental Materials

Methods

Cell culture

The immortalised murine brain endothelial cells, bEnd.3 cells (Cat no. CRL-2299, American Type Culture Collection, Manassas, VA, USA [1]) and the immortalised OPC cell line (RRID:CVCL_IZ82 [2], Merck Millipore, Burlington, MA, USA), Oli-neu cells, were cultured in Dulbecco's Modified Eagle's Medium high glucose (DMEM, Cat no. D6429, Merck Millipore) supplemented with 10% foetal bovine serum (FBS) and 100 µg/mL penicillin/streptomycin (P/S) at 37°C and in an atmosphere of 5% CO2. BEnd.3 cell between passage 26 and 28 and Oli-neu cells at passage 18 were seeded onto multi-well plates a density of $1.0x10^5$ cells/cm2 for expression studies and treated at confluency (1-3 days after seeding).

Cells were treated with recombinant Wnt7a (Cat no. SRP3296, Sigma-Aldrich, St. Louis, MO, USA), dissolved in 0.1% BSA-PBS to study the effects of paracrine mediated Wnt signalling on EC. Briefly, cells were grown to confluency and treated with control (0.1% BSA-PBS) or Wnt7a (100 ng/ml dissolved in 0.1% BSA-PBS) for 24h, lysed and used for RNA sequencing.

Wnt7a activation of β-catenin was investigated by co-administration with XAV939 (XAV, Cat no. X3004, Sigma-Aldrich), a Tankyrase inhibitor known to selectively inhibit Wnt/β-catenin mediated transcription [3, 4]. Briefly, cells were grown to confluency and treated with control, Wnt7a (100 ng/ml), XAV (10 µM) or a co-administrated of Wnt7a with XAV (100 ng/ml and 10 µM, respectively) for 24h. After 24h, cells were lysed for RNA isolation, and used for qPCR. All samples were kept at -80°C until further use.

RNA sequencing

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the TRIzol method and stored at -80°C before use. Total isolated RNA quantity was checked using Qubit 2.0 Fluorometer (Invitrogen) and RNA quality was assessed using Bioanalyzer (Cat no. RNA 6000 Nano kit; 2100 Bioanalyzer, Agilent Technologies). Purification of mRNA from total RNA (NEXTFLEX Poly(A) Beads 2.0, Cat no. NOVA-512992, PerkinElmer, Waltham, MA, USA) and directional, strand specific RNA library preparation (NEXTFLEX Rapid Directional RNA-Seq Kit 2.0, Cat no. NOVA-5198, PerkinElmer) was performed according to manufacturer's protocol. Sequencing was performed using NovaSeq 6000 Sequencing system (NovaSeq S Prime flow cell 200 cycles; NovaSeq 6000, Illumina, Inc, San Diego, CA, USA) according to manufacturer's protocol. The raw sequencing data was trimmed using *fastp*, remaining reads were then mapped against the Ensembl mouse genome (release 100) using STAR (version 2.7.3a) and quantified using RSEM (v.1.3.1). The resulting raw read counts were processed using the R package DESeq2. Genes with were not sequenced (0 reads) in more than 75% of the samples of any given condition were removed. Genes were considered differentially expressed with an adjusted p-value (false discovery rate; FDR) below 0.01. Gene ontology (GO) enrichment analysis were performed on the top 100 differentially expressed genes using g:Profiler [5] and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8. [6, 7]. The modified Fisher exact p-value (EASE score) <0.05 and FDR <0.05 were considered enriched. Gene interaction network analysis was performed using Cytoscape version 3.9.1 [8].

Quantitative PCR (qPCR)

Quality and quantity were checked using NanoDrop 1000 spectrophotometer and the RNA was reverse transcribed into cDNA using the high-capacity RNA-to-cDNA kit (Cat no. 1708891, Bio-rad laboratories, Inc., Hercules, CA, USA) according to manufacturer's manual. cDNA samples were stored at -20°C before use. QPCR was performed using Sensimix™ SYBER® & Fluorescein kit (Cat no. QT615-05, Meridian Bioscience Inc., Cincinnati, OH, USA) on the Light Cycler 480 (Roche Applied Science, Penzberg, Germany) with the following qPCR program: 10 min at 95°C followed by 55 cycles a 10s at 95°C and 20s at 60°C. Temperature was increased from 60 to 95°C for melting curve analyses. Primers were designed to cover exon-exon junctions and all possible splice variants using NCBI Primer-BLAST tool. Primers were synthesised by Eurofins Genomics (Ebersberg, Germany) and quality was ensured by testing on cell cultures, as well as by calculation of primer efficiency. At least two stable reference housekeeping genes were selected from a selection of three genes by using the GeNorm Software (Primerdesign, Southampton, NY, USA). Primers are listed in Supplementary Table 1. Gene expression analysis was performed using LinReg PCR (Ver. 2014.0) and the Light Cycler 480 data converter (Ver. 2014.1) and shown as fold change (FC) compared to control.

Statistical analysis

Data was analysed using GraphPad Prism 9 (Dotmatics). Data distribution was tested using Shapiro-Wilk test for normality. Unpaired Student t-tests were used to compare Wnt7a vs control. One-way ANOVA with post-hoc Tukey's multiple comparisons test was used to assess multiple comparisons. $P<0.05$ was considered statistically significant and data are expressed as mean±SEM.

Supplementary Table 1: Primers used in quantitative PCR.

Primer name	Primer sequence	
Rpl13a	Forward	AGCCTACCAGAAAGTTTGCTTAC
	Reverse	GCTTCTTCTTCCGATAGTGCATC
Ywhaz	Forward	GAAAAGTTCTTGATCCCCAATGC
	Reverse	TGTGACTGGTCCACAATTCCTT
Gusb	Forward	CCGACCTCTCGAACAACCG
	Reverse	GCTTCCCGTTCATACCACACC
Axin2	Forward	AACCTATGCCCGTTTCCTCTA
	Reverse	GAGTGTAAAGACTTGGTCCACC
Cxcl12	Forward	CAGAGCCAACGTCAAGCATC
	Reverse	TTCTTCAGCCGTGCAACAATC
Cxcr4	Forward	GACTGGCATAGTCGGCAATGGA
	Reverse	CAAAGAGGAGGTCAGCCACTGA

References

1. Montesano R, Pepper MS, Möhle-Steinlein U, et al (1990) Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. Cell 62:435–445. https://doi.org/10.1016/0092-8674(90)90009-4
2. Jung M, Krämer E, Grzenkowski M, et al (1995) Lines of Murine Oligodendroglial Precursor Cells Immortalized by an Activated neu Tyrosine Kinase Show Distinct Degrees of Interaction with Axons In Vitro and In Vivo. Eur J Neurosci 7:1245–1265. https://doi.org/10.1111/j.1460-9568.1995.tb01115.x
3. Huang S-MA, Mishina YM, Liu S, et al (2009) Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461:614–620. https://doi.org/10.1038/nature08356
4. Laksitorini MD, Yathindranath V, Xiong W, et al (2019) Modulation of Wnt/β-catenin signaling promotes blood-brain barrier phenotype in cultured brain endothelial cells. Sci Rep 9:1–13. https://doi.org/10.1038/s41598-019-56075-w
5. Raudvere U, Kolberg L, Kuzmin I, et al (2019) g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Research 47:W191–W198. https://doi.org/10.1093/nar/gkz369
6. Huang DW, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37:1–13. https://doi.org/10.1093/nar/gkn923
7. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57. https://doi.org/10.1038/nprot.2008.211
8. Shannon P, Markiel A, Ozier O, et al (2003) Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res 13:2498–2504. https://doi.org/10.1101/gr.1239303

Chapter 5

-

Hypoxic oligodendrocyte precursor cell-derived VEGFA is associated with blood-brain barrier impairment

Narek Manukjan, Daria Majcher, Peter Leenders, Florian Caiment, Marcel van Herwijnen, Hubert J. Smeets, Ernst Suidgeest, Louise van der Weerd, Tim Vanmierlo, Jacobus F.A. Jansen, Walter H. Backes, Robert J. van Oostenbrugge, Julie Staals, Daniel Fulton, Zubair Ahmed, W. Matthijs Blankesteijn, Sébastien Foulquier

Received: 14 April 2023

Accepted: 23 July 2023

Published: 07 August 2023

Based on Acta Neuropathologica Communications 2023, 11:128

DOI: 10.1186/s40478-023-01627-5

Abstract

Cerebral small vessel disease is characterised by decreased cerebral blood flow and blood–brain barrier impairments which play a key role in the development of white matter lesions. We hypothesised that cerebral hypoperfusion causes local hypoxia, affecting oligodendrocyte precursor cell—endothelial cell signalling leading to blood–brain barrier dysfunction as an early mechanism for the development of white matter lesions. Bilateral carotid artery stenosis was used as a mouse model for cerebral hypoperfusion. Pimonidazole, a hypoxic cell marker, was injected prior to humane sacrifice at day 7. Myelin content, vascular density, blood–brain barrier leakages, and hypoxic cell density were quantified. Primary mouse oligodendrocyte precursor cells were exposed to hypoxia and RNA sequencing was performed. *Vegfa* gene expression and protein secretion was examined in an oligodendrocyte precursor cell line exposed to hypoxia. Additionally, human blood plasma VEGFA levels were measured and correlated to blood–brain barrier permeability in normal-appearing white matter and white matter lesions of cerebral small vessel disease patients and controls. Cerebral blood flow was reduced in the stenosis mice, with an increase in hypoxic cell number and blood–brain barrier leakages in the cortical areas but no changes in myelin content or vascular density. *Vegfa* upregulation was identified in hypoxic oligodendrocyte precursor cells, which was mediated via *Hif1a* and *Epas1*. In humans, VEGFA plasma levels were increased in patients versus controls. VEGFA plasma levels were associated with increased blood–brain barrier permeability in normal appearing white matter of patients. Cerebral hypoperfusion mediates hypoxia induced VEGFA expression in oligodendrocyte precursor cells through *Hif1a/Epas1* signalling. VEGFA could in turn increase BBB permeability. In humans, increased VEGFA plasma levels in cerebral small vessel disease patients were associated with increased blood–brain barrier permeability in the normal

appearing white matter. Our results support a role of VEGFA expression in cerebral hypoperfusion as seen in cerebral small vessel disease.

Keywords: Vascular dementia; Glial biology; OPC; Angiogenesis; BBB; cSVD

Introduction

Cerebral small vessel disease (cSVD) is an umbrella term referring to pathological processes affecting the small arteries, arterioles, venules, and capillaries in the brain, leading to pathological lesions such as white matter lesions (WML) observed as white matter hyperintensities (WMH) on MRI. WML are thought to result from chronic hypoperfusion of the white matter (WM), which can in turn lead to oligodendrocyte damage and eventually the degeneration of their myelinating extensions [49]. Cerebral blood flow (CBF) in WM decreased close to WMH in patients with cSVD, whilst decreased CBF was also correlated with an increase in blood–brain barrier (BBB) leakage volume and rate. This correlation was strongest in regions close to WMH but was also found in the normal appearing white matter (NAWM) of these patients [65].

Increased BBB permeability and reduced CBF are hallmarks of a dysfunctional neurovascular unit (NVU), which results in a failure to provide sufficient oxygen and energy to glial cells and neurons. In the WM, oligodendrocyte and oligodendrocyte precursor cells (OPCs) are most vulnerable to the effects of hypoxia [24]. This vulnerability of OPCs to hypoxia might lead to demyelination and impairment in remyelination, leading to WML, however, how hypoxic OPCs can mediate changes in the WM, is currently not known. Previous findings show a decrease in the number of oligodendroglia after a transient period of hypoxia preceding lesion formation at the white–grey matter border [12, 29]. Demyelination was reduced or even eliminated by normalising oxygen levels in rats [10, 12]. Recent in vivo findings suggest that perivascular OPC density increases post stroke in an attempt to rescue demyelinated regions by recruiting progenitors of the damaged myelinating cells [29]. Interestingly, adult myelinating oligodendrocytes seem to be less vulnerable to hypoxia than OPC [4]. Taken together, this high vulnerability of OPC to

hypoxia, particularly later stage OPC, might suggest its detrimental role in the development of WML [10, 24, 29].

It remains unknown however, if cerebral hypoperfusion occurring in cSVD patients can affect OPC and how this may lead to endothelial cell (EC) dysfunction and increased BBB permeability. The aim of this study was to investigate hypoperfusion-mediated hypoxic signalling in OPC and its impact on cerebral small vessel integrity. We hypothesised that cerebral hypoperfusion causes local hypoxia, affecting the OPC–EC signalling, leading to increased BBB permeability.

We used carotid artery stenosis as model for cerebral hypoperfusion. Although this is not an intrinsic small vessel disease model, carotid occlusive disease is associated with small vessel hypoperfusion [48]. We investigated the impact of 1 week of cerebral hypoperfusion in mice that had previously undergone bilateral carotid artery stenosis (BCAS) to identify early effects on myelin content, vascular density, BBB leakages, and hypoxic cell density. We combined our in vivo investigations with the study of OPC in vitro under hypoxic conditions. Finally, we related our findings in a retrospective analysis of VEGFA levels in blood plasma samples derived from cSVD patients.

Materials and methods

Animals and tissue collection

Male C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany) between 9–10 weeks of age (weighing between 20 to 26 g) and kept on a normal 12 h day-night cycle. All mice were allowed access to water and food ad libitum. All animal experiments were ethically approved by the regulatory authority of Maastricht University and were performed in compliance with the national and European guidelines

(AVD1070020173885). Group sizes were calculated priori to ensure a statistical power of 90% using G*Power 3.1 (Heinrich Heine University, Dusseldorf, Germany; www.psycholgie.hhu.de).

To mimic cerebral hypoperfusion, a hallmark for cSVD, we used the well-established BCAS mouse model. Following a period of acclimatisation, mice of 10–12 weeks old (weighting between 20 to 30 g) were randomly allocated to undergo a BCAS (n = 13) or a Sham surgery (n = 7) as described previously [56]. Briefly, mice were anaesthetised and micro coils with an internal diameter of 0.18 mm (Sawane Spring Co., Ltd, Hamamatsu, Japan) were placed around the left and right common carotid arteries in BCAS-operated mice with a 30 min delay between both sides. The same surgery was performed in Sham-operated animals without placement of the micro coils. Animals were returned to their home cages in groups of two per cage and were closely monitored daily to ensure complete recovery until they were humanely sacrificed at day 7 post-procedure. Animals were weighted before surgery and at day 1, 2, 3 and 7 after surgery. Animals received pre- and post-operative analgesia (buprenorphine 0.05 mg/kg SC pre- and postsurgery; and buprenorphine 9 µg/mL in drinking water for the first 3 days) as advised by the named veterinary surgeon.

CBF was measured during surgery by laser doppler flowmetry (LDF, moorVMS-LDF2, Moor Instruments Inc, Axminster, UK) and measurements of the cortical CBF were additionally performed under anaesthesia before (baseline), after (d0) and 7 days after (d7) surgery using a Laser Speckle Contrast Imager (LSCI, Pericam, PSI, Perimed AB, Järfälla, Sweden). Mice with a CBF reduction exceeding 50% (measured with LDF) or with a body weight reduction exceeding 15% compared to the body weight prior to surgery were humanely sacrificed and excluded from the study. Mice were sacrificed by perfusion under terminal anaesthesia 1-week post-surgery. Pimonidazole, a hypoxic marker, was injected 1

h prior to sacrifice (60 mg/kg, I.P., HypoxyProbe Inc., Burlington MA, USA) in conscious mice before CBF measurements.

Brains were harvested post-mortem following perfusion with ice-cold PBS using a peristaltic pump. A sagittal cut was used to split the brain hemispheres and the left hemisphere was fixed overnight in 4% paraformaldehyde (PFA), washed in PBS, and transferred to PBS with 0.1% sodium azide (NaN_3) before vibratome sectioning.

Cerebral blood flow imaging and data processing

Animals were injected with buprenorphine (0.05 mg/kg, S.C.) one hour prior to surgical procedures. Anaesthesia was initiated using isoflurane (4%) before placing mice on a stereotaxic monitoring platform (Harvard Apparatus, Hollistion, MA, USA). Heart rate, breathing rate and oxygen saturation were constantly measured during the entire procedure to monitor the animal sedation and to ensure CBF was measured in physiological conditions. Animals were kept sedated via isoflurane inhalation at a constant concentration of 1.8–2.2%. Lidocaine was injected locally onto the periosteum, followed by a midline skin incision. The skull was kept moist using a light (Cat no. 163-2129, Bio-rad laboratories, Inc., Hercules, CA, USA) and a heavy (Cat no. 330760, Sigma- Aldrich, St. Louis, MO, USA) mineral oil during imaging with the LSCI. The field of view (FOV) (1 × 1 cm) was imaged at a frame rate of 44 images/sec and resolution of 0.01 mm/pixel. Colour-coded CBF images correlated with the blood flow velocity were obtained. CBF imaging was performed before (baseline), immediately after (d0), and 7 days (d7) after surgery. At the end of d0, 6 h after the initial injection, a second injection of buprenorphine (0.05 mg/kg, S.C.) was performed, and buprenorphine administration was continued via the drinking water overnight (9 μg/mL, oral) as post-operative analgesia for all animals.

CBF images were analysed in PIMSoft (Perimed AB). CBF absolute values were obtained over the whole cortical surface (222.5±1.6 mm^2) and above the third order branch from superior sagittal sinus (8.8±0.1 mm^2) to avoid large superficial vessels. CBF values were averaged over a 1 min period. Relative CBF change was calculated as percentage of baseline measurements for both regions of interest (ROI).

CBF reduction during placement of the micro coils was monitored using LDF. After identifying the bregma and lambda, a small area was superficially thinned with a dental drill on the left primary somatosensory cortex for positioning a 0.5-mm flexible fibreoptic laser probe (Moor POF500, Moor instruments Inc.), which was removed after the surgical procedure. CBF was monitored throughout the surgery using IDEEQ (M-PAQ, Development Engineering & Evaluation [IDEE], Maastricht, The Netherlands).

Immunohistochemistry (IHC)

Using a vibratome (VT1200S, Leica, Freiburg, Germany), 30 µm-thick coronal sections were cut, and the free-floating sections were permeabilised and blocked with 1% donkey serum in TBS-T (0.1–1% Triton-X). Sections were then incubated overnight with primary antibodies in blocking buffer. Sections were then incubated with secondary antibodies at RT for 2 h. Antibodies used are provided in Additional file 1: Table S1. Finally, the sections were mounted on gelatin-coated microscopic slides with a fluorescence-preserving mounting medium (Prolong gold antifade, Cat no. P36934, ThermoFisher Scientific).

IHC image acquisition and analysis

Images were acquired with the experimenter masked to the group conditions using a confocal microscopy (DMI 4000 B, TCS SPE, Leica, Amsterdam, The Netherlands) or fluorescent

slide scanner (ImageXpress Pico Automated Cell Imaging System, Molecular Devices, San Jose, CA, USA). To assess myelin integrity and the total number of hypoxic cells (Pimonidazole^{+}), hypoxic OPC (Pimonidazole^{+}/Olig2^{+}/CC1^{-}), nine FOVs of the corpus callosum (CC) (three per coronal section, with three sections [between +1.3 mm and −0.5 mm bregma]) were obtained per animal. Twenty microns thick image stacks were obtained using the confocal microscope, and processed and analysed using Fiji package [55] in ImageJ software (National Institutes of Health, Bethesda, MD, USA). The total number of hypoxic cells and hypoxic OPC were quantified in the deep cortical regions by a blinded observer and expressed in number of cells per mm^2. Myelin content and integrity were assessed within the CC. MBP grey value signal and negative areas were measured in the CC. Early myelin damage was analysed using the anti-MBP clone SMI94 to assess focal myelin degradation [7]. Hyperintense SMI94 foci in the CC were automatically detected on maximally projected volumes using the local maxima value, after thresholding. Images for Lectin/IgG were obtained at 20X magnification using the fluorescent slide scanner. BBB permeability was investigated in six sections (between +2 mm and −1 mm bregma). Sections were investigated for vascular density and extravascular leaked IgG was quantified using AngioTool [77] and ImageJ, respectively. Leakage size was measured by quantifying the area of IgG signal outside of the vascular mask delineated by the lectin signal using the Otsu threshold method. The reported total leakage size per animal corresponds to the sum of the sizes of individual leakages measured in six sections per animal (mm^2).

Cell cultures

Primary OPC cultures were prepared as previously described [47, 54, 58]. Briefly, cerebral cortices from new-born (P0) C57BL/6J mice were isolated, dissected and digested. Mixed glia

cultures were maintained in DMEM with high glucose (Cat no. D6429, Merck Millipore, Burlington, MA, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C and 8.5% CO_2 on poly-L-lysine (PLL) coated flasks. Medium was changed every 3–4 days and insulin (5 µg/ ml) was added from day 7 onwards. Cultures were maintained for 12–14 days before initial shaking on an orbital shaker (75 rpm) at 37 °C for 45 min to remove microglia. The medium was then discarded, and flasks were shaken (280 rpm) with fresh medium overnight (16–18 h) at 37 °C. Medium was collected and plated on noncoated tissue culture dishes for 30 min at 37 °C and 8.5% CO_2. The non-adherent cells (OPC) were collected and maintained in DMEM with high glucose containing 0.5% FBS, 1% P/S, 1% B27 supplement, bovine serum albumin (102 ng/ml), putrescine dihydrochloride (29.4 ng/ml), triiodothyronine (0.414 ng/ml), l-thyroxine (0.40 ng/ml), sodium selenite (5.00 pg/ml), progesterone (60.0 pg/ml), apo-transferrin (50 µg/ml), insulin (5 µg/ml) on PLL-coated plates. PDGF-AA (10 ng/ml), and FGF-2 (10 ng/ ml) was added for 24 and 48 h, respectively, to limit spontaneous OPC differentiation before experiments (Additional file 1: Fig. S4).

The immortalised OPC line, Oli-neu (RRID:CVCL_IZ82), originally obtained by transfecting primary mouse OPC with the replication defective retrovirus expressing the t-neu oncogene [27], were maintained in DMEM with high glucose containing 10% FBS and 1% P/S at 37 °C and in an atmosphere of 5% CO_2.

Hypoxic primary OPC experiments

Primary OPC cultures were exposed to either normoxic (21% oxygen) or hypoxic (2% oxygen) conditions for 24 h at 37 °C and 8.5% CO_2 in fresh PDGF-AA and FGF-AA free OPC medium. After 24 h, medium was collected, and cells were lysed and stored at −20 °C before RNA isolation.

RNA isolation and sequencing

Primary OPC were lysed using RLT buffer (Qiagen, Hilden, Germany) and frozen at −80°C. Samples were later thawed, and RNA was isolated using the RNeasy Micro kit following the manufacturer's instructions (Cat no. 74004, Qiagen). RNA quantity was checked using Qubit 2.0 Fluorometer (Invitrogen, Waltham, MA, USA) and RNA quality was assessed using Bioanalyzer (Cat no. RNA 6000 Nano kit; 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). Purification of mRNA from total RNA (NEXTFLEX Poly(A) Beads 2.0, Cat no. NOVA-512992, PerkinElmer, Waltham, MA, USA) and directional, strand specific RNA library preparation (NEXTFLEX Rapid Directional RNA-Seq Kit 2.0, Cat no. NOVA-5198, PerkinElmer) was performed according to manufacturer's protocol. Sequencing was performed using NovaSeq 6000 Sequencing system (NovaSeq S Prime flow cell 200 cycles; NovaSeq 6000, Illumina, Inc, San Diego, CA, USA) according to manufacturer's protocol.

RNA sequencing analysis

The raw sequencing data was trimmed using fastp. The remaining reads were mapped against the Ensembl mouse genome (release 100) using STAR (version 2.7.3a) and quantified using RSEM (v.1.3.1). The resulting raw read counts were processed using the R package DESeq2. Genes that were not sequenced (0 reads) in more than 75% of the samples of any given condition were removed. Genes were considered differentially expressed with an adjusted p-value (false discovery rate; FDR) below 0.01. Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched pathways, Gene ontology (GO) classification and UniProt functional annotation, terms approximating cellular component, biological process, and molecular function, were used to identify functional enriched differentially expressed genes

(DEG) using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 [22, 23]. The modified Fisher exact p-value (EASE score) <0.05 and FDR <0.05 were considered enriched.

Hypoxic Oli-neu experiments

The immortalized OPC cell line, Oli-neu, was exposed to either normoxic or hypoxic conditions (as above) for 12, 24, or 48 h at 37 °C and 5% CO_2. Medium was collected, and cells were lysed for RNA isolation and stored at −20 °C. Experiments were repeated in cells transfected with Negative control siRNA (Silencer™ Cy™3-labeled Negative Control No. 1 siRNA, Cat no. AM4621, Invitrogen), *Hif1a* siRNA (Silencer™ Select Pre-Designed mouse *Hif1a* siRNA, sequence 5'→3': Sense CCUUUACCUUCAUCGGGAAAtt; Antisense UUUCCGAUGAAGGUAAAGGag, Cat no. 4390771, Invitrogen) and/or *Epas1* siRNA (Silencer™ Select Pre-Designed mouse Epas1 siRNA, sequence 5'→3': Sense CGGAUCCACCAUUACAUUUtt; Antisense AAAUGUAAUGGUGGAUCCGgg, Cat no. 4390771, Invitrogen). Briefly, cells were transfected with 20 pmol siRNA and 6 µg/ml Lipofectamine™ 2000 Transfection Reagent (Cat no. 11668, Invitrogen) in high glucose DMEM containing 10% FBS and 10% Opti-MEM (Cat no. 31985070, ThermoFisher Scientific) for 24 h. Cells were then washed with PBS and incubated at normoxic or hypoxic conditions with normal culturing medium. After 24 h, cells were lysed and stored at −20 °C until further use.

Conditioned medium experiments

Immortalised mouse brain EC (bEnd.3) cells were cultured with culture medium diluted (1:1) with conditioned medium (CM) obtained from Oli-neu exposed to normoxic or hypoxic conditions for 24 h. After 24 h, cells were lysed for RNA isolation and stored at −20 °C until further use.

Quantitative PCR

Total RNA was isolated from Oli-neu cells using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol and stored at − 80 °C before use. Quality and quantity were checked using NanoDrop 1000 spectrophotometer and the RNA was reverse transcribed into cDNA using the high-capacity RNA-to-cDNA kit (Cat no. 1708891, Bio-rad laboratories, Inc.) according to manufacturer's manual. cDNA samples were stored at −20 °C before use. Quantitative PCR was performed using Sensimix™ SYBER® & Fluorescein kit (Cat no. QT615-05, Meridian Bioscience Inc., Cincinnati, OH, USA) on the Light Cycler 480 (Roche Applied Science, Penzberg, Germany) with the following qPCR program: 10 min at 95 °C followed by 55 cycles a 10 s at 95 °C and 20 s at 60 °C. Temperature was increased from 60 to 95 °C for melting curve analyses. Primers were designed to cover exon-exon junctions and all possible splice variants using NCBI Primer-BLAST tool. Primers were synthesised by Eurofins Genomics (Ebersberg, Germany) and quality was ensured by testing on appropriate tissue or cell cultures, as well as by calculation of primer efficiency employing a cDNA dilution curve. Two stable reference housekeeping genes (*Rpl13a* and *Ywhaz*) were selected from a selection of three genes by using the GeNorm Software (Primerdesign, Southampton, NY, USA). Primers are listed in Additional file 1: Table S2. Gene expression

analysis was performed using LinReg PCR (Ver. 2014.0) and the Light Cycler 480 data converter (Ver. 2014.1).

Enzyme-linked immunosorbent assay (ELISA)

CM was obtained from primary OPC or Oli-neu cells after exposure to either 21% or 2% O_2 for 24 h. Secreted VEGFA concentration was determined in diluted (1:1) samples using commercially available Mouse VEGF-A ELISA kit (Cat no. BMS619-2, Invitrogen) according to manufacturer's protocol.

Human brain imaging analysis

Study population

We performed a retrospective pilot study, for which we used data of an MRI study in cSVD. Eighty patients with clinically manifested cSVD (lacunar stroke or vascular cognitive impairment) and 39 age- and sex- matched controls were included. Inclusion criteria, and methods for image acquisition, processing and analysis have been described previously and is provided in Appendix A [28, 64, 65, 72].

The Medical Ethics Committee of the Maastricht University Medical Centre approved the study. All participants were included after providing written informed consent according to the Declaration of Helsinki. This study is registered on trialregister.nl (NTR number: NTR3786). Participants were included from the Maastricht University Medical Centre and Zuyderland Hospital, the Netherlands, between April 2013 and December 2014.

Structural MRI

All participants underwent structural brain imaging on a 3.0T MRI system (Achieva TX, Philips Healthcare, Best, the Netherlands) using a 32-element head coil suitable for parallel imaging. T1-weighted sequence was used for anatomical reference and T2-weighted fluid-attenuated inversion recovery (FLAIR) sequence for detecting WMH [28, 72].

Permeability MRI

Details of the scanning protocol have been published [72]. Dual-time resolution dynamic contrast enhanced (DCE)-MRI consisted of a fast and slow dynamic scan time sequence to sample properly the various timescales in the contrast enhancement curves. Scans of both sequences were acquired prior to contrast injection, followed by the fast sequence during contrast bolus injection (Gadobutrol), and then followed by the slow sequence.

Image processing and analysis

Tissue segmentation

Grey and white matter were segmented on T1-weighted images using Freesurfer [15]. NAWM and WMH were differentiated by segmenting WMH on FLAIR images automatically, with manual corrections by a trained assessor [11]. T1 and FLAIR Images were coregistered using FSL (v5.0) and the NAWM and WMH were selected [26].

Pharmacokinetic modelling

DCE-MRI data was pharmacokinetically modelled and histogram analysis was performed as previously described [17, 73]. Briefly, contrast agent tissue concentration was calculated by using the relative signal change over time and T1 mapping, using the superior sagittal

sinus as vascular input function [33]. Subsequently, the Patlak pharmacokinetic model was used to calculate the BBB leakage rate in terms of the leakage rate K_i (min^{-1}) [50].

Histogram Analysis

K_i was determined in a voxel wise manner [28, 65, 72]. For both NAWM and WMH, a K_i voxel histogram was composed, and noise correction was applied by mirroring and then subtracting negative K_i value distribution from the original K_i distribution, resulting in a histogram of K_i values reflecting detectable leakage rates. Mean leakage rate (mean K_i) was calculated by taking the average of all noise-corrected K_i values. The relative volume of leakage with respect to the tissue volume, the fractional leakage volume (v_L), was the remaining area under the noise corrected histogram curve [72].

Retrospective blood plasma VEGFA analysis

Blood samples from all participants were collected. Plasma supernatant was obtained from these samples and stored for future analysis at −80°C. Plasma VEGFA levels were determined with the Human VEGF-A ELISA kit (Cat no. BMS277-2, Invitrogen) according to manufacturer's protocol. Samples with absorption values below blank measurements were considered artefacts and excluded from further analysis. Analyses including samples with VEGFA concentrations below the detection limit are provided in Appendix A (Fig A1).

Statistical analysis

Data were analysed in GraphPad Prism 9 (Dotmatics) or SPSS version 28 (IBM Corp., San Diego, CA, USA). Unpaired Student t-tests or Mann-Whitney tests (for non-parametric data) were used to compare Sham vs BCAS, normoxia vs hypoxia exposed cells, and VEGFA in

controls vs cSVD patients. One-way ANOVA with post-hoc Tukey's multiple comparisons tests was used to assess multiple comparisons. Uni- and multivariable regression analysis was performed with group (cSVD or control) as independent and MRI measures (WMH volume, and BBB leakage K_i and V_L in NAWM and in WMH) as dependent variables, and correction for age and sex. Outliers for VEGFA plasma concentration were identified (defined as 3rd quartile + 1.5*interquartile range). The relation between VEGFA plasma concentration and MRI characteristics was assessed with uni- and multivariable regression analysis, with correction for age and sex, for both patients and controls after removal of outliers (details on outliers are provided in Additional file 1: Table S4). Analyses without exclusion of outliers are provided in the supplementary data. Estimated 95% confidence interval were obtained with standardised Z-scores. $P<0.05$ was considered statistically significant and data are expressed as mean ± SEM.

Results

BCAS led to a persistent cerebral blood flow reduction after 7 days

Three mice in the BCAS group had to be sacrificed due to post-operative complications, whereas full recovery was seen in the rest (Sham: $n = 7$; BCAS: $n = 10$; Additional file 1: Fig. S1). Blood flow was measured in a ROI containing all sizes of superficial vessels (ROI1, Fig. 1A), and a smaller ROI above the 3rd order branch from superior sagittal sinus to avoid larger superficial vessels (ROI2, Fig. 1A). A significant decrease in CBF in ROI1 was observed in BCAS mice compared to baseline measurements at d0 (− 14.7 ± 5.3%, $p = 0.02$) and at d7(− 27.2 ± 3.6%, $p < 0.0001$), while no significant changes were observed in Sham

mice (Additional file 1: Fig. S1E). CBF differed between BCAS and Sham at both d0 (p = 0.013) and d7 (p < 0.0001) (Additional file 1: Fig. S1E). At d0, CBF in ROI2 was not significantly decreased in BCAS mice (− 10.7 ± 5.7%, p = 0.089) compared to baseline measurements, while CBF was increased in Sham mice (+ 23.2 ± 8.5%, p = 0.034, Fig. 1B). At d7, CBF in ROI2 was significantly decreased in BCAS mice (− 22.3 ± 5.6%, p < 0.01) compared to baseline, while CBF was normalised in Sham mice (+ 11.4 ± 7.8%, Fig. 1B). CBF in ROI2 differed between BCAS and Sham at both d0 and d7 (p < 0.01).

Cerebral hypoperfusion did not alter myelin integrity after 7 days

Structural changes in the CC were investigated ex vivo by an immunostaining for MBP (Fig. 2A). MBP intensity and MBP negative area analysis showed no significant structural changes in the CC BCAS compared to Sham mice (p = 0.93 and p = 0.90, respectively, Fig. 2B, C). SMI94 was used to investigate early myelin changes. SMI94 detects an MBP peptide known to only be exposed during myelin degradation [39]. There was no difference in the total number of hyperintense foci between BCAS and Sham mice (p = 0.65, Fig. 2D).

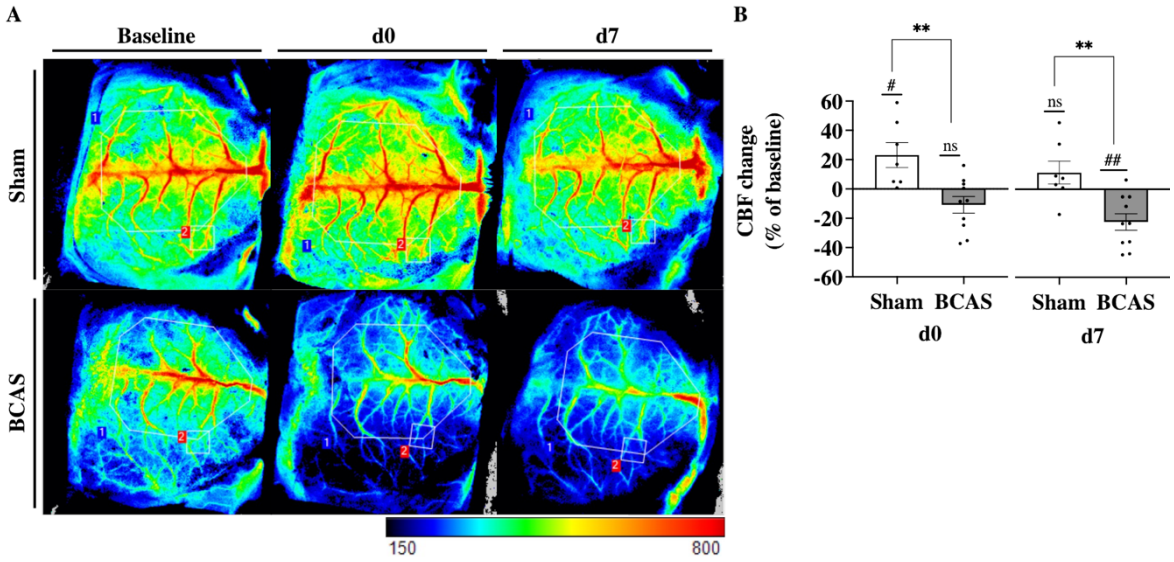


Figure 1: Bilateral carotid artery stenosis led to a persistent decrease in cerebral blood flow. (A) LSCI images showing blood flow in superficial blood vessels captured at baseline, d0, and d7. Blood flow measurements in ROI1 (blue) contained all sizes superficial blood vessels, while ROI2 (red) contained the 3rd order branch from superior sagittal sinus to avoid larger superficial vessels. Visualization of blood flow signal ranged from high flow (red) to low flow (blue). **(B)** Changes in CBF were quantified and compared to baseline measurements at d0 and d7 (ROI2). Scale bar, arbitrary value, 150 (low blood flow), 800 (high blood flow). Mean±SEM; ns=not significant; Data points (dots) indicate biological replicates; #p<0.05, ##p<0.01 vs baseline measurements; *p<0.05, **p<0.01, vs Sham; unpaired student t-test.

Cerebral hypoperfusion led to increased hypoxia in OPCs in the deep cortical regions

Immunolabeling for Olig2, CC1, and pimonidazole revealed OPCs, oligodendrocytes, and hypoxic cell densities in the deep cortical regions, respectively (Fig. 2E). A significant increase in hypoxic cells was observed in BCAS mice when compared to Sham (12.9 ± 1.3 vs 7.8 ± 1.1 cells/mm2; p = 0.013, Fig. 2F). Olig2 and CC1 signals in hypoxic cells were thereafter used to identify hypoxic OPCs and oligodendrocytes. Hypoxic CC1 cells were not detected. In contrast, hypoxic Olig2 cells were increased in abundance in the deep cortical regions of BCAS mice compared to Sham (2.3 ± 0.4 vs 0.6 ± 0.3 cells/mm^2, p = 0.004, Fig. 2G). Neither OPCs nor oligodendrocyte density was altered in BCAS mice compared to Sham (Additional file 1: Fig. S3).

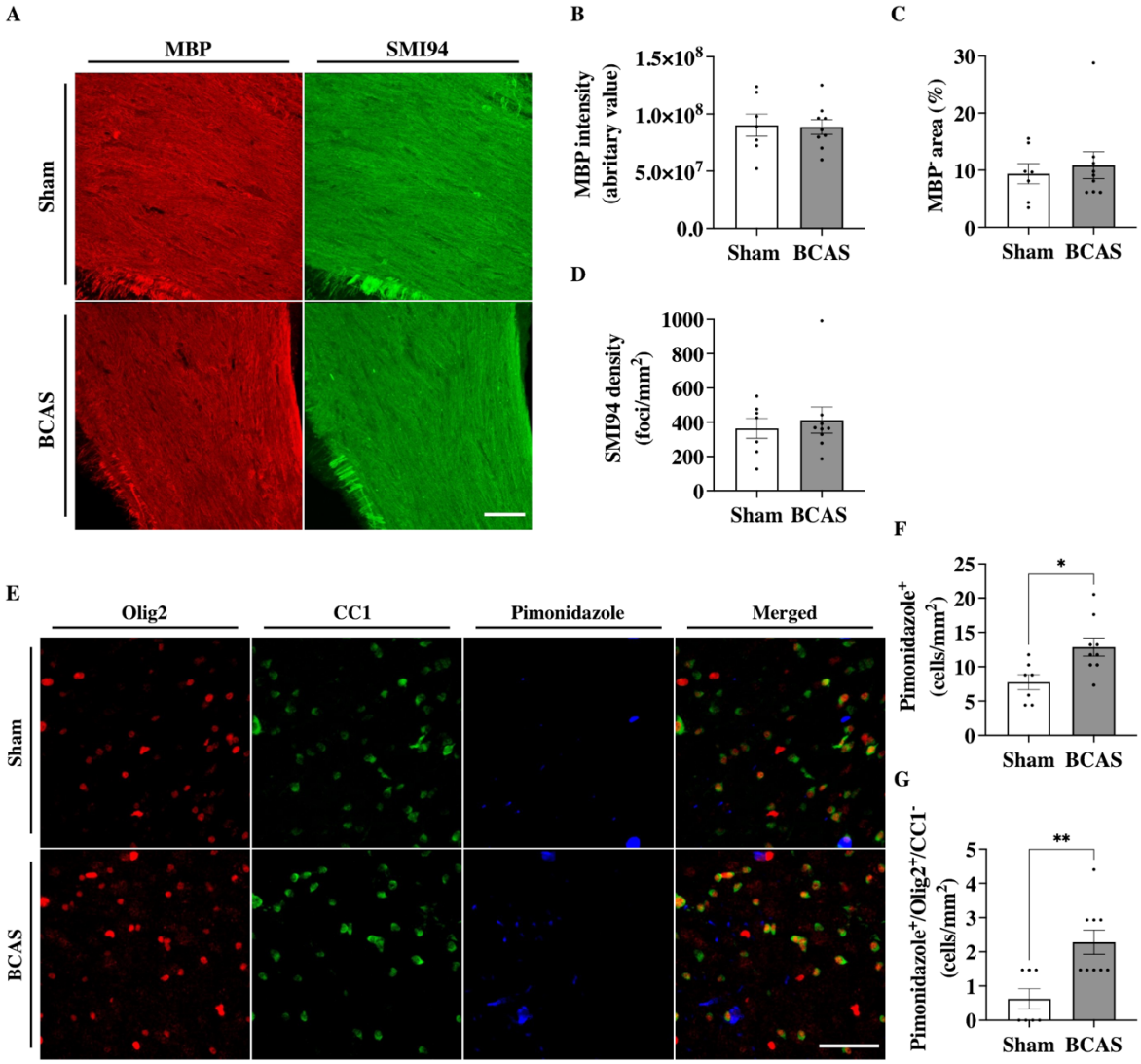


Figure 2: BCAS led to an increase in hypoxic OPC in deep cortical regions after 7 days. (A) Immunolabeling for MBP and MBP clone SMI94 in the corpus callosum. Scale bar, 50 µm. **(B)** Quantification of MBP integrity, **(C)** MBP negative area, and **(D)** Myelin degradation, quantified by intensity signal and the number of hyperintense foci, respectively, was not statistically different between BCAS and Sham mice 7 post-operative days. **(E)** immunolabeling for Olig2, CC1, and Pimonidazole-Pacific blue in the deep cortical regions. Scale bar, 50 µm. **(F)** A significant increased hypoxic cell density was observed in the deep cortical regions of BCAS animals compared to Sham animals. **(G)** BCAS mice also showed an increased number of hypoxic OPC in these regions compared to Sham animals. Mean±SEM; Data points (dots) indicate biological replicates; *p<0.05, **p<0.001; unpaired student t-test or Mann-Whitney U-test. Statistically non-significant comparisons are not shown.

Hypoxia in OPC led to increased VEGFA secretion in vitro

To further investigate the effects of hypoxia in OPC, we cultured primary OPC in hypoxic condition. We then identified DEG in normoxic vs hypoxic OPC by RNA sequencing. This

led to the identification of 417 DEG with an FDR below 0.01, of which 246 were up-regulated and 171 down-regulated (Fig. 3A). Figure 3B represents the volcano plot, with all upregulated DEGs in green and the down regulated DEGs in red. DAVID enrichment analysis was performed for the functional annotation of the identified DEG (Fig. 3C). HIF-1 signalling pathway (5.5-fold change [FC], FDR < 0.0001), positive regulation of cell migration (3.1 FC, FDR = 0.047), cell junction (2.4 FC, FDR < 0.0001), and angiogenesis (3.9 FC, FDR = 0.014) were considered as enriched pathways that might be involved in crosstalk of OPC with surrounding cells. Vascular endothelial growth factor A (*Vegfa*) was identified as a potential mediator in the mentioned enriched pathways (log2FC = 1.14; −log(FDR) = 22.4). To investigate the potential of VEGFA protein as mediator of crosstalk, secreted protein levels in the CM of these cells were measured. Hypoxia exposure led to a significant increase in VEGFA protein secretion by primary OPCs compared to normoxic condition (272.2 ± 7.9 vs 81.6 ± 7.5 pg/ml, $p < 0.0001$; Fig. 3D).

VEGFA increase in hypoxic OPCs was mediated by *Hif1a* and *Epas1*

Expression of Vegfa mRNA was measured in Oli-neu cells after 12 h, 24 h, and 48 h hypoxia exposure and showed a persistent significant increase in mRNA expression (12 h: 6.75 ± 1.40 FC, $p < 0.01$; 24 h: 11.81 ± 1.02FC, $p < 0.0001$; 48 h: 7.78 ± 0.91 FC, $p < 0.0001$; Fig. 4A). This increase in *Vegfa* expression translated in secreted VEGFA protein concentration as measured in hypoxia exposed CM at 24 h compared to normoxia (5329 ± 145.4 vs 617.8 ± 15.83 pg/ml, $p < 0.0001$; Fig. 4B).

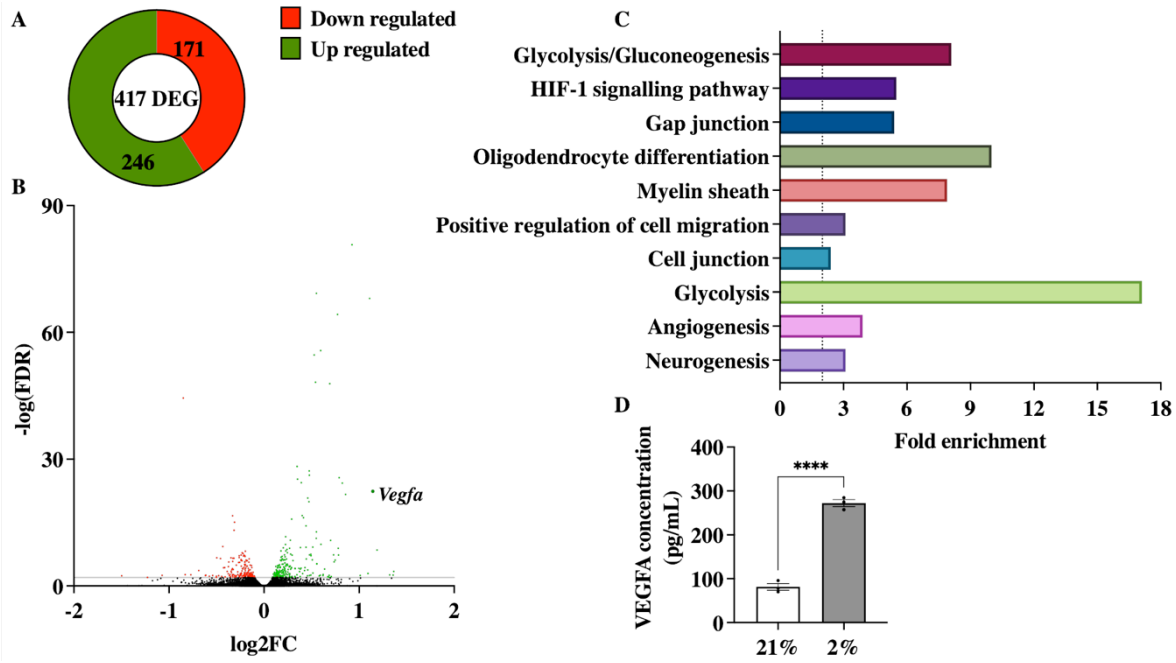


Figure 3: Hypoxia induced the expression of 417 DEGs. (A) Hypoxia (2% O_2) induced the expression of 417 differentially expressed genes (DEG) compared to normoxia (21% O_2) in OPC after 24h exposure, of which 246 were upregulated and 171 downregulated. **(B)** Volcano plot showing the 417 DEG in hypoxic OPC with genes that were downregulated in red, and upregulated genes in green. Note that hypoxia did not significantly alter expression of Wnt7a or Wnt7b in primary OPCs at this time point **(C)** DAVID enrichment analysis shows enrichment of pathways including HIF-1 signalling pathway, positive regulation of cell migration, and angiogenesis. Terms were considered significant with an EASE score <0.05 and FDR <0.05. **(D)** VEGFA was identified as a potential key mediator in these pathways and secreted protein concentration in the hypoxia exposed conditioned medium was increased compared to normoxia. Data points (dots) indicate technical replicates. Mean±SEM; ****p<0.0001, vs 21%; unpaired student t-test.

Expression of *Hif1a*, the most important gene involved in hypoxic signalling, was not significantly increased in hypoxia exposed Oli-neu cells compared to normoxic cells (1.20 ± 0.13 FC; Fig. 4C), while expression of *Epas1*, one of the DEGs involved in angiogenesis and related to hypoxia and VEGFA signalling [36], was significantly increased (2.68 ± 0.26 FC, $p < 0.0001$; Fig. 4C). Oli-neu cells transfected with negative control siRNA and exposed to hypoxia showed a significant increase of *Vegfa* expression (6.28 ± 1.38FC, $p < 0.0001$; Fig. 4D) compared to normoxia, while inhibition of *Hif1a* resulted in normalisation of *Vegfa* expression (0.75 ± 0.16 FC compared to normoxia, $p = 0.99$; Fig. 4D). *Epas1* inhibition decreased *Vegfa* in hypoxic cells significantly and was thus not significantly different from

normoxic expression (2.49 ± 0.62 FC, p = 0.54, Fig. 4D). Inhibition of expression of both genes decreased Vegfa expression by twofold compared to normoxia (0.44 ± 0.11 FC, p = 0.98, Fig. 4D).

As VEGFA is known to regulate the expression of tight junction proteins, Claudin-5 (*Cldn5*) and Occludin (*Ocln*) mRNA expressions were measured in mouse bEnd.3 ECs treated with CM from either normoxic or hypoxic Oli-neu cells [2, 3, 32, 44, 61]. Both *Cldn5* and *Ocln* expression in bEnd.3 cells were significantly decreased when treated with hypoxic CM (0.34 ± 0.05 FC, p = 0.005 and 0.47 ± 0.09 FC, p = 0.006, respectively; Fig. 4E, F).

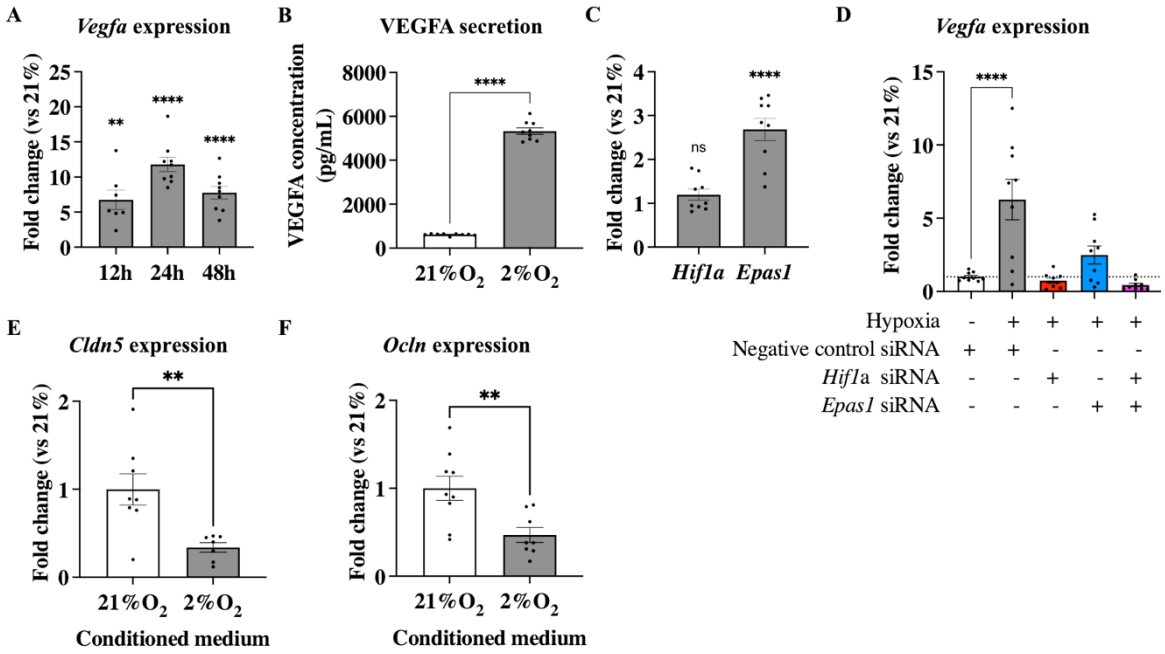


Figure 4: Hypoxia induced expression and secretion of VEGFA in Oli-neu cells. (A) The expression of *Vegfa* mRNA was constantly increased in hypoxic Oli-neu cells exposed to 2% O_2 compared to normoxic cell expression (21% O_2) from 12h to 48h, with a peak increase after 24h. **(B)** Hypoxic Oli-neu cells significantly secreted more VEGFA protein into conditioned media 24h after exposure to hypoxia (2% O_2) compared to normoxia (21% O_2). **(C)** Oli-neu cells exposed to hypoxia did not significantly increase mRNA expression of *Hif1a* after 24h exposure, while *Epas1* (aka *Hif2α*) was significantly increased compared to normoxic cell expression. **(D)** inhibition of *Hif1a* and/or *Epas1* results in inhibition of hypoxia mediated increase in *Vegfa* expression. **(E)** Both *Claudin-5* (*Cldn5*) and **(F)** *Occludin* (*Ocln*) mRNA expression was significantly decreased in brain EC treated with hypoxic OPC derived CM. Mean±SEM; ns=not significant, Data points (dots) indicate technical replicates; **p<0.01, and ****p<0.0001, vs 21%; unpaired student t-test or one-way ANOVA with Tukey's multiple comparisons test.

Hypoperfusion led to increased BBB permeability in mice after 7 days

The effects of VEGFA play a crucial role in the regulation of angiogenesis and BBB permeability [40, 70]. Thus, we investigated vascular density and BBB permeability changes in our BCAS mice. After 1 week of hypoperfusion, vascular density was not changed in the cortex (p = 0.22), deep cortex (p = 0.28), CC (p = 0.60), or striatum (p = 0.39) when comparing BCAS to Sham (Fig. 5A, B). We then assessed BBB permeability by quantifying IgG extravasation in BCAS and Sham mice (Fig. 5C). The number of leakages in BCAS mice was significantly higher compared to Sham (4.00 ± 0.93 vs 0.71 ± 0.42 leakages/brain, $p < 0.01$, Fig. 5D). Furthermore, the total leakage size was significantly higher in BCAS compared to Sham (7079 ± 3297 vs 480 ± 390 mm^2, $p < 0.01$, Fig. 5E).

Increased VEGFA plasma levels in cSVD patients correlated with increased BBB permeability in NAWM

We excluded samples considered as artefacts and 12 samples showing outliers for VEGFA concentration (eight patients and four controls) from further analysis. This resulted in 47 samples of cSVD patients and 26 controls; characteristics are presented in Table 1.

Higher WMH volume, and higher BBB leakage volumes (V_L) both in NAWM and WMH were significantly associated with cSVD patient group (Table 1). Plasma VEGFA concentration was higher in cSVD patients compared to controls (25.26 ± 3.07 vs 14.45 ± 2.08 pg/ml, p = 0.035, Fig. 6A). In cSVD patients, we found a trend in the relation between

VEGFA plasma concentration and BBB leakage rates K_i in the NAWM and WMH (p = 0.051 and p = 0.051, respectively; Table 2 and Fig. 6C, D).

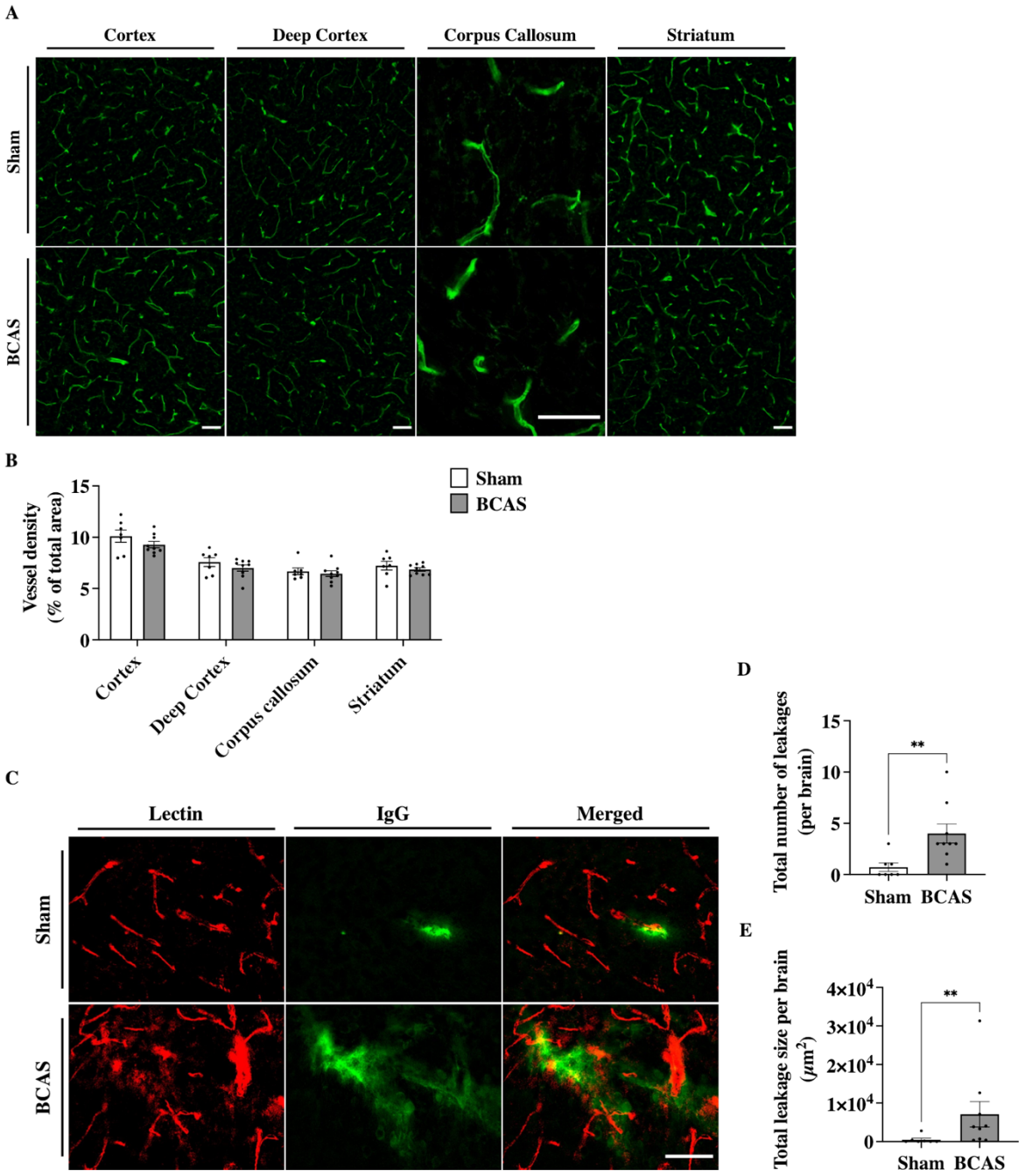


Figure 5: BCAS led to an increase in BBB permeability after 7 days without changes in vascular density. (A) immunolabeling for lectin in the cortex, deep cortex, corpus callosum, and striatum. Scale bar, 50 µm. **(B)** Quantification of vessel density in BCAS compared to Sham. No statistical differences were found in vessel density when comparing BCAS to Sham in the respective areas. **(C)** Immunolabeling for blood vessel (Lectin), and mouse IgG for BBB leakages. Scale bar, 50 µm. **(D)** The number of leakages and **(E)** the total leakage size was significantly higher in BCAS compared to Sham. Mean±SEM; Data points (dots) indicate biological replicates; **p<0.01, vs Sham; unpaired student t-test. Statistically non-significant comparisons are not shown.

Table 1: Characteristics of the clinical study population. Characteristics in Mean+SEM unless otherwise indicated. Association between WMH, BBB leakage rate (K_i) and volume (V_L) and participant group was determined using a uni- and multivariable (corrected for age and sex) analysis. Standardised Coefficients Beta (β) and *p*-value are shown for each analysis. $P<0.05$ was considered as significant.

		Controls (n=26)	cSVD Patients (n=47)	Univariable β (p-value)	Multivariable (age and sex corrected) β (p-value)
	Age (years)	67.8±2.5	69.6±1.6		
	Male (Female)	18 (8)	30 (17)		
WMH	**Relative volume, 10^{-3}**	3.5±1.8	16.1±2.4	0.390 (<0.001)	0.412 (<0.001)
BBB leakage in NAWM	**K_i, $10^{-3}min^{-1}$**	1.09±0.07	0.98±0.05	-0.176 (0.137)	-0.182 (0.129)
	v_L	0.276±0.034	0.370±0.028	0.241 (0.040)	0.253 (0.035)
BBB leakage in WMH	**K_i, $10^{-3}min^{-1}$**	0.85±0.06	0.84±0.04	-0.016 (0.893)	-0.033 (0.723)
	v_L	0.318±0.037	0.459±0.031	0.318 (0.006)	0.328 (0.005)

Abbreviations: WMH = white matter hyperintensities; BBB = blood-brain barrier; NAWM = normal appearing white matter; K_i = leakage rate; v_L = leakage volume.

However, age and sex are known to influence both VEGFA plasma levels and BBB integrity [9, 53, 62, 68]. After adjustment for these covariates we found a significant relationship between VEGFA plasma levels and BBB leakage rate (K_i) in the NAWM in patients (p = 0.044, Table 2). VEGFA plasma levels were not associated with leakage rate in the WMH, leakage volume in NAWM and WMH, or WMH volume after correcting for age and sex (Table 2). In controls, no associations were found between VEGFA plasma levels and any of the imaged variables.

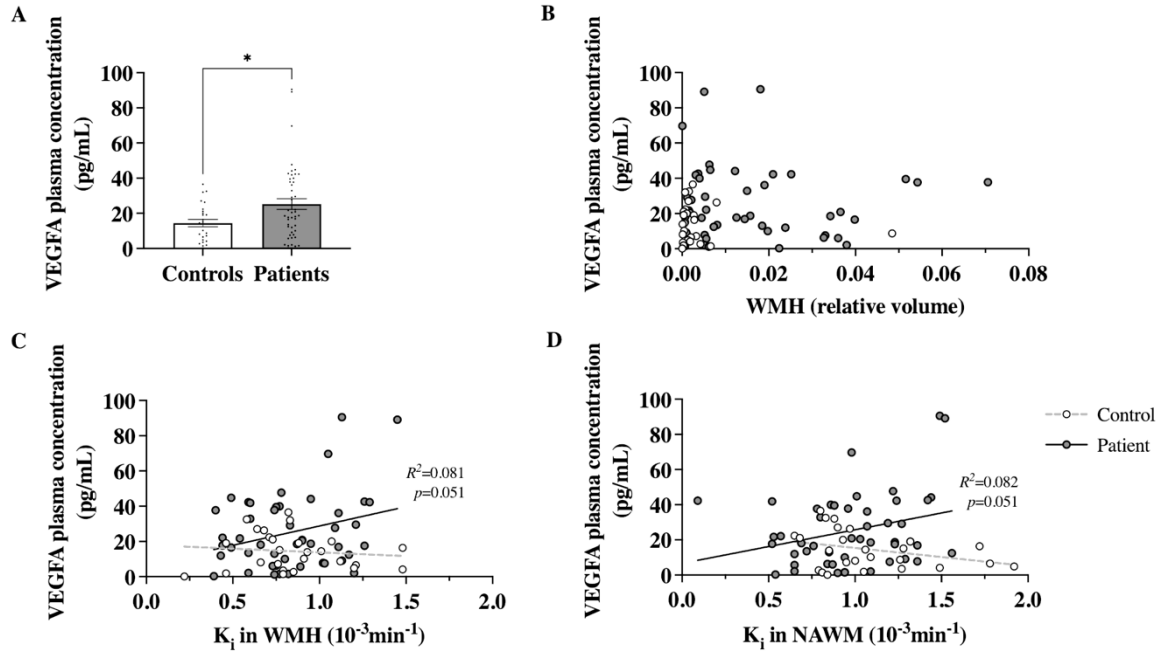


Figure 6: Increased VEGFA plasma levels in cSVD patients. (A) VEGFA blood plasma levels in cSVD patients were higher compared to age and sex-matched controls after identification and removal of outliers for VEGFA plasma concentration (defined as 3rd quartile + 1.5*interquartile range). Data points (dots) indicate biological replicates **(B)** There was no correlation between VEGFA plasma levels and WMH volume in patients or controls. A trend in the relation between VEGFA plasma levels and leakage rate in **(C)** WMH and **(D)** NAWM in patients, but not in controls, was observed (indicated by the solid line for patients and dotted line for controls). Abbreviations: WMH = white matter hyperintensities; NAWM = normal appearing white matter; K_i = leakage rate. For quantification, Mean±SEM; *p<0.05; Mann-Whitney U test.

Table 2: Association between increased VEGFA plasma levels and BBB leakage rate in the NAWM of cSVD patients. Univariable analysis was conducted with MRI characteristics as dependent and VEGFA as independent variable. Multivariable analysis was done by correcting for age and sex. Standardised Coefficients Beta (β) with the estimated 95% confidence interval (95%CI) and p-value are shown for each analysis. P<0.05 was considered significant.

		Univariable		Multivariable (age and sex corrected)	
		β [95%CI]	***p*-value**	**β [95%CI]**	***p*-value**
WMH		-0.024 [-0.324, 0.276]	0.873	0.009 [-0.277, 0.294]	0.951
BBB leakage in NAWM	**K_i**	0.286 [-0.001, 0.574]	0.051	0.307 [0.009, 0.605]	**0.044**
	v_L	-0.217 [-0.510, 0.076]	0.142	-0.255 [-0.556, 0.045]	0.093
BBB leakage in WMH	**K_i**	0.286 [-0.001, 0.574]	0.051	0.284 [-0.016, 0.585]	0.063
	v_L	-0.170 [-0.466, 0.126]	0.253	-0.208 [-0.511, 0.095]	0.174

Abbreviations: WMH = white matter hyperintensities; BBB = blood-brain barrier; NAWM = normal appearing white matter; K_i = leakage rate; v_L = leakage volume; CI = confidence interval.

Discussion

The aim of this study was to investigate the impact of cerebral hypoperfusion on early mechanisms involved in the development of BBB dysfunction and WM damage. We showed that cerebral hypoperfusion in mice, induced by carotid stenosis, leads to an increase in hypoxia in OPC residing in the deep cortical regions. Additionally, an increase in BBB permeability was observed without clear changes in vascular density and WM integrity. In vitro hypoxic conditions led to increased *Hif1a*- and *Epas1*-dependent VEGFA expression and secretion in Oli-neu cells, an OPC cell line, and downregulation of tight junction (TJ) proteins *Cldn5* and *Ocln* expression in bEnd.3 cells, a brain EC cell line. Finally, we showed a significant increase in plasma levels of VEGFA protein, derived from patients with intrinsic cSVD compared to age-matched controls. VEGFA levels correlated with BBB permeability in the NAWM of these patients, suggesting a potential role in the early mechanisms leading to BBB dysfunction and the development of WMH.

In our mouse experiments, CBF was decreased in BCAS at 7 days after surgery, while the CBF after Sham operation did not differ from baseline measurements. Similar results were found on CBF reduction at 2 h after BCAS and no changes in Sham-operated mice [19, 25, 31, 42, 46, 56, 57]. Taken together, we confirm a sustained decrease in CBF after 1 week in the BCAS model. To investigate the effects of hypoperfusion on myelin integrity and WML, we examined the MBP integrity in the CC of mice after 7 days of hypoperfusion. Our results showed no changes in myelin integrity, which is in line with earlier studies showing myelin damage due to cerebral hypoperfusion in BCAS mice only after 14 days, but not after 7 days [8, 37, 42, 56, 57].

Although myelin damage could not be detected after 7 days, an increased number of hypoxic cells were observed in the cortical region of BCAS mice. Previous findings also indicated a

hypoperfusion-mediated increase in cerebral hypoxia and hypoxia in the CC after 21 and 28 days, respectively [13, 31]. This apparent hypoxic susceptibility was also identified in a study using magnetic resonance angiography and arterial spin labelling to measure intracranial, cortical, and subcortical CBF in BCAS mice. There was a more substantial and lasting decrease in blood flow in the subcortical areas compared to the brain surface in BCAS mice [19]. We found a substantial proportion of hypoxic cells to be OPC, while no mature oligodendrocytes were found positive for the hypoxic marker. Cerebral hypoperfusion is known to induce OPC proliferation and increase OPC density at 14 postoperative days in mice, and a decrease in mature oligodendrocytes after 4 weeks [16, 37]. We did not observe any changes in OPC density after 7 days. These results suggest that the effects of hypoperfusion mediated hypoxia on OPC might precede the effects on OPC maturation and myelin integrity.

When exposing OPC to hypoxia in vitro, pathway enrichment analysis of our transcriptomic data revealed the regulation of oligodendrocyte differentiation and HIF-1 signalling as well as angiogenesis, highlighting a possible interaction between hypoxic OPC and EC. This unbiased approach helped us identify VEGFA as a potential candidate in this interaction. Of note, previous studies have suggested the regulation of other factors, such as *Wnt7a/b*, *Sox9/10*, or several matrix metalloproteinases (*MMPs*), in hypoxic OPC [1, 18, 41, 42, 69, 75]. However, we did not find these factors to be regulated in our transcriptomic data derived from hypoxic OPC. *Sox10* was differentially expressed in our RNA data but was not involved in differentiation, HIF-1 signalling, or angiogenesis. Thus, we focused on *Vegfa*, which was the only shared gene by the GO enriched HIF-1 signalling pathway, and processes angiogenesis and differentiation. VEGFA secretion was also increased in these cells, thus further strengthening the notion of hypoxia mediated interaction between OPC and brain EC.

VEGFA is a well-known regulator of angiogenesis in the CNS, which is highly expressed by neural cells including oligodendroglial cells but not by EC [52, 71]. VEGF signalling not only regulates proliferation of EC, it is also involved in the proliferation and migration of OPCs, mediated by VEGFC and VEGFA, respectively [20, 21]. This is in line with previous in vivo findings where OPC density in ischemic mice was not affected, while an increase in OPC migration in hypoxic brain areas was observed [29]. Our RNA expression data showed that when exposing in vitro Oli-neu cells to hypoxia, both VEGFA expression and secretion were upregulated in a *Hif1a* and *Epas1*-dependent manner. In vivo and in vitro stabilisation of *Hif1a* in Oli-neu cells have been shown to increase VEGFA expression, whereas *Hif1a* knockout or inhibition resulted in decreased VEGFA [1, 74]. The role of *Hif1a* and *Epas1* in VEGFA expression however seems to be cell specific [5, 6, 14]. Thus, we propose that in OPC, *Hif1a* is necessary for the expression of *Vegfa*, while *Epas1* has a regulatory role in hypoxic conditions. However, further investigation is needed to clarify the exact mechanism and cell specific response involving *Hif1a* and *Epas1*. Our findings contradict previous reports that HIF stabilization in oligodendrocyte lineage does not increase *Vegfa* expression [69]. A more recent study showed that in vivo stabilisation of HIFα by *Von Hippel-Lindau* gene deletion, which is responsible for the rapid degradation of HIFα, increased *Vegfa* expression in mouse Plp^{+} OPC as demonstrated by in situ hybridization, which was essential for CNS angiogenesis [74]. Our in vitro work using brain EC cultured in presence of hypoxic Oli-neu derived CM indicates a decrease in TJ proteins, which is required for angiogenesis. Similar in vivo findings show decreased TJ proteins mRNA expression in BCAS mice, even after 3 days of hypoperfusion [34, 51, 59, 60, 76]. Together, we suggest that hypoperfusion-induced hypoxia, leading to *Hif1a* and *Epas1* stabilisation in OPC, and subsequent VEGFA production and CNS angiogenesis. Early characteristics of angiogenesis and a decrease in

TJ proteins between ECs might lead to BBB dysfunction and increased BBB permeability [60, 76].

In line with the above, we then examined changes in vascular density and BBB permeability in BCAS mice. While vascular density was unaltered at 7 days of hypoperfusion, there was a significant increase in the number and size of cortical extravascular IgG levels. This indicates that 7 days of hypoperfusion does not lead to the formation of new vessels yet, but does affect BBB permeability, suggesting that BBB dysfunction is one of the first pathological structural changes occurring due to cerebral hypoperfusion. Similar results were obtained by other groups that only saw increased vascular density after 30 days, but not 7 days after BCAS, while first signs of BBB leakages were observed after 3 days [16, 38, 42, 51]. Finally, it is important to note that, while the only hypoxic glial cells identified in our in vivo study were OPC (Additional file 1: Fig. S2), our in vitro findings on VEGFA were not validated in vivo. Similarly, apart from IgG extravasation to assess BBB permeability in vivo, the expression of tight junction proteins was only assessed in vitro upon exposure of brain endothelial cells to conditioned medium derived from hypoxic OPC. However, a recent study showed decreased expression of Cldn5 and Ocln after 1, 3, 7, and 42 days hypoperfusion in BCAS mice, with a significant difference after 43 days compared to Sham [67]. In addition, an increased BBB permeability was observed at all timepoints in these animals [67].

Several studies have shown decreased levels of VEGFA in Alzheimer's disease, while ischemic stroke patients have elevated levels post symptom onset [30, 45, 63, 66]. Interestingly, our results show that VEGFA plasma levels were associated with BBB leakages in the NAWM in cSVD patients, while there was no association in the WMH. This emphasises the notion that VEGFA might have a determining role in the development of

vascular pathology in NAWM in an early stage, ultimately leading to the transition to WML, possibly due to subsequent neuroinflammatory reactions.

Our study presents some limitations. First, hypoxia may have affected other glial cells that could have contributed to the observed BBB dysfunction. In fact, using Cx3Cr1-GFP mice in another study, we found that microglia cells were never positive for the hypoxic marker pimonidazole (Additional file 1). However, while microglia density was not affected in BCAS vs Sham, we found that microglia acquired a pro-inflammatory phenotype in BCAS mice as shown by the increased cell area (Additional file 1: Fig. S2C) and by the increased Cx3Cr1 expression (Additional file 1: Fig. S2D). Although, microglia activation and inflammatory response in BCAS mice is well characterised, it is still largely unknown whether this is a cause or consequence of hypoperfusion and blood–brain barrier dysfunction [42, 56]. Our investigations showed that OPC were the main hypoxic glial cells affected by hypoperfusion in our BCAS model (Additional file 1: Fig. S2). This encouraged us to expose OPCs to hypoxia in vitro and to perform a transcriptomic analysis to identify key differentially expressed genes using an unbiased approach. Second, the increased VEGFA found in vitro was not validated in our in vivo model. Indeed, while the treatment of brain EC in vitro with conditioned medium derived from hypoxic OPC is valuable to assess the function in vitro, it does not help to verify the causality in our in vivo model. Therefore, we suggest future studies to verify our findings in transgenic mice lacking VEGFA selectively in OPCs. Lastly, while we did find a significant correlation between VEGFA plasma concentration and BBB leakage rate in NAWM, the low R^2 means that a considerable amount of the variance in BBB leakage is explained by other factors which we did not account for, such as increased pulsatility and impaired glymphatic drainage as a consequence of hypertension [35, 43, 48].

Conclusion

Taken together, we suggest that cerebral hypoperfusion can lead to hypoxia in the deep cortical regions, affecting OPC, the precursors of the myelinating cells. This may trigger the production of VEGFA in hypoxic OPC via HIF1α and EPAS1 signalling, with subsequent release and action on brain EC. Our in vitro findings suggests that VEGFA may then increase the BBB permeability. This may initiate a pathophysiological cascade ultimately leading to the development of WML. It was previously hypothesised that this might be the other way around, with vascular dysfunction triggering dysfunction in OPC differentiation and myelination leading to WML [76]. Thus, future studies are needed to investigate the role of OPC-derived VEGFA in the development of WML. Understanding the OPC-vascular interaction may lead to treatment strategies specifically targeting OPC-derived VEGFA in the early development of the disease. However, this must be taken with caution, as modulating VEGFA might be a double-edged sword for its role in increasing necessary blood perfusion in hypoxic areas but also causing damage by introducing BBB leakages, thus, stressing the importance of time and cell specific targeting [70].

Abbreviations

BBB	Blood–brain barrier
BCAS	Bilateral carotid artery stenosis
CBF	Cerebral blood flow
CC	Corpus callosum
Cldn5	Claudin-5
CM	Conditioned medium

cSVD	Cerebral small vessel disease
CVON	CardioVasculair Onderzoek Nederland
DAVID	Database for annotation, visualization, and integrated discovery
DCE	Dual-time resolution dynamic contrast enhanced
DEG	Differentially expressed genes
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery rate
FLAIR	Fluid-attenuated inversion recovery
FOV	Field of view
GO	Gene ontology
IHC	Immunohistochemistry
KEGG	Kyoto encyclopedia of genes and genomes
LDF	Laser doppler flowmetry
LSCI	Laser speckle contrast imager
MMPs	Matrix metalloproteinases
NAWM	Normal appearing white matter
NVU	Neurovascular unit
NWO	Netherlands Organisation for Scientific Research
Ocln	Occludin
OPC	Oligodendrocyte precursor cell
P/S	Penicillin/streptomycin

PFA	Paraformaldehyde
PLL	Poly-L-lysing
ROI	Region of interest
TJ	Tight junction
VEGFA	Vascular endothelial growth factor A
WM	White matter
WMH	White matter hyperintensity
WML	White matter lesion

Acknowledgements

Not applicable.

Authors' contributions

NM designed and performed research with input from SF. DM supported NM in performing and analysing qPCR data. NM performed animal studies and PL performed BCAS surgeries. FC and MW performed the RNA sequencing. JFAJ, WHB, RJO, and JS provided patient MRI data and JS supported with patient data analysis. NM wrote manuscript with input from all authors. All authors read and approved the final version of the manuscript. Corresponding authors are ZA, WMB, and SF.

Funding

This work was supported by a PhD studentship, jointly funded by University of Birmingham and CARIM, School for Cardiovascular Diseases Maastricht. SF has received funding from the Young Talent program "Out of the Box Ideas" of CardioVasculair Onderzoek Nederland (CVON). Additional funding for this project were received from the Netherlands

Organisation for Scientific Research (NWO, grant: 017.009.048); Hersenstichting (grant: 2013[1]-195); and Stichting de Weijerhorst foundation.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Raw data are available from the corresponding author, upon request. Supplementary material is available at *Acta Neuropathologica* online.

Declarations

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval and consent to participate

All animal experiments were approved by the regulatory authority of Maastricht University and were performed in compliance with the national and international guidelines (AVD1070020173885). All procedures performed involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre (NTR number: NTR3786). Written informed consent was obtained from all individual participants before inclusion in the study.

Consent for publication

All authors have reviewed the final manuscript and have given their consent for publication.

References

1. Allan KC, Hu LR, Scavuzzo MA, Morton AR, Gevorgyan AS, Cohn EF, Clayton BLL, Bederman IR, Hung S, Bartels CF, Madhavan M, Tesar PJ (2021) Non-Canonical Targets of HIF1a Impair Oligodendrocyte Progenitor Cell Function. Cell Stem Cell 28:257-272.e11. doi: 10.1016/j.stem.2020.09.019
2. Antonetti DA, Barber AJ, Hollinger LA, Wolpert EB, Gardner TW (1999) Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. J Biol Chem 274:23463–23467. doi: 10.1074/jbc.274.33.23463
3. Argaw AT, Gurfein BT, Zhang Y, Zameer A, John GR (2009) VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc Natl Acad Sci U S A 106:1977–1982. doi: 10.1073/pnas.0808698106
4. Back SA, Han BH, Luo NL, Chricton CA, Xanthoudakis S, Tam J, Arvin KL, Holtzman DM (2002) Selective Vulnerability of Late Oligodendrocyte Progenitors to Hypoxia–Ischemia. J Neurosci 22:455–463. doi: 10.1523/JNEUROSCI.22-02-00455.2002
5. Barteczek P, Li L, Ernst A-S, Böhler L-I, Marti HH, Kunze R (2017) Neuronal HIF-1α and HIF-2α deficiency improves neuronal survival and sensorimotor function in the early acute phase after ischemic stroke. J Cereb Blood Flow Metab 37:291–306. doi: 10.1177/0271678X15624933
6. Chavez JC, Baranova O, Lin J, Pichiule P (2006) The Transcriptional Activator Hypoxia Inducible Factor 2 (HIF-2/EPAS-1) Regulates the Oxygen-Dependent Expression of Erythropoietin in Cortical Astrocytes. J Neurosci 26:9471–9481. doi: 10.1523/JNEUROSCI.2838-06.2006
7. Cognat E, Cleophax S, Domenga-Denier V, Joutel A (2014) Early white matter changes in CADASIL: evidence of segmental intramyelinic oedema in a pre-clinical mouse model. Acta Neuropathol Commun 2:49. doi: 10.1186/2051-5960-2-49
8. Coltman R, Spain A, Tsenkina Y, Fowler JH, Smith J, Scullion G, Allerhand M, Scott F, Kalaria RN, Ihara M, Daumas S, Deary IJ, Wood E, McCulloch J, Horsburgh K (2011) Selective white matter pathology induces a specific impairment in spatial working memory. Neurobiol Aging 32:2324.e7-2324.e12. doi: 10.1016/j.neurobiolaging.2010.09.005
9. Croley AN, Zwetsloot KA, Westerkamp LM, Ryan NA, Pendergast AM, Hickner RC, Pofahl WE, Gavin TP (2005) Lower capillarization, VEGF protein, and VEGF mRNA response to acute exercise in the vastus lateralis muscle of aged vs. young women. J Appl Physiol 99:1872–1879. doi: 10.1152/japplphysiol.00498.2005
10. d'Anglemont de Tassigny X, Sirerol-Piquer MS, Gómez-Pinedo U, Pardal R, Bonilla S, Capilla-Gonzalez V, López-López I, De la Torre-Laviana FJ, García-Verdugo JM, López-Barneo J (2015) Resistance of subventricular neural stem cells to chronic hypoxemia despite structural disorganization of the germinal center and impairment of neuronal and oligodendrocyte survival. Hypoxia (Auckl) 3:15–33. doi: 10.2147/HP.S78248
11. de Boer R, Vrooman HA, van der Lijn F, Vernooij MW, Ikram MA, van der Lugt A, Breteler MMB, Niessen WJ (2009) White matter lesion extension to automatic brain tissue segmentation on MRI. Neuroimage 45:1151–1161. doi: 10.1016/j.neuroimage.2009.01.011
12. Desai RA, Davies AL, Tachrount M, Kasti M, Laulund F, Golay X, Smith KJ (2016) Cause and prevention of demyelination in a model multiple sclerosis lesion. Ann Neurol 79:591–604. doi: 10.1002/ana.24607

13. Dong Y-F, Kataoka K, Toyama K, Sueta D, Koibuchi N, Yamamoto E, Yata K, Tomimoto H, Ogawa H, Kim-Mitsuyama S (2011) Attenuation of brain damage and cognitive impairment by direct renin inhibition in mice with chronic cerebral hypoperfusion. Hypertension 58:635–642. doi: 10.1161/HYPERTENSIONAHA.111.173534
14. Downes NL, Laham-Karam N, Kaikkonen MU, Ylä-Herttuala S (2018) Differential but Complementary HIF1α and HIF2α Transcriptional Regulation. Mol Ther 26:1735–1745. doi: 10.1016/j.ymthe.2018.05.004
15. Fischl B (2012) FreeSurfer. Neuroimage 62:774–781. doi: 10.1016/j.neuroimage.2012.01.021
16. Fujita Y, Ihara M, Ushiki T, Hirai H, Kizaka-Kondoh S, Hiraoka M, Ito H, Takahashi R (2010) Early protective effect of bone marrow mononuclear cells against ischemic white matter damage through augmentation of cerebral blood flow. Stroke 41:2938–2943. doi: 10.1161/STROKEAHA.110.596379
17. Gorelick PB, Scuteri A, Black SE, DeCarli C, Greenberg SM, Iadecola C, Launer LJ, Laurent S, Lopez OL, Nyenhuis D, Petersen RC, Schneider JA, Tzourio C, Arnett DK, Bennett DA, Chui HC, Higashida RT, Lindquist R, Nilsson PM, Roman GC, Sellke FW, Seshadri S (2011) Vascular Contributions to Cognitive Impairment and Dementia. Stroke 42:2672–2713. doi: 10.1161/STR.0b013e3182299496
18. Gorter RP, Baron W (2020) Matrix metalloproteinases shape the oligodendrocyte (niche) during development and upon demyelination. Neuroscience Letters 729:134980. doi: 10.1016/j.neulet.2020.134980
19. Hattori Y, Enmi J, Iguchi S, Saito S, Yamamoto Y, Nagatsuka K, Iida H, Ihara M (2016) Substantial Reduction of Parenchymal Cerebral Blood Flow in Mice with Bilateral Common Carotid Artery Stenosis. Sci Rep 6:32179. doi: 10.1038/srep32179
20. Hayakawa K, Pham L-DD, Som AT, Lee BJ, Guo S, Lo EH, Arai K (2011) Vascular Endothelial Growth Factor Regulates the Migration of Oligodendrocyte Precursor Cells. J Neurosci 31:10666–10670. doi: 10.1523/JNEUROSCI.1944-11.2011
21. Hiratsuka D, Kurganov E, Furube E, Morita M, Miyata S (2019) VEGF- and PDGF-dependent proliferation of oligodendrocyte progenitor cells in the medulla oblongata after LPC-induced focal demyelination. J Neuroimmunol 332:176–186. doi: 10.1016/j.jneuroim.2019.04.016
22. Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57. doi: 10.1038/nprot.2008.211
23. Huang DW, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37:1–13. doi: 10.1093/nar/gkn923
24. Husain J, Juurlink BH (1995) Oligodendroglial precursor cell susceptibility to hypoxia is related to poor ability to cope with reactive oxygen species. Brain Res 698:86–94. doi: 10.1016/0006-8993(95)00832-b
25. Ihara M, Tomimoto H (2011) Lessons from a Mouse Model Characterizing Features of Vascular Cognitive Impairment with White Matter Changes. J Aging Res 2011:978761. doi: 10.4061/2011/978761
26. Jenkinson M, Bannister P, Brady M, Smith S (2002) Improved optimization for the robust and accurate linear registration and motion correction of brain images. Neuroimage 17:825–841. doi: 10.1016/s1053-8119(02)91132-8
27. Jung M, Krämer E, Grzenkowski M, Tang K, Blakemore W, Aguzzi A, Khazaie K, Chlichlia K, von Blankenfeld G, Kettenmann H, Trotter J (1995) Lines of Murine Oligodendroglial Precursor Cells Immortalized by an Activated neu Tyrosine Kinase Show Distinct Degrees of Interaction with Axons In Vitro and In Vivo. Eur J Neurosci 7:1245–1265. doi: 10.1111/j.1460-9568.1995.tb01115.x

28. Kerkhofs D, Wong SM, Zhang E, Staals J, Jansen JFA, van Oostenbrugge RJ, Backes WH (2021) Baseline Blood-Brain Barrier Leakage and Longitudinal Microstructural Tissue Damage in the Periphery of White Matter Hyperintensities. Neurology 96:e2192–e2200. doi: 10.1212/WNL.0000000000011783
29. Kishida N, Maki T, Takagi Y, Yasuda K, Kinoshita H, Ayaki T, Noro T, Kinoshita Y, Ono Y, Kataoka H, Yoshida K, Lo EH, Arai K, Miyamoto S, Takahashi R (2019) Role of Perivascular Oligodendrocyte Precursor Cells in Angiogenesis After Brain Ischemia. J Am Heart Assoc 8:e011824. doi: 10.1161/JAHA.118.011824
30. Koca S, Kiris I, Sahin S, Cinar N, Karsidag S, Hanagasi HA, Yildiz GB, Tarik Baykal A (2022) Decreased levels of cytokines implicate altered immune response in plasma of moderate-stage Alzheimer's disease patients. Neurosci Lett 786:136799. doi: 10.1016/j.neulet.2022.136799
31. Koizumi K, Hattori Y, Ahn SJ, Buendia I, Ciacciarelli A, Uekawa K, Wang G, Hiller A, Zhao L, Voss HU, Paul SM, Schaffer C, Park L, Iadecola C (2018) Apoε4 disrupts neurovascular regulation and undermines white matter integrity and cognitive function. Nat Commun 9:3816. doi: 10.1038/s41467-018-06301-2
32. Laakkonen JP, Lappalainen JP, Theelen TL, Toivanen PI, Nieminen T, Jauhiainen S, Kaikkonen MU, Sluimer JC, Ylä-Herttuala S (2017) Differential regulation of angiogenic cellular processes and claudin-5 by histamine and VEGF via PI3K-signaling, transcription factor SNAI2 and interleukin-8. Angiogenesis 20:109–124. doi: 10.1007/s10456-016-9532-7
33. Larsson HBW, Courivaud F, Rostrup E, Hansen AE (2009) Measurement of brain perfusion, blood volume, and blood-brain barrier permeability, using dynamic contrast-enhanced T(1)-weighted MRI at 3 tesla. Magn Reson Med 62:1270–1281. doi: 10.1002/mrm.22136
34. Lee EC, Hong D-Y, Lee D-H, Park S-W, Lee JY, Jeong JH, Kim E-Y, Chung H-M, Hong K-S, Park S-P, Lee MR, Oh JS (2022) Inflammation and Rho-Associated Protein Kinase-Induced Brain Changes in Vascular Dementia. Biomedicines 10:446. doi: 10.3390/biomedicines10020446
35. Li M, Kitamura A, Beverley J, Koudelka J, Duncombe J, Lennen R, Jansen MA, Marshall I, Platt B, Wiegand UK, Carare RO, Kalaria RN, Iliff JJ, Horsburgh K (2022) Impaired Glymphatic Function and Pulsation Alterations in a Mouse Model of Vascular Cognitive Impairment. Front Aging Neurosci 13:788519. doi: 10.3389/fnagi.2021.788519
36. Liang Y, Li X-Y, Rebar EJ, Li P, Zhou Y, Chen B, Wolffe AP, Case CC (2002) Activation of Vascular Endothelial Growth Factor A Transcription in Tumorigenic Glioblastoma Cell Lines by an Enhancer with Cell Type-specific DNase I Accessibility. J Biol Chem 277:20087–20094. doi: 10.1074/jbc.M201766200
37. Magami S, Miyamoto N, Ueno Y, Hira K, Tanaka R, Yamashiro K, Oishi H, Arai H, Urabe T, Hattori N (2019) The Effects of Astrocyte and Oligodendrocyte Lineage Cell Interaction on White Matter Injury under Chronic Cerebral Hypoperfusion. Neuroscience 406:167–175. doi: 10.1016/j.neuroscience.2019.03.004
38. Maki T, Ihara M, Fujita Y, Nambu T, Miyashita K, Yamada M, Washida K, Nishio K, Ito H, Harada H, Yokoi H, Arai H, Itoh H, Nakao K, Takahashi R, Tomimoto H (2011) Angiogenic and vasoprotective effects of adrenomedullin on prevention of cognitive decline after chronic cerebral hypoperfusion in mice. Stroke 42:1122–1128. doi: 10.1161/STROKEAHA.110.603399
39. Matsuo A, Lee GC, Terai K, Takami K, Hickey WF, McGeer EG, McGeer PL (1997) Unmasking of an unusual myelin basic protein epitope during the process of myelin degeneration in humans: a potential mechanism for the generation of autoantigens. Am J Pathol 150:1253–1266
40. Mayhan WG (1999) VEGF increases permeability of the blood-brain barrier via a nitric oxide synthase/cGMP-dependent pathway. Am J Physiol 276:C1148-1153. doi: 10.1152/ajpcell.1999.276.5.C1148

41. Miyamoto N, Pham L-DD, Maki T, Liang AC, Arai K (2014) A radical scavenger edaravone inhibits matrix metalloproteinase-9 upregulation and blood–brain barrier breakdown in a mouse model of prolonged cerebral hypoperfusion. Neuroscience Letters 573:40–45. doi: 10.1016/j.neulet.2014.05.005
42. Nakaji K, Ihara M, Takahashi C, Itohara S, Noda M, Takahashi R, Tomimoto H (2006) Matrix Metalloproteinase-2 Plays a Critical Role in the Pathogenesis of White Matter Lesions After Chronic Cerebral Hypoperfusion in Rodents. Stroke 37:2816–2823. doi: 10.1161/01.STR.0000244808.17972.55
43. Nam K-W, Kwon H-M, Lee Y-S (2020) Distinct association between cerebral arterial pulsatility and subtypes of cerebral small vessel disease. PLoS One 15:e0236049. doi: 10.1371/journal.pone.0236049
44. Nico B, Mangieri D, Crivellato E, Longo V, De Giorgis M, Capobianco C, Corsi P, Benagiano V, Roncali L, Ribatti D (2007) HIF Activation and VEGF Overexpression are Coupled with ZO-1 Up-phosphorylation in the Brain of Dystrophic MDX Mouse. Brain Pathol 17:399–406. doi: 10.1111/j.1750-3639.2007.00090.x
45. Nielsen HH, Soares CB, Høgedal SS, Madsen JS, Hansen RB, Christensen AA, Madsen C, Clausen BH, Frich LH, Degn M, Sibbersen C, Lambertsen KL (2020) Acute Neurofilament Light Chain Plasma Levels Correlate With Stroke Severity and Clinical Outcome in Ischemic Stroke Patients. Front Neurol 11:448. doi: 10.3389/fneur.2020.00448
46. Nishio K, Ihara M, Yamasaki N, Kalaria RN, Maki T, Fujita Y, Ito H, Oishi N, Fukuyama H, Miyakawa T, Takahashi R, Tomimoto H (2010) A Mouse Model Characterizing Features of Vascular Dementia With Hippocampal Atrophy. Stroke 41:1278–1284. doi: 10.1161/STROKEAHA.110.581686
47. O'Meara RW, Ryan SD, Colognato H, Kothary R (2011) Derivation of enriched oligodendrocyte cultures and oligodendrocyte/neuron myelinating co-cultures from post-natal murine tissues. J Vis Exp 3324. doi: 10.3791/3324
48. Onkenhout LP, Arts T, Ferro D, Oudeman EA, van Osch MJP, Zwanenburg JJM, Hendrikse J, Kappelle LJ, Biessels GJ (2022) Perforating artery flow velocity and pulsatility in patients with carotid occlusive disease. A 7 tesla MRI study. Cereb Circ Cogn Behav 3:100143. doi: 10.1016/j.cccb.2022.100143
49. Pantoni L (2010) Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. Lancet Neurol 9:689–701. doi: 10.1016/S1474-4422(10)70104-6
50. Patlak CS, Blasberg RG (1985) Graphical evaluation of blood-to-brain transfer constants from multiple-time uptake data. Generalizations. J Cereb Blood Flow Metab 5:584–590. doi: 10.1038/jcbfm.1985.87
51. Roberts JM, Maniskas ME, Bix GJ (2018) Bilateral carotid artery stenosis causes unexpected early changes in brain extracellular matrix and blood-brain barrier integrity in mice. PLOS ONE 13:e0195765. doi: 10.1371/journal.pone.0195765
52. Rosenstein JM, Krum JM, Ruhrberg C (2010) VEGF in the nervous system. Organogenesis 6:107–114. doi: 10.4161/org.6.2.11687
53. Ryan NA, Zwetsloot KA, Westerkamp LM, Hickner RC, Pofahl WE, Gavin TP (2006) Lower skeletal muscle capillarization and VEGF expression in aged vs. young men. J Appl Physiol 100:178–185. doi: 10.1152/japplphysiol.00827.2005

54. Schepers M, Paes D, Tiane A, Rombaut B, Piccart E, van Veggel L, Gervois P, Wolfs E, Lambrichts I, Brullo C, Bruno O, Fedele E, Ricciarelli R, Ffrench-Constant C, Bechler ME, van Schaik P, Baron W, Lefevere E, Wasner K, Grünewald A, Verfaillie C, Baeten P, Broux B, Wieringa P, Hellings N, Prickaerts J, Vanmierlo T (2022) Selective PDE4 subtype inhibition provides new opportunities to intervene in neuroinflammatory versus myelin damaging hallmarks of multiple sclerosis. Brain Behav Immun 109:1–22. doi: 10.1016/j.bbi.2022.12.020
55. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682. doi: 10.1038/nmeth.2019
56. Shibata M, Ohtani R, Ihara M, Tomimoto H (2004) White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion. Stroke 35:2598–2603. doi: 10.1161/01.STR.0000143725.19053.60
57. Shibata M, Yamasaki N, Miyakawa T, Kalaria RN, Fujita Y, Ohtani R, Ihara M, Takahashi R, Tomimoto H (2007) Selective Impairment of Working Memory in a Mouse Model of Chronic Cerebral Hypoperfusion. Stroke 38:2826–2832. doi: 10.1161/STROKEAHA.107.490151
58. Tiane A, Schepers M, Riemens R, Rombaut B, Vandormael P, Somers V, Prickaerts J, Hellings N, van den Hove D, Vanmierlo T (2021) DNA methylation regulates the expression of the negative transcriptional regulators ID2 and ID4 during OPC differentiation. Cell Mol Life Sci 78:6631–6644. doi: 10.1007/s00018-021-03927-2
59. Toyama K, Koibuchi N, Uekawa K, Hasegawa Y, Kataoka K, Katayama T, Sueta D, Ma MJ, Nakagawa T, Yasuda O, Tomimoto H, Ichijo H, Ogawa H, Kim-Mitsuyama S (2014) Apoptosis Signal–Regulating Kinase 1 Is a Novel Target Molecule for Cognitive Impairment Induced by Chronic Cerebral Hypoperfusion. Arterioscler Thromb Vasc Biol 34:616–625. doi: 10.1161/ATVBAHA.113.302440
60. Wallez Y, Huber P (2008) Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. Biochim Biophys Acta Biomembr 1778:794–809. doi: 10.1016/j.bbamem.2007.09.003
61. Wang W, Dentler WL, Borchardt RT (2001) VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. Am J Physiol Heart Circ Physiol 280:H434-440. doi: 10.1152/ajpheart.2001.280.1.H434
62. Weber CM, Clyne AM (2021) Sex differences in the blood-brain barrier and neurodegenerative diseases. APL Bioeng 5:011509. doi: 10.1063/5.0035610
63. Winder Z, Sudduth TL, Anderson S, Patel E, Neltner J, Martin BJ, Snyder KE, Abner EL, Jicha GA, Nelson PT, Wilcock DM (2023) Examining the association between blood-based biomarkers and human post mortem neuropathology in the University of Kentucky Alzheimer's Disease Research Center autopsy cohort. Alzheimers Dement 19:67–78. doi: 10.1002/alz.12639
64. Wong SM, Zhang CE, van Bussel FCG, Staals J, Jeukens CRLPN, Hofman PAM, van Oostenbrugge RJ, Backes WH, Jansen JFA (2017) Simultaneous investigation of microvasculature and parenchyma in cerebral small vessel disease using intravoxel incoherent motion imaging. Neuroimage Clin 14:216–221. doi: 10.1016/j.nicl.2017.01.017
65. Wong SM, Jansen JFA, Zhang CE, Hoff EI, Staals J, van Oostenbrugge RJ, Backes WH (2019) Blood-brain barrier impairment and hypoperfusion are linked in cerebral small vessel disease. Neurology 92:e1669–e1677. doi: 10.1212/WNL.0000000000007263
66. Xu K, Wu C, Wang Z, Wang H, Yin F, Li W, Liu C, Fan H (2021) VEGF Family Gene Expression as Prognostic Biomarkers for Alzheimer's Disease and Primary Liver Cancer. Comput Math Methods Med 2021:e3422393. doi: 10.1155/2021/3422393

67. Yang L, Song J, Nan D, Wan Y, Guo H (2022) Cognitive Impairments and blood-brain Barrier Damage in a Mouse Model of Chronic Cerebral Hypoperfusion. Neurochem Res 47:3817–3828. doi: 10.1007/s11064-022-03799-3
68. Yoon CW, Rha J-H, Park H-K, Park S-H, Kwon S, Kim BC, Youn YC, Jeong JH, Han HJ, Choi SH (2022) Sex differences in the progression of cerebral microbleeds in patients with concomitant cerebral small vessel disease. Front Neurol 13:1054624. doi: 10.3389/fneur.2022.1054624
69. Yuen TJ, Silbereis JC, Griveau A, Chang SM, Daneman R, Fancy SPJ, Zahed H, Maltepe E, Rowitch DH (2014) Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell 158:383–396. doi: 10.1016/j.cell.2014.04.052
70. Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, Bruggen N v, Chopp M (2000) VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. J Clin Invest 106:829–838. doi: 10.1172/JCI9369
71. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, Phatnani HP, Guarnieri P, Caneda C, Ruderisch N, Deng S, Liddelow SA, Zhang C, Daneman R, Maniatis T, Barres BA, Wu JQ (2014) An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. J Neurosci 34:11929–11947. doi: 10.1523/JNEUROSCI.1860-14.2014
72. Zhang CE, Wong SM, Haar HJ van de, Staals J, Jansen JFA, Jeukens CRLPN, Hofman PAM, Oostenbrugge RJ van, Backes WH (2017) Blood–brain barrier leakage is more widespread in patients with cerebral small vessel disease. Neurology 88:426–432. doi: 10.1212/WNL.0000000000003556
73. Zhang CE, Wong SM, Uiterwijk R, Backes WH, Jansen JFA, Jeukens CRLPN, van Oostenbrugge RJ, Staals J (2019) Blood–brain barrier leakage in relation to white matter hyperintensity volume and cognition in small vessel disease and normal aging. Brain Imaging Behav 13:389–395. doi: 10.1007/s11682-018-9855-7
74. Zhang S, Kim B, Zhu X, Gui X, Wang Y, Lan Z, Prabhu P, Fond K, Wang A, Guo F (2020) Glial type specific regulation of CNS angiogenesis by HIFα-activated different signaling pathways. Nat Commun 11:2027. doi: 10.1038/s41467-020-15656-4
75. Zhang S, Wang Y, Xu J, Kim B, Deng W, Guo F (2021) HIFα Regulates Developmental Myelination Independent of Autocrine Wnt Signaling. J Neurosci 41:251–268. doi: 10.1523/JNEUROSCI.0731-20.2020
76. Zhao Y, Zhu W, Wan T, Zhang X, Li Y, Huang Z, Xu P, Huang K, Ye R, Xie Y, Liu X (2022) Vascular endothelium deploys caveolin-1 to regulate oligodendrogenesis after chronic cerebral ischemia in mice. Nat Commun 13:6813. doi: 10.1038/s41467-022-34293-7
77. Zudaire E, Gambardella L, Kurcz C, Vermeren S (2011) A Computational Tool for Quantitative Analysis of Vascular Networks. PLOS ONE 6:e27385. doi: 10.1371/journal.pone.0027385

Supplementary materials

Animals and tissue collection

All animal experiments were approved by the regulation authority of Maastricht University and were performed in compliance with the national and European guidelines. Cx3Cr1GFP/GFP mice (Jackson Lab 005582) were crossed with Thy-1YFP/0 mice (Jackson Lab 003782) to generate Cx3Cr1GFP/WT x Thy1YFP/0 mice (abbreviated Tg mice) for microglial (GFP) and neuronal (YFP) visualisation. Animals were kept on a normal 12h day-night cycle. All mice were allowed access ad libitum to water and food. Male Tg mice underwent a bilateral carotid artery stenosis (BCAS) or a Sham surgery. Briefly, microcoils with an internal diameter of 0,18mm (Sawane) were placed around the left and right common carotids in BCAS operated mice, while the same surgery was performed in Sham operated animals without placement of the microcoils. The cerebral blood flow was measured during the surgery by Laser Doppler flowmetry and was significantly decreased in BCAS vs Sham (BCAS -42±4 vs Sham +2±2, $p<0,0001$). BCAS operated mice were sacrificed under anaesthesia after 1 week (n=4) and 2 weeks (n=3) and Sham operated mice were sacrificed after 2 weeks (n=10). The hypoxic marker pimonidazole was injected i.p one hour before sacrifice in conscious mice (only 1week BCAS group) and a vascular tracer (70kDa-dextran-TexasRed, Thermofisher D1864, 200µL at 2,5 mg/mL) was injected i.v in isoflurane-anaesthetized mice (vena cava) 15 minutes prior to the sacrifice by decapitation. Brains were harvested directly after death without perfusion and fixed overnight in 4% paraformaldehyde (PFA). The brains were then transferred to a solution containing phosphate-buffered saline (PBS) and 1% sodium azide (NaN3) before slicing.

Immunohistochemistry

Brain slicing

Fifty µm thick coronal sections were prepared using a vibratome (VT1200S, Leica). Five consecutive series of 11-12 slices were prepared. Free-floating sections were used to stain hypoxic areas/cells (pimonidazole positive cells).

Hypoxic cells staining

Six identical brain slices per brain were chosen for the staining. Briefly, sections blocked in a TBS-T + 1% bovine serum albumin (BSA) solution for 2h at room temperature (RT). samples were then incubated with primary antibody overnight at 4 °C. The following day, samples were then incubated with secondary antibody and anti-pimonidazole-pacific blue antibody (Hypoxyprobe Pacific blue kit; 1:100) in TBS-T + 1% BSA for 2h at 4 °C. Finally, the slices were washed and mounted on gelatin-coated microscopic slides with a fluorescent anti-fading mounting medium (Thermofisher, Prolong gold antifade P10144).

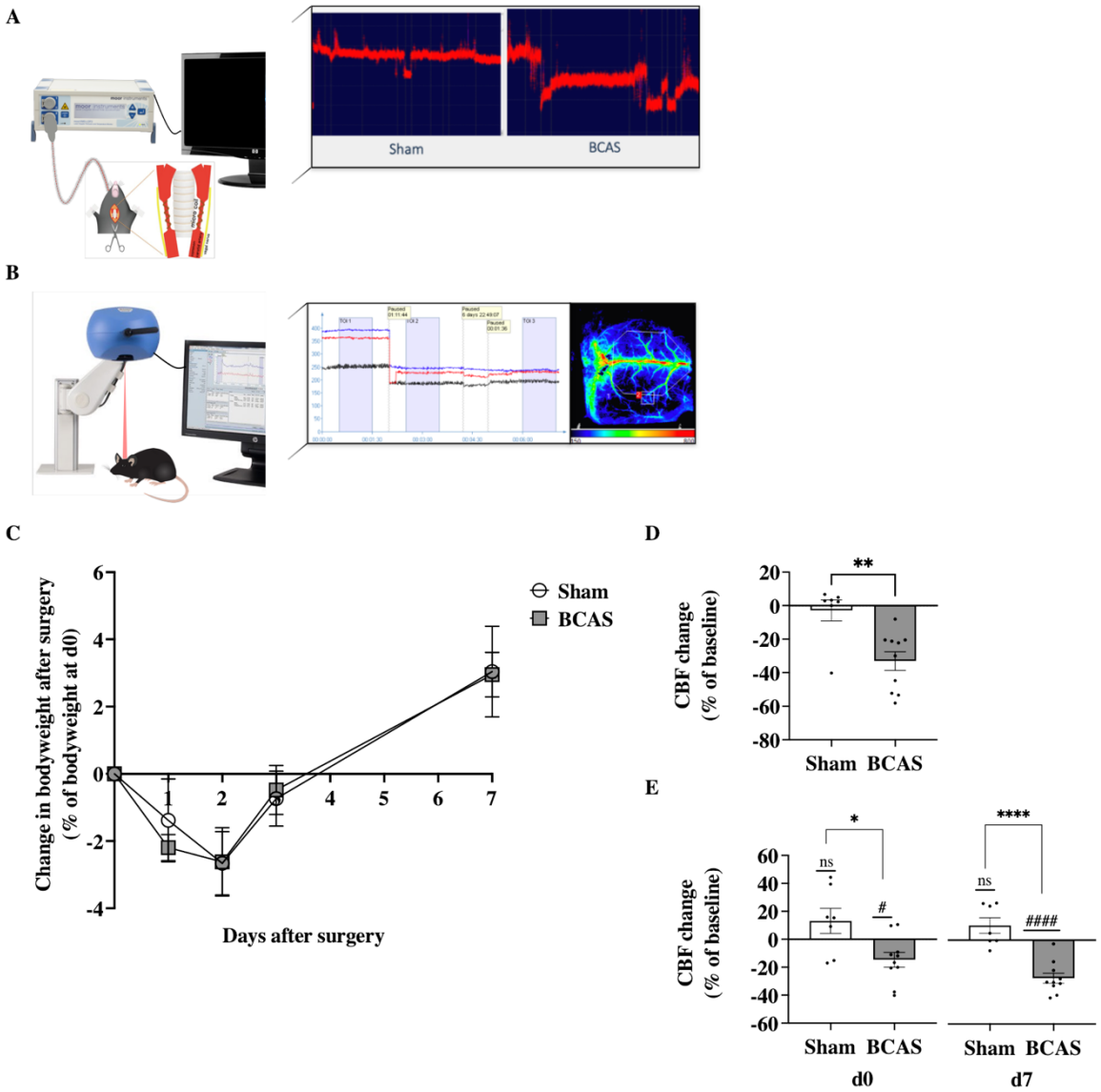
Image acquisition and analysis

Hypoxic cells and microglia

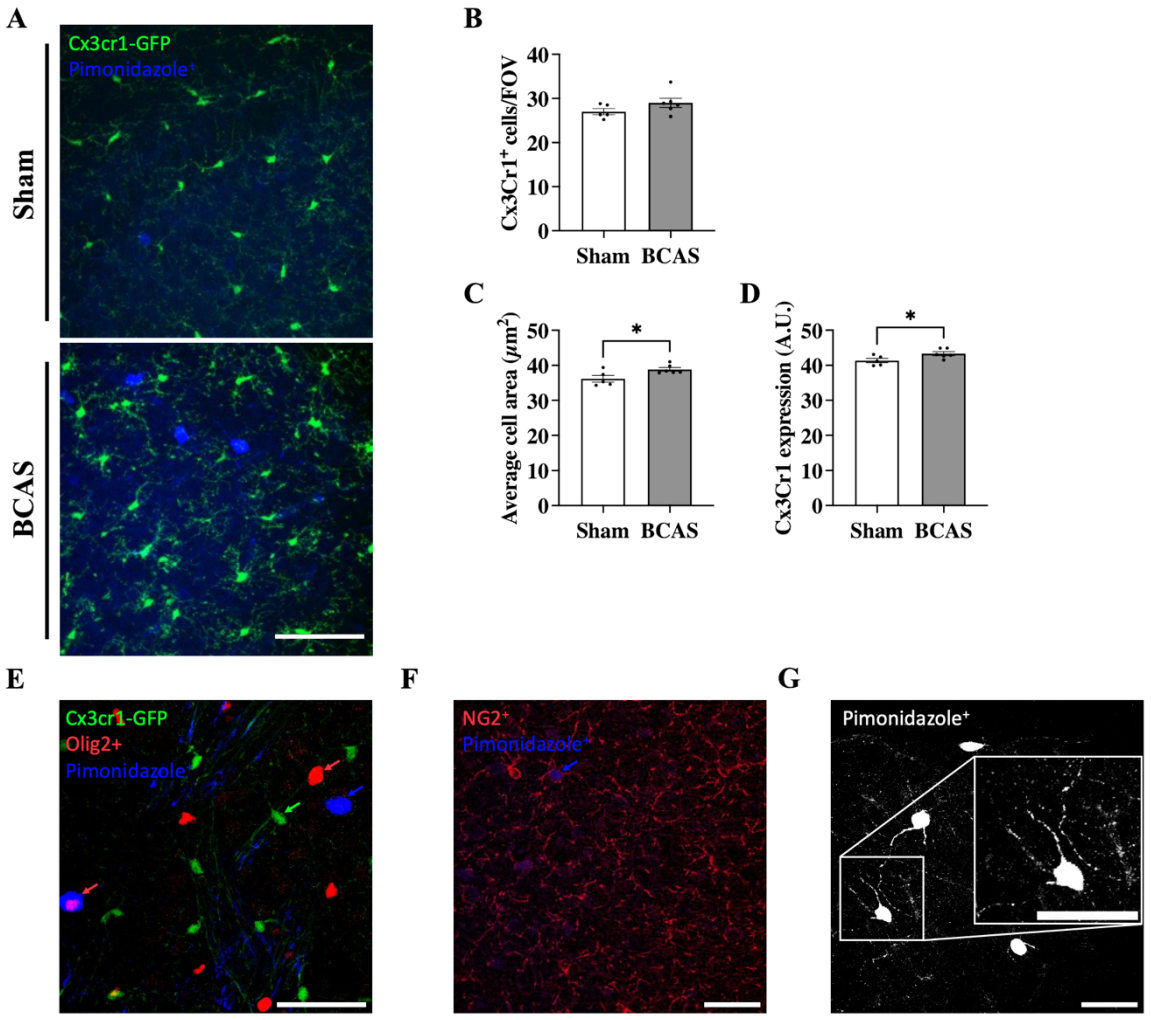
Pimonidazole-positive cells from the corpus callosum and adjacent deep cortical and striatal areas were imaged by confocal microscopy (ACS APO 40.0 x 1.15 objective, Leica DMI 4000 microscope). Image stacks (30 µm; step size: 2 µm) including microglia (GFP) OPC (Olig2/CC1/NG2), and the hypoxic marker (Pacific blue) were acquired at a resolution of 512 x 512 pixels. Stained sections were screened for the detection of hypoxic cells within the corpus callosum and the adjacent cortical areas.

Microglia quantification

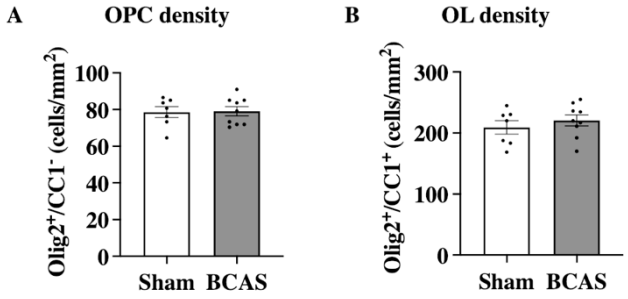
For the quantification of microglia density and the evaluation of morphological changes the software WIS-NeuroMath was used. Briefly, 1 μm was defined as 1,86 pixels and the settings were defined as the following: the noise level was set to 1.5, the min. cell intensity 10, min. area 10, max area 3850, min. diameter 2, max. axial ratio 10 and min. neurite length 5 μm.



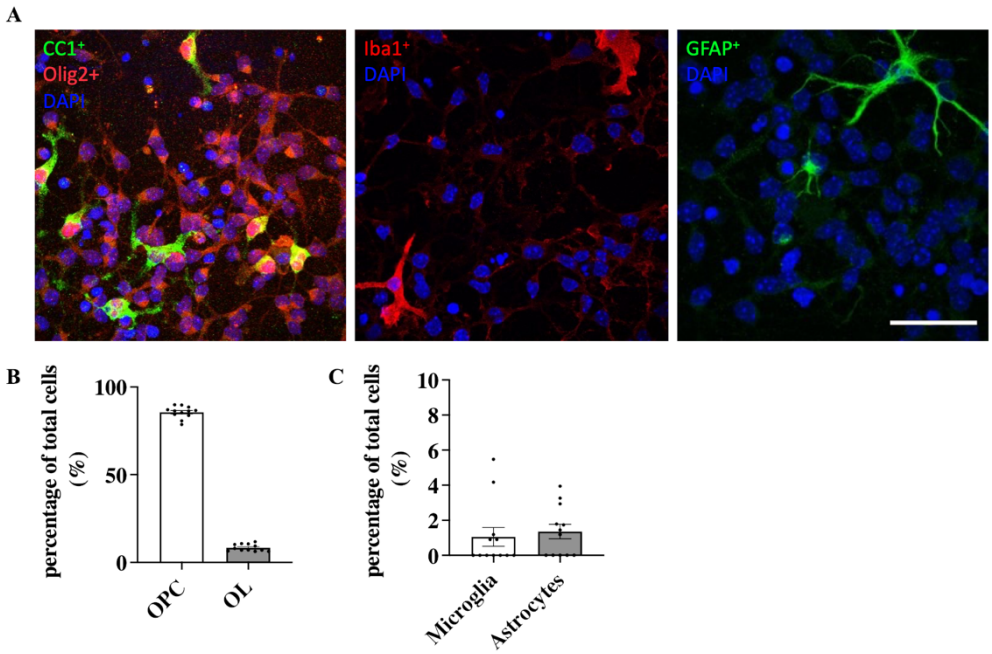
Supplementary Figure 1: Schematic overview of in vivo methods. (A) Laser Doppler Imaging (LDI) method of CBF measurement during surgical procedures. **(B)** Laser speckle contrast imaging (LSCI) method of CBF measurements before, right after and 7 days after surgical procedures. **(C)** Bodyweight changes after surgery. **(D)** CBF changes measured with LDI during surgery. **(E)** CBF was quantified and compared to baseline measurements at d0 and d7 in the large vessels. A significant decrease of 14.7±5.3% in BCAS mice compared to baseline measurements at d0 and 27.2±3.6% at d7, with no significant changes in Sham mice at both d0 (13.1±9.0%) and d7 (10.5±5.5%). A significant difference in blood flow change between the two groups was observed at both d0 and d7. Mean±SEM; ns=not significant; #p<0.05, ####p<0.0001 vs baseline measurements; Data points (dots) indicate biological replicates; *p<0.05, **p<0.01, ****p<0.0001, vs Sham; unpaired student t-test.



Supplementary Figure 2: Hypoxia affected the oligodendrocytes and neurons in the deep cortical regions. (A) Immunohistochemistry for microglial (Cx3cr-GYP reporter) and hypoxic cells (Pimonidazole$^+$). Scale bar, 50 µm. **(B)** 7 days of hypoperfusion did not lead to changes in microglia density, but **(C)** did acquire a pro-inflammatory phenotype in BCAS mice as shown by the increased cell area and **(D)** and by the increased Cx3Cr1 expression. **(E)** Although microglia (Cx3cr1-GFP, indicated by green arrow) were not identified as hypoxic (Pimonidazole$^+$, indicated by blue arrow), the hypoperfusion induced in BCAS mice did have an impact on other glial cells (Olig2$^+$, indicated by red arrow). **(F)** Hypoxic OPC were initially identified by double positive cells for OPC marker NG2 and Pimonidazole (indicated by blue arrow) **(G)** The cellular shape of pimonidazole$^+$ cells (zoomed panel) that did not express Olig2 or CC1 suggested that most hypoxic cells were in fact of neuronal origin. Scale bar, 50 µm. Mean±SEM; (n=3); Data points (dots) indicate biological replicates; *p<0.05, unpaired student t-test. Statistically non-significant comparisons are not shown.



Supplementary Figure 3: BCAS does not lead to oligodendrocyte (precursor) cell density changes after 7 days. (A) No differences in OPC density, quantified by the number of $Olig2^+/CC1^-$ immunolabelled cells, were found when comparing BCAS to Sham (p=0.91). **(B)** OL density, quantified by the number of $Olig2^+/CC1^+$ immunolabelled cells, was also unaltered in BCAS compared to Sham (p=0.43). Mean±SEM; Data points (dots) indicate biological replicates; Unpaired student t-test. Statistically non-significant comparisons are not shown.



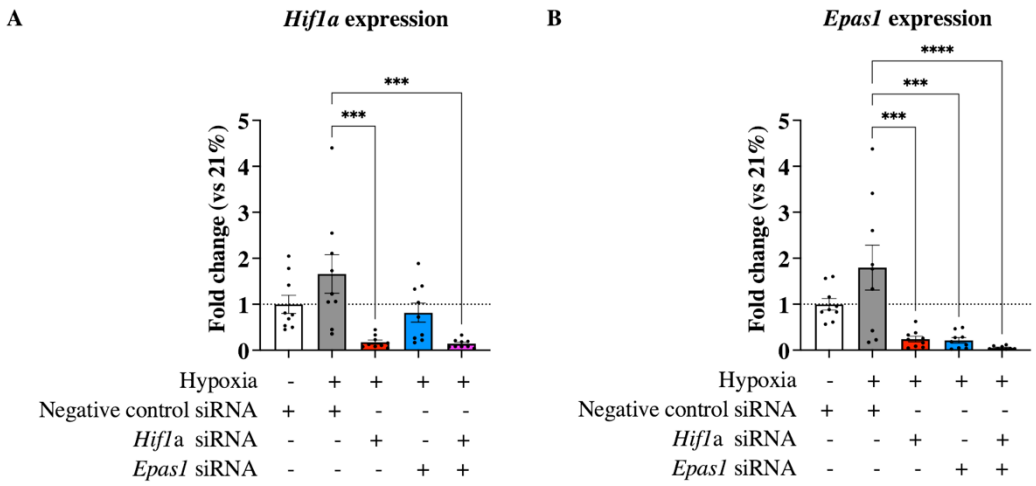
Supplementary Figure 4: Primary OPC culture purity (A) Immunocytochemistry of primary OPC cultures. Oligodendroglial (CC1 and Olig2), microglia (Iba1), and astrocyte (GFAP) were identified in the cell cultures to determine contamination after OPC isolation from mixed glial cultures by shaking. Scale bar, 50 µm. **(B)** Quantification of number of OPC ($CC1^-/Olig2^+$, 85.6%) and mature oligodendrocytes ($CC1^+/Olig2^+$, 8.4%) as a percentage of the total number of cells ($DAPI^+$). **(C)** Quantification of number of microglia ($Iba1^+$, 1.1%) or astrocytes ($GFAP^+$, 1.4%) as a percentage of the total number of cells ($DAPI^+$). Mean±SEM. Data points (dots) indicate technical replicates.

Supplementary Table 1: Primary and secondary used for immunohistochemistry.

Primary antibody	**Host**	**Dilution**	**Cat no.**	**Supplier**
Anti-Pimonidazole conjugated to Pacific Blue fluorophore	Rat	1:100	HPI15	Hypoxyprobe Inc.
Anti-NG2	Rabbit	1:250	AB5320	Sigma-Aldrich
Anti-Olig2	Rabbit	1:200	AB9610	Sigma-Aldrich
Anti-APC, clone CC1	Mouse	1:400	MABC200	Sigma-Aldrich
Anti-myelin basic protein (MBP)	Rat	1:500	MAB386	Sigma-Aldrich
Anti-MBP, clone SMI94	Mouse	1:1000	836504	Biolegend
Anti-Iba1	Rabbit	1:500	019-19741	Wako Chemicals
Anti-Glial Fibrillary Acidic Protein (GFAP)	Mouse	1:400	G3893	Sigma-Aldrich
Lycopersicon esculentum (Tomato) Lectin Texas Red	Donkey	1:100	TL-1176-1	Vector Labs
Anti-mouse IgG Alexa Fluor 488	Donkey	1:200	A-21202	Invitrogen
Secondary antibody				
Anti-mouse IgG Alexa Fluor 488	Donkey	1:200	A-21202	Invitrogen
Anti-rabbit IgG Alexa Fluor 594	Donkey	1:200	A-21207	Invitrogen

Supplementary Table 2: Primers used in quantitative PCR.

Primer name	Primer sequence	
Rpl13a	Forward	AGCCTACCAGAAAGTTTGCTTAC
	Reverse	GCTTCTTCTTCCGATAGTGCATC
Ywhaz	Forward	GAAAAGTTCTTGATCCCCAATGC
	Reverse	TGTGACTGGTCCACAATTCCTT
Vegfa	Forward	GCACATAGAGAGAATGAGCTTCC
	Reverse	CTCCGCTCTGAACAAGGCT
Hif1a	Forward	AATGAAGTGCACCCTAACAAGCCG
	Reverse	TGGCCCGTGCAGTGAAGC
Epas1	Forward	TCACTCATCCTTGCGACCAT
	Reverse	TTCCCAAAACCAGAGCCGTT
Cldn5	Forward	CCACGGCCAATGGCGATTAC
	Reverse	TCGTCATCCACACACGGCTT
Ocln	Forward	CCTCGGTACAGCAGCAATGG
	Reverse	TAGTGGTCAGGGTCCGTCCT



Supplementary Figure 5: Efficiency of Hif1a and Epas1 siRNA. (A) *Hif1a* expression was significantly decreased by *Hif1a* siRNA, while *Epas1* siRNA did not significantly decrease *Hif1a* expression. **(B)** *Epas1* expression was inhibited by both *Hif1a* and *Epas1* siRNA. Treatment with both siRNA led to a significant decrease in both *Hif1a* and *Epas1* expressions. For quantification, Mean±SEM; Data points (dots) indicate technical replicates; ***p<0.001, and ****p<0.0001, vs 2% + negative control siRNA; one-way ANOVA with Tukey's multiple comparisons test. Statistically non-significant comparisons are not shown.

Supplementary Table 3: Cohort characteristics of all included participants without exclusion of identified outliers for VEGFA plasma concentration.

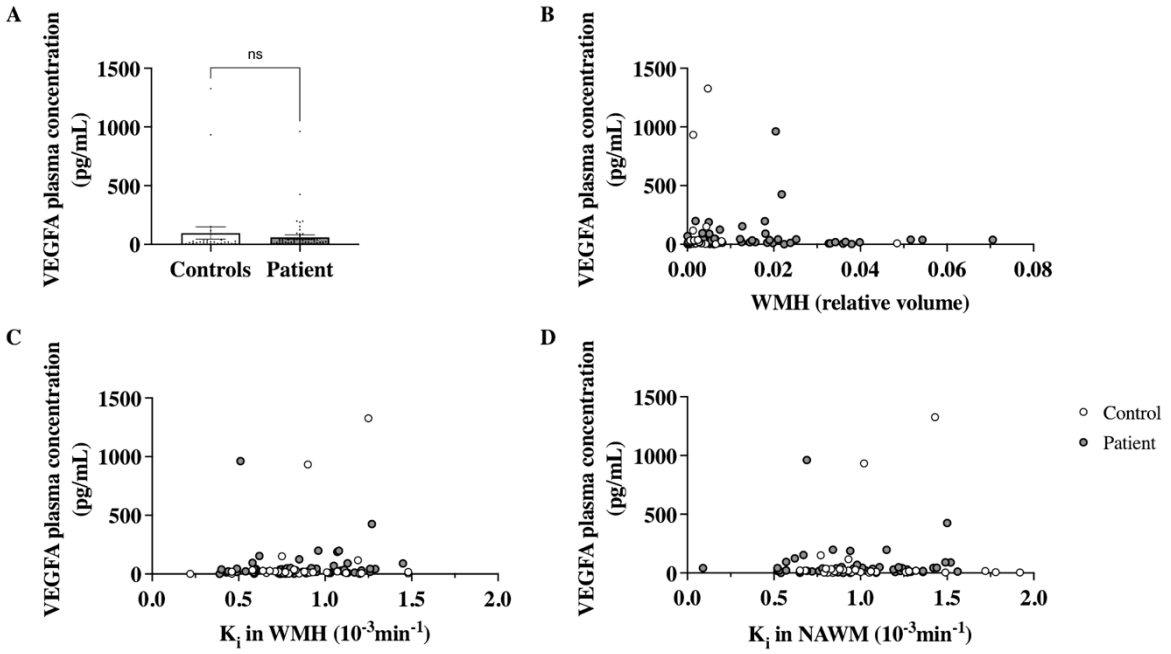
		Control (*n*=30)	Patient (*n*=55)	Univariable β (p-value)	Multivariable (age and sex corrected) β (p-value)
	Age	68.1±2.3	70.4±1.4		
	Male (Female)	19 (11)	35 (20)		
WMH	**Relative volume, 10^{-3}**	3.5±1.6	15.4±2.1	0.391 (<0.001)	0.402 (<0.001)
BBB leakage in NAWM	**K_i, $10^{-3}min^{-1}$**	1.09±0.06	0.96±0.04	-0.187 (0.086)	-0.193 (0.079)
	v_L	0.298±0.034	0.386±0.026	0.215 (0.048)	0.216 (0.050)
BBB leakage in WMH	**K_i, $10^{-3}min^{-1}$**	0.87±0.05	0.84±0.04	-0.048 (0.659)	-0.071 (0.531)
	v_L	0.333±0.036	0.459±0.028	0.285 (0.008)	0.281 (0.010)

Abbreviations: WMH = white matter hyperintensities; BBB = blood brain barrier; NAWM = normal appearing white matter; K_i = leakage rate; v_L = leakage volume.

Supplementary Table 4: Cohort characteristics of excluded participants due to measurements identified as outliers for VEGFA plasma concentration.

		Outliers (*n*=12)	
		Mean±SEM	**Min – Max**
	Age	73.7±2.6	53 – 82
	Male (Female)	6 (6)	
WMH	**Relative volume, 10^{-3}**	8.6±2.2	1.3 – 2.2
BBB leakage in NAWM	**K_i, $10^{-3}min^{-1}$**	0.93±0.088	0.6 – 1.5
	v_L	0.466±0.061	0.110 – 0.712
BBB leakage in WMH	**K_i, $10^{-3}min^{-1}$**	0.92±0.076	0.5 – 1.3
	v_L	0.449±0.061	0.146 – 0.739
VEGFA plasma conc.	**pg/ml**	405.8±121.7	94.9 – 1326.3

Abbreviations: WMH = white matter hyperintensities; BBB = blood brain barrier; NAWM = normal appearing white matter; K_i = leakage rate; v_L = leakage volume; conc. = concentration; Min = minimum; Max = maximum.



Supplementary Figure 6: No correlation between VEGFA blood plasma levels and WMH volume or leakage rate in WMH or NAWM prior to exclusion of outliers. (A) VEGFA blood plasma levels in cSVD were not different compared to age and sex-matched controls (data points [dots] indicate biological replicates) and no correlation was seen between VEGFA plasma concentration and **(B)** WMH volume or leakage rate in **(C)** WMH and **(D)** NAWM in cSVD patients prior to exclusion of identified outliers. Abbreviations: WMH = white matter hyperintensities; NAWM = normal appearing white matter; K_i = leakage rate. For quantification, mean±SEM; ns = not significant; Mann-Whitney U-test.

Supplementary Table 5: Associations between VEGFA plasma levels and MRI imaged cSVD characteristics in cSVD patients prior to exclusion of outliers. No correlations were found between VEGFA plasma levels and sSVD parameters in the cSVD patient population prior to exclusion of outliers. $P<0.05$ was considered significant.

		Univariable		Multivariable	
		β	***p*-value**	β	***p*-value**
WMH		0.012	0.933	-0.053	0.689
BBB leakage in NAWM	K_i	-0.015	0.913	-0.028	0.846
	v_L	-0.038	0.785	-0.025	0.862
BBB leakage in WMH	K_i	-0.007	0.959	-0.012	0.935
	v_L	-0.048	0.728	-0.038	0.792

Abbreviations: WMH = white matter hyperintensity; BBB = blood brain barrier; NAWM = normal appearing white matter; K_i = leakage rate; v_L = leakage volume.

Appendix A

Inclusion and exclusion criteria cSVD study population

Patients included lacunar stroke and mild vascular cognitive impairment (mVCI) patients. Lacunar stroke patients had first-ever acute lacunar syndrome with a compatible recent small subcortical infarct defined as a small (<20mm) lesion in the brain stem, thalamus, basal ganglia, internal capsule or white matter, and hyperintense on diffusion weighted (DW) MRI and either normal or hyperintense on T2 and fluid-attenuated inversion recovery (FLAIR) MRI and were included 3 months after the acute stroke to avoid acute stroke phase changes. Patients with no visible lesions on imaging were included based on clinical criteria such as unilateral motor and/or sensory signs involving the whole of at least two of the three body parts (face, arm, leg) without disturbance of consciousness, visual fields, language, or other cortical functions. Patients were excluded when presented with a potential cardiac embolic source or stenosis of ≥ 50% of one or both internal carotid arteries. MVCI patients presented subjective cognitive complaints, failure in one or more cognitive domains determined by neuropsychological assessment, and extensive MRI abnormalities associated with cSVD, i.e. white matter hyperintensities (WMHs) Fazekas score 2 or 3, and/or with microbleeds, and/or lacunes and no other cognitive deficits. Patients with suspected neurodegenerative disease other than vascular cognitive impairment (e.g. Alzheimer's disease), another neurological or psychiatric disease interfering with cognitive testing, or with severe cognitive impairment defined as Mini Mental State Examination <20 and/or Clinical Dementia Rating >1, were excluded. Controls participants were age- and sex-matched with no symptoms of stroke or cognitive impairments. Participants with a history of cerebrovascular disease, other diseases of the central nervous system or with MRI contraindications were excluded.

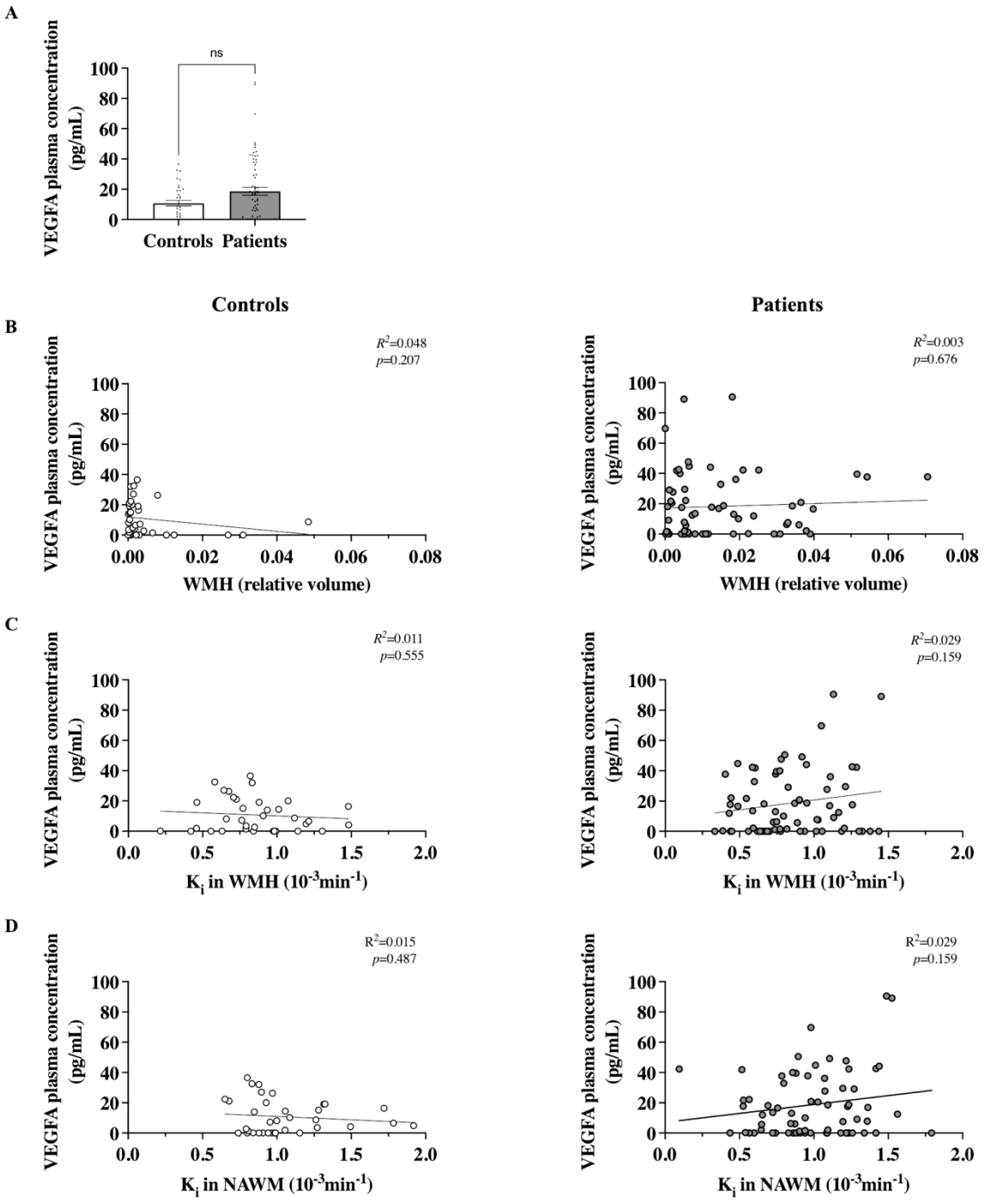


Figure A1: No correlation was found between VEGFA blood plasma levels and leakage rate in WMH or NAWM prior to exclusion of samples with undetectable levels of VEGFA. (A) VEGFA blood plasma levels in cSVD patients were not different compared to controls (data points [dots] indicate biological replicates) and no correlation was seen between VEGFA plasma concentration and **(B)** WMH volume or leakage rate in **(C)** WMH and **(D)** NAWM in cSVD patients prior to exclusion of samples with undetectable levels of VEGFA. Abbreviations: WMH = white matter hyperintensities; NAWM = normal appearing white matter; K_i = leakage rate. For quantification, mean±SEM; ns = not significant; Mann-Whitney U-test.

Chapter 6

-

Summary and general discussion

Pathological features of cerebral small vessel disease (cSVD) include impairment of the blood-brain barrier (BBB) and the progression of white matter lesions (WMLs) amongst other structural lesions, leading to the clinical manifestations of cSVD such as progressive cognitive decline and gait and other motor impairments. Hypertension, which is one of the most prominent risk factors for cSVD, can result in chronic hypoperfusion in the brain [1]. This can lead to a series of events causing pathology such as microbleeds and WMLs. The BBB functions to protect and maintain the homeostasis in the brain, with endothelial cells (ECs) as a major component of this structure. These ECs are characterised by specific tight junction proteins such as Claudin-5 (Cldn5) and Occludin (Ocln) forming the endothelial barrier and disruption of these cells might lead to BBB dysfunction. ECs interact with several cell types in the brain, such as astrocytes, microglia, and oligodendrocytes, to not only provide structural and functional support for proper brain function but also develop and maintain the BBB [2].

As for oligodendrocytes, they are responsible for myelinating axons in the central nervous system (CNS), protecting them from damage and facilitating the fast transduction of action potentials. However, oligodendrocytes and oligodendrocyte precursor cells (OPCs) are not only important for these functions, but they also interact with their surrounding cells to provide trophic support. Crosstalk between ECs and OPCs has emerged in recent literature as an important player in the developing brain vasculature, but also in the adult BBB and white matter (WM) integrity [3]. A body of evidence suggest that this interaction is mediated by signalling through the Wnt/β-catenin pathway. The Wnt/β-catenin signalling pathway is an important regulator of vascular development and tight junction (TJ) protein expression and subsequent barrier function [4]. In the developed adult brain, Wnt signalling seems to be involved in angiogenesis and BBB integrity and OPCs seem to secrete Wnt proteins in

response to hypoxic injury [4–7]. Hypoxic insult caused by hypoperfusion in the brain might cause myelin damage in the brain WM leading to recruitment of OPCs in an attempt to repair the damage. These recruited OPCs utilise the vasculature to migrate to the site of injury, which seems to be driven by Wnt signalling in the interaction of the ECs and OPCs [8]. Thus, dysfunctional EC-OPC interaction due to aberrant Wnt signalling may limit remyelination of WMLs and render BBB impairments, thereby initiating a vicious neuroinflammatory cycle leading to the development of cSVD.

We hypothesised that cerebral chronic hypoperfusion leads to local hypoxia in the brain, affecting the OPCs, which lead to abnormal OPC signalling and an impaired interaction with brain ECs, ultimately causing disruption of the BBB and unresolved WM damage due to compromised remyelination. Understanding these early pathogenic mechanisms in cSVD can lead to the development of novel diagnostic tools and therapeutic strategies. Thus, the overall aim of this thesis was to identify and investigate molecular signalling mediators of the OPC-EC crosstalk that might be underlying early mechanisms leading to the development of cSVD. To test our hypothesis, we used several experimental in vitro brain endothelial and OPC models, and an in vivo model for cerebral hypoperfusion. Additionally, a retrospective human study was used to support our in vivo and in vitro findings.

In this general discussion, we intend to discuss some of the main findings and limitations of our studies. We will particularly address: **(1)** The involvement of Wnt7a in the crosstalk between OPCs and ECs and their role in cSVD; **(2)** The effects of Wnt7a stimulation on TJ protein expression and endothelial barrier function; and **(3)** cell migration in brain ECs; and lastly **(4)**, we explore the role of hypoperfusion induced response of OPCs affecting the vascular ECs and BBB permeability and WM integrity.

Summary of main findings

Chapter 2 provides an overview of the knowledge on Wnt signalling for the endothelial – oligodendroglial interaction and its relevance for the pathophysiology of cSVD. Our systematic review revealed that Wnt signalling plays a key role in OPC recruitment and migration in hypoxia in an attempt to recover damaged WM [3, 5, 8–10]. Furthermore, a reciprocal interaction between ECs and OPCs has been suggested, as endothelial dysfunction led to WM damage and decreased OPC densities [11, 12]. This is strengthened by data from in vivo studies showing that EC dysfunction precedes OPC and WM abnormalities in cSVD pathology, and that WM damage can lead to increased Wnt expression by OPCs, resulting in the recruitment of new OPCs via the Cxcl12-Cxcr4 mediated interaction with the vasculature [8, 10]. Once OPCs reach their destination, Wnt signalling is downregulated to allow OPC detachment from the vasculature and initiate their proliferation and differentiation [8].

Dysfunction of the Wnt signalling pathway can lead to the inhibition of the detachment and differentiation of OPCs, leading to clustering of perivascular OPCs, ultimately leading to BBB impairments [10, 13]. In Chapter 2, the systematic review identified Wnt7a as a potential mediator of the interaction between ECs and OPCs in response to hypoxic injury. Hypoxic conditions can initiate stabilisation of hypoxia-inducible factor (HIF)1/2α in OPCs, which then increases Wnt7a and Wnt7b expression and secretion. In vitro studies suggest that Wnt7a triggers tip sprouting and tube formation in mouse brain ECs mediated by β-catenin, while in vivo studies in mice showed β-catenin mediated angiogenesis mediated by Wnt7a/b secretion by OPCs [5, 6, 14]. Overexpression of Wnt7a is suggested to underlie the OPC response to hypoxic injury, which can lead to the above-mentioned pathological

response seen in cerebral hypoxia [5, 6, 15]. Thus, we focused on the Wnt7a-mediated changes in brain ECs.

In **Chapter 3**, we aimed to investigate the pathways involved in the in vitro effects of Wnt7a stimulation mediated by β-catenin signalling on brain endothelial barrier integrity. Our results revealed that 100 ng/ml Wnt7a stimulation led to an increase in β-catenin expression and nuclear translocation, which was reversed by the selective Wnt signalling inhibitor XAV939. Wnt7a stimulation led to a β-catenin mediated decrease in *Cldn5* and *Ocln* expression, while Wnt7a stimulation only decreased protein levels of Cldn5. In line with the decrease in TJ proteins, Wnt7a stimulation also led to decreased endothelial barrier formation assessed with the in vitro trans-endothelial electrical resistance (TEER) assay. In addition, transcriptomic data of the Wnt7a treated bEnd.3 cells revealed the enrichment of the angiogenesis pathway and upregulation of *Hif1a* and vascular endothelial factor A (*Vegfa)*. The upregulation of *Hif1a* and *Vegfa* was mediated by β-catenin, indicating an interaction between the Wnt and HIF1α signalling pathways. However, inhibition of *Hif1a* with siRNA did not affect protein levels of Cldn5 or Ocln but did inhibit Wnt7a-mediated decrease in *Ocln* mRNA expression. Thus, our results suggest that Wnt7a stimulation leads to the β-catenin mediated downregulation of TJ protein Cldn5 and decrease of endothelial barrier function, which might be modulated by HIF1α signalling.

In **Chapter 4**, we utilised the transcriptomic regulation of Wnt7a stimulated brain ECs to uncover cell migration pathways linked to OPCs. Gene ontology analysis revealed cell migration as one of the top enriched pathways in response to Wnt7a stimulation. Gene analysis identified *Cxcl12* as the highest regulated gene in the Wnt/β-catenin pathway. We demonstrated that the Wnt7a-mediated decrease in *Cxcl12* was not dependent on β-catenin signalling. It is known that OPCs migrate by utilising the vasculature via Cxcl12-Wnt-Cxcr4

signalling [8, 16]. We therefore investigated the possible effect of Wnt7a on the expression of *Cxcl12* in ECs and the expression of its receptor, *Cxcr4*, in OPCs. While *Cxcr4* was unchanged in OPCs upon Wnt7a stimulation, our study warrants further study of the possible role of Wnt7a-mediated decrease of Cxcl12 in brain ECs, as this can consequently lead to decreased OPC migration and impair the WM remyelination capacity after ischaemic damage.

Finally, the aim of **Chapter 5** was to investigate early in vivo mechanisms leading to impaired OPC-EC interaction due to chronic hypoperfusion of the brain. To mimic the decreased brain perfusion observed in the WM of cSVD patients, we induced cerebral hypoperfusion via bilateral carotid artery stenosis (BCAS) in mice [17]. Our results showed a persistent decrease in cerebral blood flow (CBF) in BCAS mice compared to Sham mice after 1 week of stenosis. Reduced CBF led to an increase in hypoxic subcortical OPCs, without any changes in WM integrity at this early disease stage. This was associated with an increased BBB permeability. In vitro, we assessed the transcriptomic profile of primary OPCs exposed to hypoxia. Based on our earlier systematic review, we expected Wnt7a/b to be regulated in this condition. However, both *Wnt7a* and *Wnt7b* mRNA expression remained unchanged (data not shown). We revealed *Vegfa* as one of the top regulated genes in OPCs in response to hypoxia with gene ontology and gene expression analysis. Increase in *Vegfa* expression in response to hypoxia was regulated by both *Hif1a* and *Epas1* (*Hif2a*), and was validated in an OPC line. Treatment of brain ECs with conditioned medium from hypoxic OPCs revealed the downregulation of *Cldn5* and *Ocln* expression. To assess the translational relevance of our in vitro and in vivo findings, we retrospectively investigated VEGFA protein levels in blood plasma samples from cSVD patients and controls. VEGFA plasma levels were increased in patients versus controls and were associated with increased blood-

brain barrier permeability in normal appearing white matter of patients. These findings indicate that VEGFA associated pathological mechanisms might only be present in the early development of WM damage in cSVD patients and not in the developed lesions. Taken together, our findings suggest a role of OPC-derived VEGFA as an early mechanism in BBB impairment and WM damage in cSVD pathology. A schematic summary of our main findings is illustrated in Figure 1.

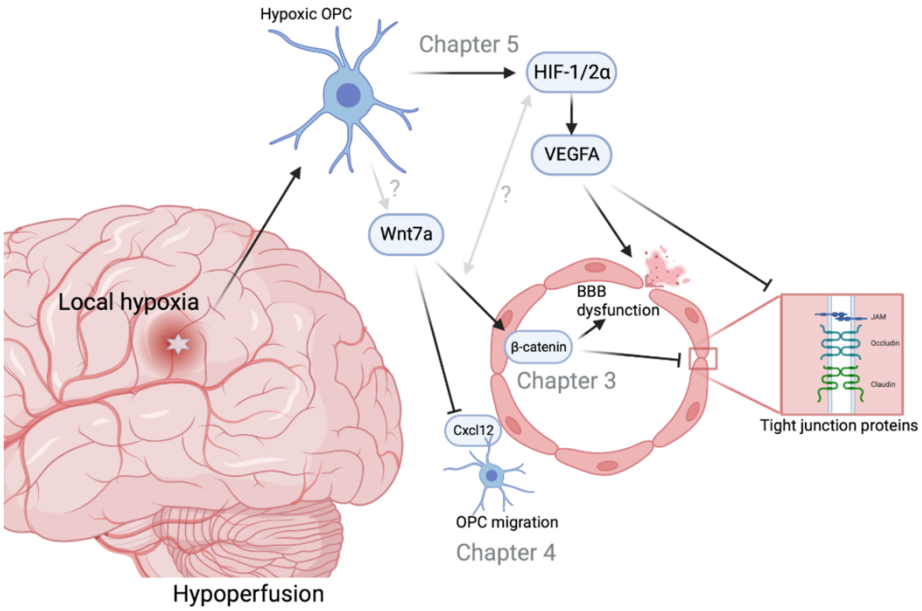


Figure 1: schematic summary of main findings of this thesis. Hypoxia induced Wnt7a led to a β-catenin dependent decrease in tight junction proteins and increased BBB permeability (*Chapter 3*). Wnt7a stimulation also led to the decrease in Cxcl12 that is involved in the migration of OPCs via the vasculature (*Chapter 4*). Hypoperfusion mediated hypoxia led to hypoxic OPCs in the deep cortical regions. Hypoxic OPCs show increased HIF-1/2α dependent expression of VEGFA, which in turn can lead to increased blood-brain barrier (BBB) dysfunction and decrease in tight junction proteins (*Chapter 5*). This might be the underlying pathology in the development of WM damage seen in cerebral small vessel disease.

Interpretation of main findings

We discussed the Wnt mediated crosstalk between ECs and OPCs in **chapter 2** and established Wnt7a as a potential molecular mediator of this interaction. It is suggested that hypoxia exposed OPCs increase their production of Wnt7a protein thought HIF1α signalling,

which results in an increase in proliferation and tube formation of ECs [5]. These are important processes in brain vascular angiogenesis [18]. Wnt7a acts through activation of its receptor Frizzled (Fzd) 4 and coreceptors low-density lipoprotein receptor (Lrp) 5 or 6 to induce the formation of endothelial tip cells that sprout from the vasculature to direct the proliferation and formation of new vessels during angiogenesis [18–20]. A study in zebrafish identified the interaction of Wnt co-activators, Reck and the Gpr124, as being essential for Wnt7a/b-specific brain angiogenesis via the regulation of these tip cells [21]. Thus, indicating that the angiogenic response of ECs does not solely depend on Wnt7a signalling. In hypoxic conditions, in vitro pluripotent stem cells increase their Wnt7a expression and differentiate into microvascular ECs, while in vivo hypoxia leads to angiogenesis and higher vascular density in the WM through regulation of EC sprouting [15, 22, 23]. In mice, OPCs were able to interact with sprouting ECs and facilitate the vascularisation in the WM, while hypoxia resulted in hypervascularisation of the WM accompanied by hypomyelination and decreased number of mature oligodendrocytes [23].

Histological analysis of human hypoxic ischaemic encephalopathy brain lesions suggests that these OPCs express Wnt7a and increase Wnt/β-catenin target genes, *Apcdd1* and *Axin2*, in the ECs of the hypoxia damaged WM, without affecting the BBB [23]. However, our results in **chapter 3** did not show the increase in *Axin2* expression in brain ECs stimulated with Wnt7a. We did see a significant decrease in TJ protein Cldn5 and decreased endothelial barrier formation. Another study that reported an increase in the number of perivascular OPCs in response to ischaemic insult, showed a similar upregulation of Wnt7a expression in hypoxic OPCs exposed to hypoxia [15]. However, these cells were also deprived from glucose and oxygen tension was 0.1%, indicating that such factors might have a modulating role in the response of Wnt/β-catenin signalling [15]. Key players in these pathways might

be *Hif1a* and *Vegfa*, as *Vegfa* expression was also increased in these hypoxic OPCs, which is in accordance with our results showing a β-catenin dependent increase in both *Hif1a* and *Vegfa* in response to Wnt7a signalling [15]. Several other studies have shown the interplay between Wnt/β-catenin and HIF1α/VEGFA pathways [22, 24, 25]. In vitro exposure of human pluripotent stem cells (hPSCs) to hypoxia upregulated both Wnt7a and HIF1α [22]. Similarly, hypoxia exposure in hepatocellular carcinoma activated β-catenin, while β-catenin enhanced the hypoxia induced effects by increasing HIF1α activity [24, 25]. This suggests that the observed EC-OPC crosstalk mediated by OPC-derived Wnt7a under hypoxia conditions may be a consequence of the interplay between Wnt/HIF1α signalling rather than direct Wnt/β-catenin involvement. However, we were not able to demonstrate the role of HIF1α signalling in the Wnt/β-catenin effect on TJ proteins expression in Chapter 3, as suggested by other studies showing the interplay between these signalling pathways [6, 26–30]. Thus, the precise role of Wnt/HIF1α signalling remain uncertain, requiring further investigation to address this knowledge gap (Figure 1).

Wnt7a signalling can also exert effects independently of β-catenin signalling. In **Chapter 4,** our results suggest the β-catenin independent regulation of *Cxcl12* expression by Wnt7a in brain ECs. *Cxcl12-Cxcr4* mediated migration of OPCs via the vasculature has previously been linked to Wnt signalling, with clustered Wnt-activated perivascular OPCs showing an upregulation of *Cxcr4* in mice, while treatment with the *Cxcl12-Cxcr4* antagonist, AMD3100, led to the dissociation of the OPCs from the vessels [8]. Although our results do not reflect the regulation of *Cxcr4*, we could demonstrate the regulation of *Cxcl12* in ECs upon Wnt7a stimulation. These findings together indicate the importance of this chemokine interaction in the migration of OPCs in response to Wnt signalling. However, a study in bone marrow stromal cells showed the downregulation of Cxcl12 by Wnt3a mediated by β-catenin

signalling, while our results indicate that the *Cxcl12* decrease was independent of β-catenin activation [31]. These apparent contradictory results indicate the complexity and versatility of the Wnt/β-catenin signalling pathways and its modulators and their exact role in vascular and glial interaction. This might also explain the lack of evidence to directly link Wnt7a to cSVD. However, a recent human genome-wide association study (GWAS) suggests an indirect link between Wnt7a and cSVD pathological features [32]. In this GWAS study with more that 40,000 participants, two independent polymorphisms were identified at the Wnt7a locus (chr3 p25.1) and were associated with extensive perivascular space (PVS) burden in the WM. PVS are spaces surrounding blood vessels as they penetrate into the brain parenchyma, and enlargement of these spaces is one of the earliest MRI detectable markers for cSVD [32, 33]. However, these findings do not demonstrate a causal link of the involvement of Wnt7a in PVS burden, but rather show an association with polymorphisms near the Wnt7a gene.

Considering the significance of OPC-derived Wnt in maintaining WM integrity [23], these results suggest that dysfunction of Wnt7a in OPCs might be an underlying early mechanism in the development of cSVD. However, our in vitro results in **Chapter 5** did not indicate a hypoxia mediated regulation of Wnt7a in OPCs. Our hypoperfusion model in mice showed that OPCs were the first glial cells to be affected by hypoxia, before the occurrence of WMLs. In line with this findings, other studies found that Wnt7a is not affected in OPCs by hypoxic signalling pathways [7, 34–36]. Furthermore, HIF1α signalling did not lead to the regulation of Wnt7a in cells of the oligodendroglial lineage, and blocking Wnt signalling did not affect regulation of OPC differentiation and myelination [7]. The discrepancy in the literature might be a consequence of methodological inconsistency. A methodological consideration that could explain the different OPC response to hypoxia might be the

definitions of hypoxia. Wnt7a regulation was found in a study that exposed OPCs to glucose deprivation in addition to hypoxic insult [6], while other groups use only oxygen deprivation [5, 15]. Metabolic changes have huge consequences on the OPC transcriptomic profile and could thus result in discrepancy between results from different groups. To test our hypothesis, we used hypoxic in vitro conditions without glucose deprivation to mimic the in vivo and in human conditions [17, 37]. Another factor might be the purity of the OPC cultures, as these can be contaminated with other cell types such as astrocytes and microglia due to method of isolation. Contamination of primary OPC cultures with astrocytes might result in Wnt7a upregulation upon hypoxic exposure [36, 38, 39], while in vivo microglia are known to express Wnt7a in their anti-inflammatory M2c state [40]. This discrepancy is also seen in in vivo models. HIF1α stabilisation in astroglia in transgenic mice increased Wnt/β-catenin signalling mediated angiogenesis in ECs, while HIF1α stabilisation in OPC led to increased VEGFA mediated angiogenesis [36]. In addition, it seems that the Wnt and VEGFA signalling pathways might also interact with each other [41]. Thus, these signalling pathways might easily be confused (Figure 1) [5, 36].

VEGF regulates angiogenesis in the brain by stimulating EC proliferation and migration, and can also affect the oligodendroglial cells. For example, VEGFA increases OPC migration without affecting proliferation, while another member of the VEGF family, VEGFC, promotes the OPC proliferation [42–44]. Our results in **Chapter 5** indicate that hypoxia induced VEGFA expression was regulated by both *Hif1a* and *Epas1* in OPCs, which is in line with other in vitro studies that show the increase in VEGFA in OPCs after hypoxia exposure [15, 35] and in vivo BCAS hypoperfusion mice [45, 46]. We also found no changes in OPC density in BCAS, which might reflect the role of VEGFA in promoting OPC migration without affecting proliferation. Additionally, VEGFA is known to downregulate

Cldn5 and Ocln in microvascular ECs, which was similar to the downregulation of these TJ proteins observed in our in vitro ECs that were treated with conditioned medium from OPCs exposed to hypoxia [47]. These findings suggest that VEGFA signalling might play an important role in hypoxia mediated EC-OPC interaction [3, 48]. OPCs in hypoxic WM might secrete VEGFA in an attempt to increase oxygen levels, as activated ECs migrate towards the VEGFA gradient during angiogenesis [49]. We found a correlation between blood plasma VEGFA levels and BBB leakages in the normal appearing white matter (NAWM) in cSVD patients, which may be the consequence of increased BBB permeability due to VEGFA before the initiation of the WMLs. Similarly, other studies have found a correlation between VEGFA levels and WM damage. VEGFA levels were higher in the WM and CSF of patients with vascular dementia [50, 51]. A recent study that investigated the association between WMLs and neuroinflammatory markers also found that higher VEGFA levels in the cerebrospinal fluid (CSF) was associated with more WMLs in mild cognitive impaired patients [52]. Thus, VEGFA is a pleiotropic mediator in the endothelial-oligodendroglial interaction in response to hypoxia; OPCs respond to early hypoxic injury by secreting VEGFA, which induces angiogenesis and EC proliferation, leading to increased BBB permeability in areas with high VEGFA concentrations, while also attracting OPCs to repair damaged WM [53].

Methodological considerations

Wnt7a as a stand-alone active compound

Several studies have suggested that the use of recombinant Wnt7a shows little to no effect on Wnt signalling pathway, with Wnt7a being prone to enzymatic degradation [54]. However, the co-activator Reck can bind and protect Wnt7a, resulting in enhanced Wnt7a

signalling [27, 55, 56]. We acknowledge the fact that Wnt7a did not induce expression of the β-catenin signalling target gene, *Axin2*. However, our immunocytochemistry and transcriptomic data clearly showed the activation of β-catenin and expression of wide range of genes, indicating that Wnt7a clearly acts as an active compound and activates both β-catenin mediated and non-β-catenin mediated signalling in our cultured brain ECs. Although using bEnd.3 cells might offer a consistent and low-cost model to investigate endothelial barrier function, it does not reflect the full anatomical function [57]. Using hPSCs that are differentiated into brain microvascular ECs (ihBMECs) shows superior barrier properties, offering a more suitable model to study brain endothelial barrier function [57].

Physiological hypoxia in vitro

As mentioned earlier, the use of in vitro models provides an ethical and cost- and time-efficient alternative for in vivo models. However, a wide range of variables must be considered, such as composition of cell culture media, medium exchange frequency, culture surface, and cell confluency. The cells must also be maintained at the correct temperature (37 ºC), atmospheric air (21% O_2) enriched by 5% CO_2, and appropriate humidity [58]. Due to partial pressures of CO_2 and water vapour, the incubator atmosphere reaches 18.6% O_2 in the conventional standard culturing conditions, which is considered as normoxic [59]. These standard culturing conditions however do not always offer the right environment for all cell types. As the oxygen demand is variable depending on the cell type, the pericellular oxygen levels are very different from the atmosphere levels. In addition, the physiological range of oxygen levels in different tissue is variable [58]. Thus, making it difficult to mimic the exact environmental physiological conditions in culture. The atmosphere oxygen level commonly described in literature as hypoxia, ranges from 1% to 10% [58]. In our model, we used 2%

and 5% oxygen levels to investigate the ideal hypoxic condition (data not shown). Oxygen levels of 2% resulted in the biggest transcriptomic response and were chosen as the appropriate oxygen levels for hypoxia. We used 21% atmospheric oxygen levels as standard normoxic condition, which might reflex a hyperoxic environment for some cell types. Areas in the deep WM might not be well vascularised and might thus have lower oxygen tension in normoxic brain tissue. Thus, we used the transcriptomic data of 2%, 5%, and 21% oxygen levels to determine the normoxic (21% O_2) and hypoxic (2% O_2) culturing conditions in our setup, but we do acknowledge that this must be taken with caution.

BCAS as a model for small vessel pathology

The BCAS mouse model is a valuable and commonly used model for vascular cognitive impairment and to some extent for cSVD as it presents several pathological characteristics of chronic hypoperfusion. Investigations in this model report decreased CBF, increase BBB permeability, endothelial dysfunction, inflammation, and WMLs [60]. However, the model has some major limitations, such as the global and abrupt CBF reduction and decrease in blood flow pulsatility [61–63]. Patients with cSVD present a gradual decrease in CBF and only present pathological features in late stages of disease. Additionally, a study that investigated pulsatility in cSVD found increased pulsatility associated with higher cSVD burden, while BCAS mice present a decrease in pulsatility [62–64].

Although the BCAS model is a well-established hypoperfusion model, there is no animal model that mimics the local hypoperfusion induced cSVD. It is thus important to consider other models to validate our findings, such as longitudinal studies in humans to investigate the involvement of VEGFA in the development of BBB impairments and WMLs.

VEGFA in human blood plasma samples

Our investigations in the cohort of cSVD patients show a correlation between plasma VEGFA levels and BBB impairments in the NAWM. However, these findings have some limitation. The study was conducted retrospectively, blood plasma protein levels were not a primary outcome of the initial study design. This may have influenced the protein levels in the samples as sample collection and preservation time, handling, and freeze/thaw cycle might vary between the samples, resulting in a high number of outliners that were identified and excluded from the study. Another concern might be the source of VEGFA leading to the peripheral protein level increase. The healthy functioning BBB is a tight structure preventing large molecules, such as VEGFA, to cross from the brain to the periphery, or vice versa. However, dysfunctional BBB in disease, such as in cSVD, might allow molecules to cross in both directions, thus leading to measurable changes in the periphery and a valuable non-invasive diagnostic tool [2].

Lastly, the experimental setup of our present study did not allow us to validate OPCs as the source of increased VEGFA levels measured in blood plasma levels. However, associations between non-invasive peripheral measurements and brain pathologic could lead to valuable diagnostic biomarker for early detection of patients at risk. Thus, future studies should focus on replicating our findings in a large cSVD cohort with blood plasma protein levels as primary outcome measure, and post-mortem brain tissue analysis to support our OPC-derived VEGFA hypothesis.

Conclusion

In conclusion, we investigated molecular mediators of the OPC-EC crosstalk involved in the early mechanisms that lead to the development of cSVD. We identified Wnt7a as a Wnt signalling mediator potentially underlying disease pathology. Studies suggest that hypoperfusion mediated hypoxia in the brain leads to aberrant Wnt7a secretion by OPCs, which in turn affects OPC migration and differentiation in an autocrine manner, causing OPC clustering. On the other hand, paracrine effects lead to abnormal angiogenesis and BBB impairments, ultimately leading to WMLs. In vitro Wnt7a stimulation of brain ECs led to downregulation of TJ proteins, Cldn5 and Ocln, mediated by Wnt/β-catenin signalling. Decreased TJ protein expression in these cells could underlie decreased EC barrier function due to Wnt7a stimulation.

We also identified HIF1α as a potential modulator of this Wnt/β-catenin signalling pathway, as expression of *Hif1a* and *Vegfa* were both increased by Wnt7a stimulation through β-catenin signalling. Although TJ protein levels of Cldn5 and Ocln was not affected by silencing *Hif1a*, affected gene expression of *Ocln* indicated the crosstalk between Wnt and HIF1α signalling. Wnt7a stimulation also leads to β-catenin independent decreased expression of *Cxcl12*, a vascular chemokine involved in OPC migration, without affecting the expression of *Cxcr4*, the receptor for Cxcl12, in OPCs. Although Wnt7a stimulates all these effects that might underlie early cSVD pathology, we did not find regulation of Wnt7a in hypoxic OPCs. Chronic hypoperfusion does lead to hypoxia in OPCs in mice, affecting the BBB permeability before the development of WMLs.

In contrast to our original hypothesis that Wnt7a was upregulated in hypoxic OPCs, we identified the upregulation of the angiogenesis mediator VEGFA in OPCs exposed to hypoxia. Expression of VEGFA in hypoxic OPCs is mediated by *Hif1a* and *Epas1* and

causes a decrease in TJ protein expression in ECs. VEGFA levels are increased in cSVD patients and correlate with BBB impairment in NAWM. Thus, our results show OPC-derived VEGFA as a potential mediator of dysfunctional OPC-EC crosstalk and driver in the early development of BBB impairments leading to WMLs. Future studies validating these findings in large cSVD cohorts could lead to the development of rapid, low-cost, and non-invasive diagnosis of cSVD, which is currently not possible. Additionally, investigating the potential of VEGFA signalling mediating drugs for cSVD prevention or treatment could offer novel treatment options to improve the quality of life of patients and decrease the global burden of vascular dementia.

References

1. Pantoni L (2010) Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. Lancet Neurol 9:689–701. https://doi.org/10.1016/S1474-4422(10)70104-6
2. Kadry H, Noorani B, Cucullo L (2020) A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity. Fluids and Barriers of the CNS 17:69. https://doi.org/10.1186/s12987-020-00230-3
3. Miyamoto N, Pham L-DD, Seo JH, et al (2014) Crosstalk between cerebral endothelium and oligodendrocyte. Cell Mol Life Sci 71:1055–1066. https://doi.org/10.1007/s00018-013-1488-9
4. Liebner S, Corada M, Bangsow T, et al (2008) Wnt/β-catenin signaling controls development of the blood–brain barrier. Journal of Cell Biology 183:409–417. https://doi.org/10.1083/jcb.200806024
5. Yuen TJ, Silbereis JC, Griveau A, et al (2014) Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell 158:383–396. https://doi.org/10.1016/j.cell.2014.04.052
6. Wang L, Geng J, Qu M, et al (2020) Oligodendrocyte precursor cells transplantation protects blood–brain barrier in a mouse model of brain ischemia via Wnt/β-catenin signaling. Cell Death Dis 11:9. https://doi.org/10.1038/s41419-019-2206-9
7. Zhang S, Wang Y, Xu J, et al (2021) HIFα Regulates Developmental Myelination Independent of Autocrine Wnt Signaling. J Neurosci 41:251–268. https://doi.org/10.1523/JNEUROSCI.0731-20.2020
8. Tsai H-H, Niu J, Munji R, et al (2016) Oligodendrocyte precursors migrate along vasculature in the developing nervous system. Science 351:379–384. https://doi.org/10.1126/science.aad3839
9. Miyamoto N, Tanaka R, Shimura H, et al (2010) Phosphodiesterase III inhibition promotes differentiation and survival of oligodendrocyte progenitors and enhances regeneration of ischemic white matter lesions in the adult mammalian brain. J Cereb Blood Flow Metab 30:299–310. https://doi.org/10.1038/jcbfm.2009.210
10. Niu J, Tsai H-H, Hoi KK, et al (2019) Aberrant oligodendroglial–vascular interactions disrupt the blood–brain barrier, triggering CNS inflammation. Nat Neurosci 22:709–718. https://doi.org/10.1038/s41593-019-0369-4
11. Tong X-K, Trigiani LJ, Hamel E (2019) High cholesterol triggers white matter alterations and cognitive deficits in a mouse model of cerebrovascular disease: benefits of simvastatin. Cell Death Dis 10:1–14. https://doi.org/10.1038/s41419-018-1199-0
12. Iijima K, Kurachi M, Shibasaki K, et al (2015) Transplanted microvascular endothelial cells promote oligodendrocyte precursor cell survival in ischemic demyelinating lesions. J Neurochem 135:539–550. https://doi.org/10.1111/jnc.13262
13. Shivanna S, Harrold I, Shashar M, et al (2015) The c-Cbl ubiquitin ligase regulates nuclear β-catenin and angiogenesis by its tyrosine phosphorylation mediated through the Wnt signaling pathway. J Biol Chem 290:12537–12546. https://doi.org/10.1074/jbc.M114.616623
14. Li C, Zheng X, Han Y, et al (2018) XAV939 inhibits the proliferation and migration of lung adenocarcinoma A549 cells through the WNT pathway. Oncol Lett 15:8973–8982. https://doi.org/10.3892/ol.2018.8491
15. Kishida N, Maki T, Takagi Y, et al (2019) Role of Perivascular Oligodendrocyte Precursor Cells in Angiogenesis After Brain Ischemia. J Am Heart Assoc 8:e011824. https://doi.org/10.1161/JAHA.118.011824

16. Tian Y, Yin H, Deng X, et al (2018) CXCL12 induces migration of oligodendrocyte precursor cells through the CXCR4-activated MEK/ERK and PI3K/AKT pathways. Mol Med Rep 18:4374–4380. https://doi.org/10.3892/mmr.2018.9444
17. Wong SM, Jansen JFA, Zhang CE, et al (2019) Blood-brain barrier impairment and hypoperfusion are linked in cerebral small vessel disease. Neurology 92:e1669–e1677. https://doi.org/10.1212/WNL.0000000000007263
18. Daneman R, Agalliu D, Zhou L, et al (2009) Wnt/β-catenin signaling is required for CNS, but not non-CNS, angiogenesis. PNAS 106:641–646. https://doi.org/10.1073/pnas.0805165106
19. Olsen JJ, Pohl SÖ-G, Deshmukh A, et al (2017) The Role of Wnt Signalling in Angiogenesis. Clin Biochem Rev 38:131–142
20. Siemerink MJ, Klaassen I, Van Noorden CJF, Schlingemann RO (2013) Endothelial Tip Cells in Ocular Angiogenesis. J Histochem Cytochem 61:101–115. https://doi.org/10.1369/0022155412467635
21. Vanhollebeke B, Stone OA, Bostaille N, et al Tip cell-specific requirement for an atypical Gpr124- and Reck-dependent Wnt/β-catenin pathway during brain angiogenesis. eLife 4:e06489. https://doi.org/10.7554/eLife.06489
22. Park T-E, Mustafaoglu N, Herland A, et al (2019) Hypoxia-enhanced Blood-Brain Barrier Chip recapitulates human barrier function and shuttling of drugs and antibodies. Nat Commun 10:2621. https://doi.org/10.1038/s41467-019-10588-0
23. Chavali M, Ulloa-Navas MJ, Pérez-Borredá P, et al (2020) Wnt-Dependent Oligodendroglial-Endothelial Interactions Regulate White Matter Vascularization and Attenuate Injury. Neuron 108:1130-1145.e5. https://doi.org/10.1016/j.neuron.2020.09.033
24. Zhang Q, Bai X, Chen W, et al (2013) Wnt/β-catenin signaling enhances hypoxia-induced epithelial-mesenchymal transition in hepatocellular carcinoma via crosstalk with hif-1α signaling. Carcinogenesis 34:962–973. https://doi.org/10.1093/carcin/bgt027
25. Xu W, Zhou W, Cheng M, et al (2017) Hypoxia activates Wnt/β-catenin signaling by regulating the expression of BCL9 in human hepatocellular carcinoma. Sci Rep 7:40446. https://doi.org/10.1038/srep40446
26. Paolinelli R, Corada M, Ferrarini L, et al (2013) Wnt Activation of Immortalized Brain Endothelial Cells as a Tool for Generating a Standardized Model of the Blood Brain Barrier In Vitro. PLOS ONE 8:e70233. https://doi.org/10.1371/journal.pone.0070233
27. Gastfriend BD, Nishihara H, Canfield SG, et al (2021) Wnt signaling mediates acquisition of blood–brain barrier properties in naïve endothelium derived from human pluripotent stem cells. eLife 10:e70992. https://doi.org/10.7554/eLife.70992
28. Taddei A, Giampietro C, Conti A, et al (2008) Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat Cell Biol 10:923–934. https://doi.org/10.1038/ncb1752
29. Karnati HK, Panigrahi M, Shaik NA, et al (2014) Down Regulated Expression of Claudin-1 and Claudin-5 and Up Regulation of B-Catenin: Association with Human Glioma Progression. CNS Neurol Disord Drug Targets 13:1413–1426
30. Wang Y, Cho C, Williams J, et al (2018) Interplay of the Norrin and Wnt7a/Wnt7b signaling systems in blood–brain barrier and blood–retina barrier development and maintenance. Proceedings of the National Academy of Sciences 115:E11827–E11836. https://doi.org/10.1073/pnas.1813217115
31. Tamura M, Sato MM, Nashimoto M (2011) Regulation of CXCL12 expression by canonical Wnt signaling in bone marrow stromal cells. Int J Biochem Cell Biol 43:760–767. https://doi.org/10.1016/j.biocel.2011.01.021

32. Duperron M-G, Knol MJ, Le Grand Q, et al (2023) Genomics of perivascular space burden unravels early mechanisms of cerebral small vessel disease. Nat Med 29:950–962. https://doi.org/10.1038/s41591-023-02268-w
33. Deramecourt V, Slade JY, Oakley AE, et al (2012) Staging and natural history of cerebrovascular pathology in dementia. Neurology 78:1043–1050. https://doi.org/10.1212/WNL.0b013e31824e8e7f
34. Kleszka K, Leu T, Quinting T, et al (2020) Hypoxia-inducible factor-2α is crucial for proper brain development. Sci Rep 10:19146. https://doi.org/10.1038/s41598-020-75838-4
35. Allan KC, Hu LR, Scavuzzo MA, et al (2021) Non-Canonical Targets of HIF1a Impair Oligodendrocyte Progenitor Cell Function. Cell Stem Cell 28:257-272.e11. https://doi.org/10.1016/j.stem.2020.09.019
36. Zhang S, Kim B, Zhu X, et al (2020) Glial type specific regulation of CNS angiogenesis by HIFα-activated different signaling pathways. Nat Commun 11:2027. https://doi.org/10.1038/s41467-020-15656-4
37. Martinez Sosa S, Smith KJ (2017) Understanding a role for hypoxia in lesion formation and location in the deep and periventricular white matter in small vessel disease and multiple sclerosis. Clin Sci (Lond) 131:2503–2524. https://doi.org/10.1042/CS20170981
38. Zhang Y, Chen K, Sloan SA, et al (2014) An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. J Neurosci 34:11929–11947. https://doi.org/10.1523/JNEUROSCI.1860-14.2014
39. Vanlandewijck M, He L, Mäe MA, et al (2018) A molecular atlas of cell types and zonation in the brain vasculature. Nature 554:475–480. https://doi.org/10.1038/nature25739
40. Mecha M, Yanguas-Casás N, Feliú A, et al (2020) Involvement of Wnt7a in the role of M2c microglia in neural stem cell oligodendrogenesis. Journal of Neuroinflammation 17:88. https://doi.org/10.1186/s12974-020-01734-3
41. Li Y, Baccouche B, Olayinka O, et al (2021) The Role of the Wnt Pathway in VEGF/Anti-VEGF-Dependent Control of the Endothelial Cell Barrier. Invest Ophthalmol Vis Sci 62:17. https://doi.org/10.1167/iovs.62.12.17
42. Hayakawa K, Pham L-DD, Som AT, et al (2011) Vascular Endothelial Growth Factor Regulates the Migration of Oligodendrocyte Precursor Cells. J Neurosci 31:10666–10670. https://doi.org/10.1523/JNEUROSCI.1944-11.2011
43. Hayakawa K, Seo JH, Pham L-DD, et al (2012) Cerebral endothelial derived vascular endothelial growth factor promotes the migration but not the proliferation of oligodendrocyte precursor cells in vitro. Neurosci Lett 513:42–46. https://doi.org/10.1016/j.neulet.2012.02.004
44. Le Bras B, Barallobre M-J, Homman-Ludiye J, et al (2006) VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. Nat Neurosci 9:340–348. https://doi.org/10.1038/nn1646
45. Yu W, Jin H, Sun W, et al (2021) Connexin43 promotes angiogenesis through activating the HIF-1α/VEGF signaling pathway under chronic cerebral hypoperfusion. J Cereb Blood Flow Metab 41:2656–2675. https://doi.org/10.1177/0271678X211010354
46. Maki T, Ihara M, Fujita Y, et al (2011) Angiogenic and vasoprotective effects of adrenomedullin on prevention of cognitive decline after chronic cerebral hypoperfusion in mice. Stroke 42:1122–1128. https://doi.org/10.1161/STROKEAHA.110.603399
47. Argaw AT, Gurfein BT, Zhang Y, et al (2009) VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc Natl Acad Sci U S A 106:1977–1982. https://doi.org/10.1073/pnas.0808698106

48. Maki T, Morancho A, Segundo PM-S, et al (2018) Endothelial progenitor cell secretome and oligovascular repair in a mouse model of prolonged cerebral hypoperfusion. Stroke 49:1003–1010. https://doi.org/10.1161/STROKEAHA.117.019346
49. Gerhardt H (2008) VEGF and endothelial guidance in angiogenic sprouting. Organogenesis 4:241–246. https://doi.org/10.4161/org.4.4.7414
50. Tarkowski E, Issa R, Sjögren M, et al (2002) Increased intrathecal levels of the angiogenic factors VEGF and TGF-beta in Alzheimer's disease and vascular dementia. Neurobiol Aging 23:237–243. https://doi.org/10.1016/s0197-4580(01)00285-8
51. Tayler H, Miners JS, Güzel Ö, et al (2021) Mediators of cerebral hypoperfusion and blood-brain barrier leakiness in Alzheimer's disease, vascular dementia and mixed dementia. Brain Pathology 31:e12935. https://doi.org/10.1111/bpa.12935
52. Gertje EC, Janelidze S, Westen D van, et al (2023) Associations Between CSF Markers of Inflammation, White Matter Lesions, and Cognitive Decline in Individuals Without Dementia. Neurology 100:e1812–e1824. https://doi.org/10.1212/WNL.0000000000207113
53. Girolamo F, Coppola C, Ribatti D, Trojano M (2014) Angiogenesis in multiple sclerosis and experimental autoimmune encephalomyelitis. Acta Neuropathologica Communications 2:84. https://doi.org/10.1186/s40478-014-0084-z
54. Foulquier S, Daskalopoulos EP, Lluri G, et al (2018) WNT Signaling in Cardiac and Vascular Disease. Pharmacol Rev 70:68–141. https://doi.org/10.1124/pr.117.013896
55. Eubelen M, Bostaille N, Cabochette P, et al (2018) A molecular mechanism for Wnt ligand-specific signaling. Science 361:eaat1178. https://doi.org/10.1126/science.aat1178
56. Cho C, Wang Y, Smallwood PM, et al (2019) Molecular determinants in Frizzled, Reck, and Wnt7a for ligand-specific signaling in neurovascular development. eLife 8:e47300. https://doi.org/10.7554/eLife.47300
57. Sun J, Ou W, Han D, et al (2022) Comparative studies between the murine immortalized brain endothelial cell line (bEnd.3) and induced pluripotent stem cell-derived human brain endothelial cells for paracellular transport. PLOS ONE. 17:e0268860. https://doi.org/10.1371/journal.pone.0268860
58. Pavlacky J, Polak J (2020) Technical Feasibility and Physiological Relevance of Hypoxic Cell Culture Models. Frontiers in Endocrinology 11:
59. Woo KW, Yeo SI (1995) Dalton's Law vs, Amagat's Law for the Mixture of Real Gases. SNU Journal of Education Research 5:127–134
60. Bink DI, Ritz K, Aronica E, et al (2013) Mouse models to study the effect of cardiovascular risk factors on brain structure and cognition. J Cereb Blood Flow Metab 33:1666–1684. https://doi.org/10.1038/jcbfm.2013.140
61. Kim KJ, Diaz JR, Presa JL, et al (2021) Decreased parenchymal arteriolar tone uncouples vessel-to-neuronal communication in a mouse model of vascular cognitive impairment. GeroScience 43:1405–1422. https://doi.org/10.1007/s11357-020-00305-x
62. Li M, Kitamura A, Beverley J, et al (2022) Impaired Glymphatic Function and Pulsation Alterations in a Mouse Model of Vascular Cognitive Impairment. Front Aging Neurosci 13:788519. https://doi.org/10.3389/fnagi.2021.788519
63. Wang M, Qin C, Luo X, et al (2019) Astrocytic connexin 43 potentiates myelin injury in ischemic white matter disease. Theranostics 9:4474–4493. https://doi.org/10.7150/thno.31942
64. Nam K-W, Kwon H-M, Lee Y-S (2020) Distinct association between cerebral arterial pulsatility and subtypes of cerebral small vessel disease. PLoS One 15:e0236049. https://doi.org/10.1371/journal.pone.0236049

Chapter 7

-

Impact

Widespread risk factors such as hypertension can lead to functional and structural changes of the cerebral small blood vessels, ultimately leading to development of cerebral small vessel disease (cSVD) [1]. It is difficult to estimate the exact prevalence of cSVD, as the disease is often asymptomatic, underdiagnosed, and heterogeneous, and often presents with several comorbidities. The disease contributes to approximately 25% of strokes and 45% of dementia cases, causing a substantial global social and economic burden [2]. After ageing, hypertension as well as most of the other risk factors associated with cSVD are modifiable (e.g. smoking, diabetes, physical inactivity), which gives rise to a window of opportunity for potential prevention and treatment [3, 4]. However, primary prevention therapies are lacking as cSVD progression is usually asymptomatic and diagnosis relies on MRI markers, while primary treatment is based on late-stage symptom relief [3]. Thus, the aim of this thesis was the identification of cellular and molecular mechanisms that may ultimately be used for the prevention and treatment of cSVD. This thesis particularly focuses on the interplay between oligodendrocytes precursor cells (OPCs) and vascular endothelial cells (ECs). Unravelling mechanisms involved in this interplay not only clarifies current knowledge gaps, but also paves the way for new diagnostic strategies, including fluid biomarkers, and treatment options to tackle the growing global cSVD burden.

Scientific impact

In Chapter 2, our systematic review revealed Wnt7a as a potential mediator of OPC-EC crosstalk in cSVD pathology. Studies demonstrated that hypoxia increases the expression and secretion of Wnt7a by hypoxic OPCs and thereby mediates EC proliferation and angiogenesis [5–9]. Furthermore, Wnt7a is also known to promote the migration of OPCs by utilising the vasculature through inducing Cxcl12-Cxcr4 signalling pathway [5, 10, 11].

However, our findings in Chapter 3 and 4 are partly contradicting these previous findings. In Chapter 3, Wnt7a decreased the expression of TJ proteins and increased blood-brain barrier (BBB) permeability through activation of β-catenin, which contradicts studies showing the Wnt/β-catenin mediated increase in these TJ proteins and the decrease in BBB permeability [12, 13]. It is thus important that future studies investigate these findings and unravel its involvement in pathological mechanisms leading to BBB impairments and white matter lesions (WMLs) seen in cSVD patients [14].

In vitro studies utilising human pluripotent stem cells (hPSCs) hold the potential to elucidate these discrepancies, as they provide a valuable tool for disease modelling. Additionally, OPC migration via the vasculature has previously been shown to be mediated by increased Cxcl12-Cxcr4 interaction as a consequence of Wnt7a stimulation [10], which also contradict our findings in Chapter 4 where we found decreased Cxcl12 expression. Genetic mouse models for Wnt7a knockdown can elucidate these findings in vivo. A previous study generated a Wnt7a/b-deficient mutant mouse by intercrossing Olig2-Cre to conventional Wnt7a null and conditional Wnt7b (*fl*/*fl*) alleles to study the role of OPC-derived Wnt7a/b in development and hypoxic injury [8]. Inducing hypoperfusion with BCAS in similar models could lead to better understanding the role of OPC-derived Wnt7a in BBB permeability and OPC migration, and its implication in hypoxic brain injury.

Lastly, our in vitro findings in Chapter 5 indicate that hypoperfusion mediated hypoxia in OPCs leads to increased VEGFA rather than Wnt7a expression, supporting other studies that did not find Wnt7a regulation in OPCs under hypoxic conditions [15, 16].

Although these findings may identify novel molecular pathways relevant to cSVD development, addressing knowledge gaps regarding the underlying dysfunctional mechanisms contributes to the complexity of cSVD pathology.

Clinical implications

Highlighting the clinical implications of research on cSVD is crucial given that treatment strategies primarily focus on symptom relief rather than addressing the underlying cause. Efficacy of current treatments may be compromised due to the presence of multiple risk factors and the various conditions contributing to disease development. Understanding molecular mechanisms in cSVD can advance diagnosis with biomarkers, improve cognitive outcomes through novel prevention and treatment strategies, and enable personalised therapies targeting high-risk individuals. Current approaches for the prevention and treatment of cSVD encompass a combination of pharmacotherapy and lifestyle modifications. Pharmacotherapy involves the use of various medications, blood pressure-, cholesterol-, and glucose-lowering drugs, aimed at limiting the impact of cardiovascular risk factors. Additionally, anti-dementia drugs, like the NMDA-receptor partial antagonist memantine and cholinesterase inhibitors, as well as drugs such as cytidine-diphosphocholine and dl-3-n-butylphthalidle may be utilised [17]. Memantine can improve cognitive function by antagonising glutamate-induced neurotoxicity [18, 19], while cholinesterase inhibitors increase the availability of acetylcholine, an important neurotransmitter associated with memory, in neuromuscular junctions by inhibiting its enzymatic breakdown [20, 21]. Cytidine-diphosphocholine show neuroprotective effects by increasing noradrenaline and dopamine levels in the central nervous system (CNS) [22]. Dl-3-n-butylphthalidle can improve outcome after stroke due to its vasodilative effects by promoting NO production of ECs, its anti-thrombotic effects, or most importantly, by upregulating VEGFA and HIF1α expression [23].

More recently, clinical trials assessing efficacy of drugs in restoring cerebral blood flow (CBF) and limiting blood flow pulsatility, with the use of e.g. cilostazol, isosorbide

mononitrate, tadalafil, and pentoxifylline, are currently ongoing. In conjunction with pharmacotherapy, lifestyle modifications play a significant role in cSVD management. Quitting smoking, maintaining a healthy diet, and engaging in aerobic exercise are crucial for reducing risk factors and promoting cardiovascular well-being [24]. Taken together, this emphasising the importance of fundamental research in elucidating the link between vascular pathology and cognitive impairment in cSVD.

Wnt7a signalling modulation

Modulating the Wnt7a signalling pathway might be a potential therapeutic strategy, as our results show that Wnt7a stimulation could lead to EC permeability and suggest a role in OPC migration. Modulating these processes post-stroke might be beneficial for patients to reduce ischaemic injury and facilitate remyelination in damaged regions. Recently, clinical trials have explored novel approaches to modulate Wnt signalling pathways by combining technologies to cope with the pleiotropic nature of Wnt, which plays diverse roles in various biological processes [25]. Moreover, pre-clinical advancements demonstrate promising outcomes by utilising Wnt7a derivatives as a potential approach to mitigate CNS disorders by repairing the BBB. These derivatives specifically target Gpr124/Reck signalling and show neurovascular protective properties in stroke and glioblastoma models in mice without Wnt activation in other tissue [26]. However, Wnt signalling-targeting drugs are not clinically available yet because of associated side effects, and thus other therapeutic strategies must be considered.

VEGFA signalling modulation

As previously mentioned, the mechanism of action of dl-3-n-butylphthalidle includes modulation of ECs and VEGFA and HIF1α expression [23]. Thus, offering a potential target to promote vascular repair, inhibit brain inflammation, and prevent white matter (WM) damage by exploring the role of VEGFA in the interaction between ECs and OPCs. Following up on our retrospective findings in Chapter 5 with large cohort prospective studies that measure BBB permeability in different WM regions and VEGFA blood plasma concentration overtime could reveal an association between WML development and VEGFA blood plasma concentration. Blood plasma levels of VEGFA might then potentially be used as a biomarker for the early diagnosis for risk of WML development. It would also present a potential target for modulating VEGFA to decrease the risk of WML development. However, it is essential to acknowledge the potential dual nature of modulating VEGFA signalling. While beneficial angiogenic properties may facilitate compensatory vascular network growth in the brain, reducing hypoxia and CBF deficits, there is a risk of increased BBB permeability due to VEGFA ability to loosen tight junctions, which might ultimately contribute to inflammation, and WM damage [27, 28]. Another potentially adverse aspect of stimulating angiogenesis by systemic interventions is the risk of promoting the vascularisation of any malignancies that may be present in the patient. A solution would be the local delivery to the brain or specific modulation of VEGF receptors in the brain. The use of VEGFA isoforms, which elicit different biological effects, has been suggested as a potential solution for these unwanted side effects [28]. Thus, the therapeutic potential of VEGFA must be considered carefully.

Timing of VEGFA administration is also critical. Angiogenesis induction in the early silent stages of cSVD may help prevent ischaemic injury, while post-stroke stimulation of new

vessel formation and increased BBB permeability could enhance the inflammatory response and contribute to WML progression and cognitive decline, with previous studies indicating an association between VEGFA levels and stroke severity [29, 30]. Preclinical studies involving VEGFA administration have shown promising effects on stroke recovery, such as reducing lesion size, decreasing infarct size, promoting angiogenesis, and improving cognitive function. However, the effectiveness of VEGFA modulation in humans is yet to be proven [28, 31, 32].

Future directions

Future studies should prioritise the validation of the hypothesis regarding OPC-derived VEGFA in the interaction between OPCs and ECs in cSVD. By investigating the specific intracellular signalling pathways associated with VEGFA, such as HIF1α/VEGFA and Wnt/VEGFA, we can gain insight into the underlying mechanisms driving VEGFA-related changes in the brain vasculature and WM, which contribute to the development of cSVD. Manipulating these pathways using more accurate and sophisticated preclinical in vivo and in vitro models will allow the identification of novel biomarkers and the development of targeted therapeutic strategies. To facilitate the translation of preclinical findings into clinical applications, further research should focus on conducting well-designed clinical trials to test the safety and efficacy of VEGFA signalling modulation-based therapies. Ultimately, these efforts have the potential to improve patients' quality of life.

References

1. Pantoni L (2010) Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. Lancet Neurol 9:689–701. https://doi.org/10.1016/S1474-4422(10)70104-6
2. Cannistraro RJ, Badi M, Eidelman BH, et al (2019) CNS small vessel disease: A clinical review. Neurology 92:1146–1156. https://doi.org/10.1212/WNL.0000000000007654
3. Chojdak-Łukasiewicz J, Dziadkowiak E, Zimny A, Paradowski B (2021) Cerebral small vessel disease: A review. Adv Clin Exp Med 30:349–356. https://doi.org/10.17219/acem/131216
4. Parodi L, Mayerhofer E, Narasimhalu K, et al (2023) Social Determinants of Health and Cerebral Small Vessel Disease: Is Epigenetics a Key Mediator? J Am Heart Assoc 12:e029862. https://doi.org/10.1161/JAHA.123.029862
5. Yuen TJ, Silbereis JC, Griveau A, et al (2014) Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell 158:383–396. https://doi.org/10.1016/j.cell.2014.04.052
6. Wang Y, Cho C, Williams J, et al (2018) Interplay of the Norrin and Wnt7a/Wnt7b signaling systems in blood–brain barrier and blood–retina barrier development and maintenance. Proceedings of the National Academy of Sciences 115:E11827–E11836. https://doi.org/10.1073/pnas.1813217115
7. Kishida N, Maki T, Takagi Y, et al (2019) Role of Perivascular Oligodendrocyte Precursor Cells in Angiogenesis After Brain Ischemia. J Am Heart Assoc 8:e011824. https://doi.org/10.1161/JAHA.118.011824
8. Chavali M, Ulloa-Navas MJ, Pérez-Borredá P, et al (2020) Wnt-Dependent Oligodendroglial-Endothelial Interactions Regulate White Matter Vascularization and Attenuate Injury. Neuron 108:1130-1145.e5. https://doi.org/10.1016/j.neuron.2020.09.033
9. Wang L, Geng J, Qu M, et al (2020) Oligodendrocyte precursor cells transplantation protects blood–brain barrier in a mouse model of brain ischemia via Wnt/β-catenin signaling. Cell Death Dis 11:9. https://doi.org/10.1038/s41419-019-2206-9
10. Tsai H-H, Niu J, Munji R, et al (2016) Oligodendrocyte precursors migrate along vasculature in the developing nervous system. Science 351:379–384. https://doi.org/10.1126/science.aad3839
11. Niu J, Tsai H-H, Hoi KK, et al (2019) Aberrant oligodendroglial–vascular interactions disrupt the blood–brain barrier, triggering CNS inflammation. Nat Neurosci 22:709–718. https://doi.org/10.1038/s41593-019-0369-4
12. Hussain B, Fang C, Huang X, et al (2022) Endothelial β-Catenin Deficiency Causes Blood-Brain Barrier Breakdown via Enhancing the Paracellular and Transcellular Permeability. Front Mol Neurosci 15:895429. https://doi.org/10.3389/fnmol.2022.895429
13. Paolinelli R, Corada M, Ferrarini L, et al (2013) Wnt Activation of Immortalized Brain Endothelial Cells as a Tool for Generating a Standardized Model of the Blood Brain Barrier In Vitro. PLOS ONE 8:e70233. https://doi.org/10.1371/journal.pone.0070233
14. Zhang CE, Wong SM, Uiterwijk R, et al (2019) Blood–brain barrier leakage in relation to white matter hyperintensity volume and cognition in small vessel disease and normal aging. Brain Imaging Behav 13:389–395. https://doi.org/10.1007/s11682-018-9855-7
15. Allan KC, Hu LR, Scavuzzo MA, et al (2021) Non-Canonical Targets of HIF1a Impair Oligodendrocyte Progenitor Cell Function. Cell Stem Cell 28:257-272.e11. https://doi.org/10.1016/j.stem.2020.09.019
16. Zhang S, Wang Y, Xu J, et al (2021) HIFα Regulates Developmental Myelination Independent of Autocrine Wnt Signaling. J Neurosci 41:251–268. https://doi.org/10.1523/JNEUROSCI.0731-20.2020

17. Peng D (2019) Clinical practice guideline for cognitive impairment of cerebral small vessel disease. Aging Med (Milton) 2:64–73. https://doi.org/10.1002/agm2.12073
18. Wilcock G, Möbius HJ, Stöffler A, MMM 500 group (2002) A double-blind, placebo-controlled multicentre study of memantine in mild to moderate vascular dementia (MMM500). Int Clin Psychopharmacol 17:297–305. https://doi.org/10.1097/00004850-200211000-00005
19. Orgogozo J-M, Rigaud A-S, Stöffler A, et al (2002) Efficacy and safety of memantine in patients with mild to moderate vascular dementia: a randomized, placebo-controlled trial (MMM 300). Stroke 33:1834–1839. https://doi.org/10.1161/01.str.0000020094.08790.49
20. Fukatsu T, Miyake-Takagi K, Nagakura A, et al (2002) Effects of nefiracetam on spatial memory function and acetylcholine and GABA metabolism in microsphere-embolized rats. Eur J Pharmacol 453:59–67. https://doi.org/10.1016/s0014-2999(02)02360-9
21. Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. Cochrane Database Syst Rev 2006:CD005593. https://doi.org/10.1002/14651858.CD005593
22. Secades JJ, Frontera G (1995) CDP-choline: pharmacological and clinical review. Methods Find Exp Clin Pharmacol 17 Suppl B:1–54
23. Cui L, Zhu Y, Gao S, et al (2013) Ninety-day administration of dl-3-n-butylphthalide for acute ischemic stroke: a randomized, double-blind trial. Chinese Medical Journal 126:3405. https://doi.org/10.3760/cma.j.issn.0366-6999.20123240
24. Shindo A, Ishikawa H, Ii Y, et al (2020) Clinical Features and Experimental Models of Cerebral Small Vessel Disease. Front Aging Neurosci 12:109. https://doi.org/10.3389/fnagi.2020.00109
25. Park W-J, Kim MJ (2023) A New Wave of Targeting 'Undruggable' Wnt Signaling for Cancer Therapy: Challenges and Opportunities. Cells 12:1110. https://doi.org/10.3390/cells12081110
26. Martin M, Vermeiren S, Bostaille N, et al (2022) Engineered Wnt ligands enable blood-brain barrier repair in neurological disorders. Science 375:eabm4459. https://doi.org/10.1126/science.abm4459
27. Shibuya M (2013) Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. J Biochem 153:13–19. https://doi.org/10.1093/jb/mvs136
28. White AL, Bix GJ (2023) VEGFA Isoforms as Pro-Angiogenic Therapeutics for Cerebrovascular Diseases. Biomolecules 13:702. https://doi.org/10.3390/biom13040702
29. Slevin M, Krupinski J, Slowik A, et al (2000) Serial measurement of vascular endothelial growth factor and transforming growth factor-beta1 in serum of patients with acute ischemic stroke. Stroke 31:1863–1870. https://doi.org/10.1161/01.str.31.8.1863
30. Lennmyr F, Ata KA, Funa K, et al (1998) Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. J Neuropathol Exp Neurol 57:874–882. https://doi.org/10.1097/00005072-199809000-00009
31. Henry TD, Annex BH, McKendall GR, et al (2003) The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation 107:1359–1365. https://doi.org/10.1161/01.cir.0000061911.47710.8a
32. Anttila V, Saraste A, Knuuti J, et al (2023) Direct intramyocardial injection of VEGF mRNA in patients undergoing coronary artery bypass grafting. Molecular Therapy 31:866–874. https://doi.org/10.1016/j.ymthe.2022.11.017

Dutch summary

-

Nederlandse samenvatting

Samenvatting

Cerebral small vessel disease (cSVD) is een medische aandoening waarbij de kleine bloedvaatjes in de hersenen zijn aangetast. Deze bloedvaatjes vervoeren bloed naar verschillende hersengedeeltes, dat belangrijk is voor het bezorgen van essentiële voedingsstoffen en zuurstof. Wanneer deze vatten beschadigd of vernauwd raken door bijvoorbeeld veroudering, diabetes, of hoge bloeddruk, kunnen deze essentiële stoffen en zuurstof bepaalde delen niet bereiken. Dit kan leiden tot problemen waarbij gedeeltes van de hersenen zelfs kunnen afsterven. Een tekort aan zuurstof in de hersenen kan zeer nadelige gevolgen hebben voor de gezondheid en leiden tot hersenbloedingen, problemen met geheugen en stemming, en lichamelijke klachten zoals problemen met lopen. Een andere karakteristiek en belangrijke diagnostische beeld van cSVD is het aangetaste “witte stof”. Dit onderdeel van de hersenen is vooral opgebouwd uit de zogeheten oligodendrocyten. Deze cellen zorgen voor een beschermlaag van de zenuwcellen in de hersenen (de neuronen), die verantwoordelijk zijn voor alle signalen die van de hersenen naar het lichaam gaan. Omdat de oligodendrocyten een laagje isolerend vet om de neuronen vormen, kan de elektrische signaal sneller en efficiënter verlopen. Dit laagje vet zorgt voor een witte kleur, vandaar de term “witte stof”. Bij patiënten met cSVD wordt een deel van de witte stof aangetast waardoor de hersenen niet optimaal meer functioneren.

De onderliggende oorzaak voor deze afwijkingen zijn grotendeels onbekend. Wel is bekend dat de cellen die deze bloedvaatjes opbouwen (de zogeheten endotheelcellen) en onvolwassen isolerende cellen (oligodendrocyte precursor cellen, ook wel OPC genoemd) een belangrijke rol spelen. Hoewel een verstoorde wisselwerking tussen deze

endotheelcellen en OPCs tot schade aan de witte stof zou kunnen leiden, is de exacte rol van deze interactie onvoldoende bestudeerd in de context van cSVD.

In hoofdstuk 2 van dit proefschrift hebben we door middel van een grondig onderzoek van de recente literatuur Wnt7a geïdentificeerd als een factor die mogelijk een oorzaak kan zijn van deze verstoorde wisselwerking. Wnt7a zou als gevolg van een zuurstoftekort door de OPCs als signaal kunnen dienen die de endotheelcellen aanspoort om meer bloedvaatjes te maken als compensatie om meer zuurstof aan te voeren. In hoofdstuk 3 van dit proefschrift hebben wij aangetoond dat Wnt7a doormiddel van het verzwakken van bindingen tussen endotheelcellen ervoor kan zorgen dat de beschermede barrièrefunctie van bloedvaatjes verminderd wordt, wat tot hersenbloedingen kan leiden. In hoofdstuk 4 tonen we aan dat Wnt7a tevens de functie van endotheelcellen als platform voor verplaatsing van OPCs vermindert. OPCs hechten aan zo genoemde ankers (Cxcl12) om zich zo door het brein te verplaatsen om schade te herstellen, en Wnt7a zorgt voor een afname van deze ankers. Ten slotte hebben we aangetoond dat verlaging van de hersen bloedtoevoer bij muizen daadwerkelijk leidt tot een zuurstoftekort in OPCs en een vermindering van de barrièrefunctie van hersenbloedvaatjes. Onderzoeken met OPCs die aan lage zuurstofgehaltes blootgesteld werden in hoofdstuk 5 resulteerden in verhoging van Vegfa, eerder dan Wnt7a. Dit stofje zorgt voor een signaal dat de endotheelcellen aanspoort om zich te vermenigvuldigen en nieuwe vaatjes te vormen. Ook in het bloed van cSVD patiënten bleek dit stofje verhoogd te zijn vergeleken met een gezonde controlegroep. Deze verhoging kon in verband worden gebracht met een verlaging van de barrièrefunctie van hersenbloedvaatjes in de onaangetaste witte stof van deze cSVD patiënten.

Uit onze bevindingen concluderen we dat (1) Wnt7a de barrièrefunctie van hersenbloedvaatjes en OPC-transport vermindert en (2) dat er een rol voor Vegfa, meer dan voor Wnt7a, is weggelegd in de reactie op zuurstoftekort, in de wisselwerking tussen endotheelcellen en OPC die kan leiden tot de hersenschade die ten grondslag ligt aan het ontwikkelen van cSVD.

Acknowledgements

-

Dankwoord

After almost 5 years of hard work, moving to the UK and back to the Netherlands, meeting amazing new people, spending evenings, nights, and weekends in the lab or in front of my laptop, struggling with experiments and deadlines, contemplating quitting on multiple occasions, and literally falling flat on my face, I have reached the finish line. Some say, it is not the finish line that matters, but the journey you go on to get there. And what a journey it has been. This PhD and all the brilliant minds that I had the privilege to work with taught me so much, such as new skills in the lab to become a better scientist, but more importantly, also how to be a better colleague, person, and friend. It has therefore been a life changing journal. However, I have to say, reaching the finish line does feel satisfactory.

I would like start of by expressing my sincere gratitude to all the members of the assessment committee for taking their time to read and assess my thesis.

It has been a unique opportunity to work in both the Neuroscience and Ophthalmology Department at University of Birmingham and the Pharmacology and Toxicology Department at Maastricht University. And I would like to thank everyone at both departments.

Thank you to the people at the office in Birmingham for the warm welcome. You all made me feel at home immediately. Especially my dear friends Maryam, Ahmad, and Gibran. Birmingham will always feel like home thanks to you guys. I would also like to thank Leah Bailey (PGR Administrator) for always answering my questions quickly, even though it was always last minute and urgent.

And then my dear colleagues in Maastricht. Although many have already left the department, I am grateful for meeting every one of you. It was always a delight to work in the same lab

with you all. You were always there to show me around because I have the memory of a goldfish. Hence, I would ask at least every other week how the PCR machine works. To all the technicians, thank you for your hard work and for making our lives a lot easier. I would also like to show my gratitude to the technicians at the Toxicogenomics and Neuroscience Department for always being there to help. Philippe and Margarita, thank you both for being my dear colleagues, even though you were not my colleagues. To my colleagues in our group: Stefan, Melissa, Eline, Daria, Laura, and Sara. To Stefan, Melissa, and Eline, you guys were great teammates, and always made our group activities memorable. Daria, it was a pleasure working with you, and your help in the lab was indispensable. To Sara and Laura, who I have spent most of my office days with, thanks for always being a listening ear. You guys always checked up on me and always made sure by double checking. Also, many sincere thanks to all the co-authors of the different chapters of this thesis. Publishing our exciting work would not have been possible without your input.

To my loved ones, my friends, thank you all for always supporting me and trying to understand my project and my weird schedule. Ik zal het in het Nederlands doen voor degene die geen Engels kunnen (you know who you are). Dank jullie wel voor al jullie steun en begrip al deze jaren. Ookal vroegen jullie elke keer weer: Ben je nou nog steeds aan het studeren? Ben je nou eindelijk klaar? Wanneer ga je je verdediging doen? Ik heb ondertussen een standaardantwoord voor al deze vragen. Nee, ik studeer niet meer. Het is een PhD. Nee, ik ben nog niet klaar. Hopelijk snel. Ik weet het nog niet, ik moet eerst in Engeland verdedigen. Dus bij deze, ook mijn dank voor jullie geduld. Niet alleen voor mijn project, maar ook voor jullie geduld met mijn als persoon. Ik heb dankzij jullie ook meer geduld met mezelf gekregen.

Aan mijn geliefde zussen, Lian en Aroes. Dank jullie wel dat jullie mij altijd steunen en altijd vol lof en trots over jullie kleine broertje spreken. Ik zou nooit de man zijn die ik nu ben zonder jullie als mijn grote zussen.

Իմ սիրելի հայրիկ և մայրիկ: Շնորհակալություն ամեն ինչի համար: Որպես երեք մանկահասակ երեխա ունեցող միգրանտներ, դուք այնքան շատ եք հանձնվել, որ մենք կարողանանք ունենալ այն հնարավորությունները, որոնք դուք երբեք չեք ունեցել: Հայրիկ, չնայած դու դեռ մտածում ես, որ ես «բժիշկ» եմ դառնալու և հարցնում ես, թե ինչպես է ընթանում ուսումը, քո աջակցությունն ու նվիրվածությունն իմ կյանքը հեշտացնելուն, որպեսզի ես կարողանամ կենտրոնանալ իմ ուսման և աշխատանքի վրա, անգնահատելի են: Եվ առանց քեզ ես երբեք չէի ունենա այն հնարավորությունները, որոնք ինձ տանում էին դեպի այս ճանապարհը: Եվ մայրիկ, շնորհակալ եմ, որ միշտ իմ կողքին ես: Դուք աջակցում եք իմ կատարած յուրաքանչյուր ընտրությանը, նույնիսկ եթե միշտ չէ, որ համաձայն եք դրա հետ: Դուք ինձ աջակցում եք ամեն միջով : Ես հպարտ եմ ձեզանով և շնորհակալ եմ այն ամենի համար, ինչ արելեք մեզ համար:

Last, but definitely not least, my supervisors: Daniel, Zubair, Matthijs, and Sébastien. First of all, thank you for giving me the change to do a PhD. I remember being on the other side of the world and thinking I hope I get this position. What an opportunity it would be. Working in two countries, two labs, two departments, combining two fields, and obtaining a degree from two universities. Thank you all for believing in me, even at times I did not believe in myself. I always felt we could discuss anything and we would find a solution for everything. With a team like you, there are no problems, only solutions. Daniel and Zubair, you took good care of me in Birmingham and I don’t know if I would be writing this if it

wasn’t for you guys helping me in my difficult times. Matthijs, your personal and professional input and knowledge were essential for the direction of my project. A big special thanks to Sebastien. You always took your time to discuss the direction of my project and came up with ideas that taught me so much. Even when you were busy, I could always knock on your door to discuss anything and ask my silly questions.