Blood Vessels, Biomarkers, and Broken Barriers: Investigations of the Brain Vasculature in Models of Neonatal Brain Injury

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Cover illustration: a maximum intensity z-projection of hippocampal blood vessels acquired by Axel Andersson in a confocal microscope. Duplicated, mirrored, and illustrated by Isabell Kirstinä.

Author portrait taken by Isabell Kirstinä.

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"The more you do stuff the better you get at dealing with how you still fail at it a lot of the time" – John Mulaney (supposedly)

ABSTRACT

Brain injury during the perinatal period can lead to lifelong impairment in cognitive and motor function, or an early death. Term neonatal encephalopathy and preterm germinal matrix haemorrhage are two conditions that can irreversibly injure the brain, but clinical tools for diagnosing and treating these pathologies are lacking. It is known that the cerebrovasculature, i.e. the blood vessels of the brain and blood/brain barrier (BBB) plays a role during the course and recovery of injury.

This thesis investigated the cerebrovascular involvement in two animal models of neonatal brain injury in an attempt to elucidate injury mechanisms, find potential new treatments, and identify biomarkers for brain vascular dysfunction. Using rodent models for hypoxic/ischemic encephalopathy (HIE) and germinal matrix-intraventricular haemorrhage (GM-IVH) we found raised levels of tight-junction proteins claudin-5 and occludin, two integral components of the BBB, in blood plasma and cerebrospinal fluid at different time points. In the HIE model, levels of tight-junction proteins in the circulation were sex-dependent and the amount of claudin-5 in CSF correlated with the severity of brain injury. These proteins thus have potential as biomarkers for early detection of cerebrovascular insults.

In addition, we did in-depth assessments of the BBB function in both models and detailed the temporal and regional increases of barrier permeability after injury by measuring the extravasation of radiolabelled sucrose, visible dyes, and molecular tracers. Studies of the cerebral vasculature and angiogenesis after HI showed that the density of proliferating endothelial cells were largely unaffected after injury, but the number of growing endothelial tip cells were strongly reduced in the entire brain, accompanied by changes in the expression of angiogenesis genes.

The thesis also includes the first trial of endogenous RNAse A as a neuroprotective treatment for neonatal brain injury, a treatment that has shown promise in adult models of other pathologies with cardiovascular aspects. We found significant reductions of grey and white matter tissue loss after RNAse A administration in HI animals, but no protection of BBB function or evidence for a reduced neuroinflammatory response. Taken together, this thesis contains new insights into several aspects of the vascular mechanisms involved in the pathogenesis of two major forms of injury that can occur in the neonatal brain.

Keywords: neonatal brain injury, hypoxia/ischemia, germinal matrix haemorrhage, blood/brain barrier, tight-junctions, blood vessels

SAMMANFATTNING PÅ SVENSKA

Hjärnskador i den perinatala perioden kan leda till livslånga kognitiva och motoriska handikapp, eller en mycket förtida död. Hypoxisk/ischemisk encefalopati (HIE) och hjärnblödningar i germinalmatrix är två tillstånd som permanent kan skada hjärnan, men kliniska verktyg för att diagnostisera och behandla dessa patologier saknas. Det är välkänt att hjärnans blodkärl och den skyddande blod/hjärnbarriären (BHB) spelar en viktig roll vid både utvecklingen och återhämtningen av hjärnskador.

Denna avhandling gjorde en närmare granskning av hur hjärnans vaskulatur är inblandade i dessa sjukdomar i ett försök att kartlägga skademekanismer, hitta potentiella nya behandlingar och identifiera biomarkörer för skador i hjärnans blodkärl. Med hjälp av gnagarmodeller som efterliknar HIE och blödningar i germinalmatrix fann vi förhöjda nivåer av claudin-5 och occludin, två nödvändiga komponenter i de täta fogarna som håller samman BHB, i blodplasma och cerebrospinalvätska (CSV) vid olika tidpunkter. I HIE-modellen var nivåerna av dessa proteiner i cirkulationen könsberoende och mängden claudin-5 i CSV korrelerade med nivån av hjärnskadans omfattning. Dessa proteiner har således potential som biomarkörer för tidig upptäckt av skador i hjärnans blodkärl.

Vi gjorde även grundliga undersökningar av BHB i båda modellerna och detaljerade de tidsmässiga och regionala ökningarna av barriärens genomsläpplighet genom att mäta nivåerna av radiomärkt sackaros, synliga färgmedel, och molekylära spårämnen efter skada. Studier av hjärnans blodkärlstillväxt efter HI visade att prolifererande endotelceller i stort sett var opåverkade efter skada, men mängden växande endotelspetsceller minskade kraftigt i hela hjärnan samtidigt som uttrycket av angiogenesrelaterade gener förändrades.

Avhandlingen inkluderar också den första prekliniska prövningen av RNAse A som en neuroprotektiv behandling för neonatala hjärnskador, en behandling som har visats lovande i modeller av andra patologier med kardiovaskulära aspekter. Vi fann signifikant skydd av grå och vit substans efter RNAse A-behandling i HI-djur, men inget skydd av BHB-funktionen eller bevis för minskad neuroinflammation. Sammantaget innehåller denna avhandling nya insikter i flera aspekter av de blodkärlsrelaterade mekanismer som är involverade i de patologiska processer som ligger bakom två vanliga former av skador som kan uppstå i den mycket unga hjärnan.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I.

Circulating Tight-junction Proteins are Potential Biomarkers for Blood-Brain Barrier Function in a Model of Neonatal Hypoxic/Ischemic Brain Injury

Andersson EA, Mallard C, Ek CJ. *Fluids Barriers CNS*. 2021 Feb 10;18(1):7. doi: 10.1186/s12987-021-00240-9.

II.

Function and Biomarkers of the Blood-Brain Barrier in a Neonatal Germinal Matrix Haemorrhage Model

Andersson EA, Rocha-Ferreira E, Hagberg H, Mallard C, Ek CJ. *Cells*. 2021 Jul 2;10(7):1677. doi: 10.3390/cells10071677.

III.

Dysregulation of Angiogenesis in a Mouse Model of Neonatal Hypoxia/Ischemia

Andersson EA, Chumak T, Shahbazi N, Mallard C, Ek CJ.
In manuscript.

IV.

Treatment with RNAse A Alleviates Brain Injury, but Not Neuroinflammation, in a Mouse Model of Neonatal Hypoxia/Ischemia

Andersson EA, Anderberg R, Ek CJ, Mallard C.
In manuscript.

CONTENTS

ABBREVIATIONS

AJ	adherens junctions
BBB	blood/brain barrier
BCSFB	blood/cerebrospinal fluid barrier
BrdU	bromodeoxyuridine
CBF	cerebral blood blow
CD31	cluster of differentiation 31
CLDN5	claudin-5
CSF	cerebrospinal fluid
EB	Evans blue
ELISA	enzyme-linked immunosorbent assay
eRNA	extracellular ribonucleic acid
GM	germinal matrix
GM-IVH	germinal matrix-intraventricular haemorrhage
HI	hypoxia/ischemia or hypoxic/ischemic
HIE	hypoxic/ischemic encephalopathy
i.p	intraperitoneal
IF	immunofluorescence
IHC	immunohistochemistry
MAP2	microtubule-associated protein 2
MBP	myelin basic protein
MRI	magnetic resonance imaging
NE	neonatal encephalopathy
NVU	neurovascular unit
OCLN	occludin
PND#	post-natal day #
RNA	ribonucleic acid
TH	therapeutic hypothermia
TJ	tight junction
VEGF	vascular endothelial growth factor
ZO-1	zonula occludens-1

1 INTRODUCTION

1.1 NEONATAL BRAIN INJURIES

Insults to the developing brain during the perinatal period (defined as gestational week 22 up to one month after birth in humans), such as neonatal stroke, cerebral hypoxia-ischemia and germinal matrix and intraventricular haemorrhages (GM-IVH) can result in different types of brain pathologies. Examples include grey matter injury, periventricular leukomalacia (i.e. distinct white matter injury) and diffuse white matter injury, or a mixture of these. Although the underlying pathologies differ, they share common risk factors and aspects of disease development and progression[1]. One necessary component in paving the way towards prevention of neonatal death and disability is knowledge of pathophysiological factors, improved treatments, and clinical diagnostic tools. This thesis summarizes a series of preclinical investigations into cerebrovascular aspects involved in the pathogenesis, treatment, and diagnosis of neonatal brain injuries based on animal models of near-term and term HI as well as preterm GM-IVH. The four papers that forms the basis of the thesis are referred to in the text by their roman numerals (**I-IV**).

1.1.1 Neonatal encephalopathy and hypoxic/ischemic brain injuries in term infants

It is estimated that up to 3 per 1000 term live births (i.e. infants born between weeks 37 and 42[2]) will be afflicted with neonatal encephalopathy (NE), a syndrome of acute neurological dysfunction characterized by altered level of consciousness, abnormal muscle tone, seizures and affected vital signs including respiration[3,4]. While NE is a global problem, the condition is more prevalent in low- and middle-income countries compared to higher-income countries[5]. There are many potential predisposing factors for NE. On the maternal side, increased age and lower social-economic as well as a family history of neurological conditions increased the risk of NE in an Australian population-study[6]. Maternal conditions such as preeclampsia[7], thyroid disease[8], and hypertension[9] during pregnancy are also associated with an increased risk of NE. Antenatal and intrapartum risk factors associated with NE development includes placental insufficiency, umbilical cord complications, intrauterine growth restriction, maternal infections and delivery by caesarean section[10-14]. However, a MRI-based study of term

infants presenting with NE showed that most infants had acute injuries, which strongly suggests that perinatal events in direct association with birth were more important for the development of NE than antenatal events[15]. NE is in turn a major risk factor for serious complications and it is predicted that up to one fifth of all affected neonates will die in the first weeks of life while an additional fourth will develop permanent neurodevelopmental deficits[16]. Such complications include cerebral palsy, epilepsy, and intellectual disability [17,18].

There are some important terminological considerations to discuss in relation to NE. The condition has historically been attributed to perinatal/birth asphyxia and referred to as neonatal hypoxic/ischemic encephalopathy (HIE) as the assumed cause was hypoxia/ischemia (HI), i.e. reductions of oxygen supply and blood flow to the brain [19]. Today, it is evident that NE can have other, sometimes unknown, aetiologies unrelated to HI[20,21]. Despite calls for a standardization of correct nomenclature to use in clinical and research settings, improper usage of the terms are still common[22]. Neonatal HIE is instead recognized as a subset of NE, estimated to occur in 1.5 per 1000 live births[23], with its own diagnostic criteria[24]. As the animal models used in **Papers I, III, & IV** are based on HI, the pathophysiology of neonatal HIE will be discussed in more detail.

1.1.2 The pathophysiology of neonatal hypoxic/ischemic encephalopathy

Irrespective of the underlying aetiology, neonatal HIE has a multifactorial pathophysiology wherein several different temporal processes work in tandem from the onset of HI to neuronal death occurs and brain lesions develop. Impaired cerebral blood flow (CBF) leading to a reduction of brain oxygen and glucose supply forces a shift to anaerobic metabolism which impairs the production of high-energy phosphates, initiating the primary phase of energy failure[25]. This phase is characterized by failure of transcellular transporters leading to intracellular accumulation of sodium and calcium cations and cytotoxic oedema formation as well as excessive neurotransmitter release resulting in excitotoxicity[26,27]. The anaerobic conditions also induce production of damaging oxygen free radicals[28]. After the acute insult, a recovery characterized by partly restored CBF, mitochondrial respiration, and reoxygenation is observed while electroencephalogram readings often remain abnormal[29-31].

The length of this recovery period is variable, with a shorter duration seen after more severe HI insults[32]. Milder cases can see a full recovery at this stage, but in patients with a severe primary HI insult this partial recovery is followed by a secondary energy failure where mitochondrial activity further deteriorates[30]. This cascade of energy failure, excitotoxicity, acidosis, and oxidative stress triggers mitochondrial permeabilization and mass brain cell death via apoptosis or necrosis or both, so called necroptotic pathways[33-35]. Injury is further exacerbated by neuroinflammation[36,37] and blood/brain barrier (BBB) dysfunction[38,39]. The primary energy failure and partial recovery is ongoing up to six hours after the onset of HI and the secondary energy failure typically occurs within the coming six to 15 hours, a period where seizures are common[40]. If the infant survives these first days, a long-term tertiary phase of brain injury persists for months, during which late cell death and brain tissue repair and remodelling occurs under conditions of chronic neuroinflammation[37,41].

1.1.3 Diagnosing neonatal hypoxic/ischemic encephalopathy

Clinically available diagnostic tools for identification and prognostication of neonatal HIE are generally only applicable post-partum[24]. There is no test that quickly and reliably can diagnose suspected HIE and a number of different tests are employed if an infant presents with symptoms. During high-risk deliveries, umbilical cord blood gases can be analysed for acid/base-balance to determine whether the foetus has been exposed to intrauterine hypoxia[42]. Metabolic factors, such as lactate, and pH in cord and early postnatal samples have been shown to correlate with neurological outcomes after NE[43,44]. After birth, infants are assessed by Apgar-scoring based on Appearance, Pulse, Grimace, Activity and Respiration to summarise the health of the infant, a persistently low score five minutes after birth is an indication of NE and a risk for disadvantageous outcomes[45]. While Apgar-scoring can identify infants that need medical attention it has less value for prediction of long-term outcomes. Infants born with suspected HIE can thus be further assessed by the Sarnat grading scale in which neonatal HIE is classified in three different grades, ranging from mild to severe[46]. Another commonly used bedside tool is the monitoring of brain function with amplitude integrated electroencephalogram (aEEG) as persisting abnormal readings are a strong predictor for HIE outcome[24]. The combination of metabolic analyses, Apgar scoring, Sarnat grading, and aEEG is used to select infants likely to develop moderate or severe HIE warranting therapeutic hypothermia (see 1.1.4)[24].

Apart from these immediate tools that can distinguish infants in need for extended care, the severity of the brain injury can at a later stage be evaluated with imaging modalities such as magnetic resonance imaging (MRI) to predict long-term outcome[47]. However, a recent study showed that an unexpectedly large number of infants diagnosed with mild HIE, thus not eligible for hypothermia, had abnormal outcomes and showed signs of brain injury on MRI[48]. There is therefore a need for better diagnostic tools to accurately select infants in need of early treatment. Many studies have focused on finding fluid biomarkers to facilitate early diagnosis and risk assessment, both in different animal models and in the cerebrospinal fluid (CSF), blood, saliva, and urine of infants with NE[49]. A good biomarker should be easily and safely accessible to analyse and confer high specificity and sensitivity. Ideally, it should be able to assess the location and severity of the injury as well as give long-term prognosis and be able to monitor clinical outcome and eventual deterioration.

Putative biomarkers for neonatal HI brain injuries can generally be divided into two distinct categories. Markers for brain injury, such as astrocyte-related glial fibrillary acidic protein[50,51] and protein S100B[52,53] are expressed in the brain and elevated in the circulation following damage to the brain. Inflammation-related markers, such as IL-6 and IL-8 among others[54], are highly expressed and circulated during inflammatory conditions and could potentially be used to monitor the inflammatory cascade seen during HI. However, despite many studies no fluid biomarker for neonatal brain injuries has yet to be translated into the clinic. Common suggestions for improvements of studies investigating potential biomarkers include using larger cohorts with better-documented outcomes and a standardization of both the sample collection timing and the techniques used for analysis to ensure better assessment of the validity of the findings[49,55,56].

1.1.4 Treating neonatal hypoxic/ischemic encephalopathy

The only treatment for neonatal HI in current use is therapeutic hypothermia (TH) in which either the whole body or head of afflicted newborns are cooled down to 33.5 ± 0.5°C (systemic) or 34.5 ± 0.5°C (head) for two to three days to alleviate brain damage[57]. Although the exact key mechanisms of TH have not been identified, the treatment reduces the demand for oxygen[58] and associated oxidative stress[59], supresses excitotoxicity[60], acts anti-inflammatory[61], anti-apoptotic[62,63], and reduces the BBB permeability and subsequent oedema formation[64]. It is generally thought TH should be initiated within six hours after the

injury before the secondary energy failure occurs, but the length of this therapeutic window and efficacy of the treatment is inversely related to the extent of the injury[32,65]. Results from studies wherein TH has been initiated later, up to 24 hours after birth, have been inconclusive[66,67]. Serious medical complications due to TH is uncommon, but the treatment can still necessitate additional sedation and increase the risk of infections[68]. Several multi-centre trials have shown that TH is associated with a reduction in death and neurodevelopmental problems, however, TH is only partially effective and many afflicted and surviving infants will still develop severe disabilities[69,70].

As hypothermia is only partially effective, recent preclinical research efforts have focused on finding appropriate adjuvant neuroprotective treatments, which can potentially have beneficial effects even beyond the therapeutic window for TH[30]. Several treatments have been shown to mediate better neurodevelopmental outcomes in different animal models of HI, some examples will be mentioned here. Apoptosis in the neonatal brain seems to depend more on the apoptosis inducing factor (AIF) pathway compared to adult brains[34], and blockage of this pathway reduces HI-induced brain injury in neonatal mice[71,72]. The proangiogenic cytokine erythropoietin has been shown to be neuroprotective and regenerative, even with a delayed administration, in a range of different animal models of neonatal brain injuries[73-76] as well as during clinical trials in HIE infants[77,78]. Another example of a putative treatment that shows great potential in preclinical studies is injections of mesenchymal stem cells[79-81].

1.2 PRETERM BRAIN INJURIES

Preterm infants, that is infants born before gestational week 37, are at high risk of developing serious complications; prematurity is the main cause of mortality during the first month of life and the second leading cause of death in children aged under five[82]. In Sweden, around 3.3 per 1000 infants are born extremely premature (i.e. before week 28)[83]. The incidence of preterm birth decreases in conjunction with decreased gestational age as the majority of preterm births are near term (week 34-36). It is estimated that that 11% of all births globally (corresponding to 15 million infants) are preterm, 90% of which occur in low- and middle-income countries[84]. Improvement of neonatal care has increased the survival of extremely preterm infants in particular, but the neonatal morbidity remains high[83,85]. Male preterm infants have worse outcomes

and a higher incidence of brain injuries compared to preterm females. The reasons for the sex differences are not yet fully understood but is thought to be due to a combination of genetic, hormonal, and immunological factors[86,87]. Extremely premature infants have a high risk of developing brain injuries; 7% of these infants born in Sweden 2004-2007 had cerebral palsy and 58% had some kind of disability 2.5 years after birth[88]. Examples of major neurological pathologies include germinal matrix and intraventricular haemorrhages (GM-IVH), venous haemorrhagic infarctions and diffuse white matter injury[1,89]. These injuries are commonly associated with impaired cerebral autoregulation[90].

1.2.1 Germinal matrix haemorrhage

Germinal matrix-intraventricular haemorrhage is a serious complication of preterm birth. The germinal matrix (GM) is a vascular niche in the developing brain wherein precursors to neuronal and glial cells proliferate and the blood vessel density is high[91]. These vessels consist mainly of immature capillaries with thin walls and a shortage of surrounding connective tissue to properly stabilize the GM, an area that have a higher rate of angiogenesis than other brain regions[1,92,93]. A shortage of astrocyte end-feet and endothelial pericytes together with a basal lamina lacking in fibronectin have been implicated in contributing to the reduced structural integrity of the germinal matrix[94,95]. This fragility inherent in the GM makes these vessels especially vulnerable to dysregulation of CBF[96,97]. Thus, a fluctuating CBF induced by, for example, HI or ventilator-induced hypercapnia can cause the vessels of the GM to rupture, inducing GM-IVH[98,99]. The increased intracranial pressure and impaired drainage of CSF following severe GM-IVH can induce brain damage and lead to the formation of hydrocephalus[100]. The brain injury is further exacerbated in a secondary phase where a combination of ischemia-reperfusion injury, blood component toxicity, and neuroinflammation increases the lesion[101-103].

The GM is vascularized from gestational week 7-8 and starts to regress by week 24 until it disappears around gestational week 36, making GM-IVH a condition predominantly seen in preterm infants[104,105]. Intraventricular haemorrhages in term infants instead usually emanate from the choroid plexus[106]. The severity of the haemorrhage is usually graded on a four-stage scale proposed by Papile et al in 1978[107]. Grade I refers to a bleeding restricted to the GM, larger bleeds are characterised based on whether the bleed reaches the ventricles without (grade II) or with (grade III)

ventricular dilation, while grade IV refers to a secondary venous haemorrhagic infarction within the brain parenchyma as a result of medullary vein compression[105]. Around 10% of extremely preterm infants develop GM-IVH III-IV and the incidence is inversely related to gestational age at birth[94,108]. GM-IVH develops early post-partum with bleeds emerging within the first 24 hours of life in around half of the affected infants; very few cases are seen after post-natal day five[109]. Advances in healthcare has decreased the mortality of severe GM-IVH, but Infants with grade III-IV GM-IVH has a high risk of developing major neurodevelopmental disabilities such as cerebral pares[110].

1.2.2 Diagnosing and treating germinal matrix haemorrhage

GM-IVH is diagnosed via ultrasonography of the brain where the bleed presents as hyperechogenic regions, the caudothalamic groove is used as an orientation marker to distinguish the bleed from the equally echogenic choroid plexus[109,111]. A majority of GM-IVH cases are asymptomatic and the low-grade bleeds are instead often found during routine screenings, more sensitive tests would therefore be advantageous[112]. Examples of investigated potential biomarkers includes protein S100B[113] and activin[114], both of which are elevated as an early indicator of GM-IVH development. Treatment for post-haemorrhagic ventricular dilation, a possible consequence following grade II-IV GM-IVH, generally revolves around draining out the accumulated CSF by lumbar punctures or by inserting a reservoir for intermittent CSF tapping. In the most serious cases, a long-term ventriculoperitoneal shunt can be implanted, which is associated with a high risk of serious complications[115]. Trials are also undergoing to prevent the toxic effects of blood products after the bleed. A recently published 10-year follow up study showed that DRIFT (the combination of drainage, irrigation, and fibrinolytic therapy) reduced cognitive impairments after GM-IVH, by a combination of ventricular drainage, artificial CSF irrigation, and treatment with recombinant tissue plasminogen activator, more successfully than conventional CSF tapping[116]. Promising preclinical findings related to novel GM-IVH interventions include stem-cell based therapies[117] and treatment with insulin-like growth factor 1[118,119].

1.3 THE NEONATAL CEREBRAL VASCULATURE AND BLOOD/BRAIN BARRIER

1.3.1 The composition, physiology, and formation of blood vessels

The vasculature and the blood contained therein acts, in the simplest description, as the body's supply and waste transport chain. Oxygen and vital nutrients are transported to where it is needed while waste products are removed and white blood cells dispatched to deal with potential disruptions in a never-ending logistic flow built to maintain homeostasis. The basis of the vascular system is the endothelial cells that line all blood vessels of the body in a single layer, the tunica intima, and acts as a semi-selective barrier for nutrient and gas exchange. Larger vessels also have smooth muscle cells of the tunica media and connective tissue of the tunica adventitia to support the endothelium and complete the vessel structure. The microcirculation, i.e. capillaries and venules, are instead supported by pericytes, contractile cells that envelopes the smaller vessels and regulates the capillary blood flow[120].

The two main processes of neovascularization are vasculogenesis and angiogenesis. The former refers to formation of new blood vessels from differentiating endothelial precursor cells. Under healthy conditions this process is restricted to the embryonal stage where the newly formed vessels lay the foundation of the vasculature, but findings have implied vasculogenesis as a mechanism involved in different postnatal pathological and reparative processes[121,122]. The primitive embryonic vascular plexuses are later in development further modified and organized into a functional vascular network by angiogenesis. This process is dependent on the balance of different angiostatic and angiogenic factors, pro-angiogenic stimuli includes hypoxia and growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor[123]. Angiogenesis involves an angiogenic factor-induced detachment of endothelial cells in the precursor vessel. These cells migrate, proliferate, and sprout new vessels, which are remodelled based on the metabolic needs of the surrounding tissue. After initiation, the activated endothelial cells of the precursor vessel release matrix metalloproteinases that degrades the basement membrane and allows the cells to proliferate and migrate via integrin-mediated haptotaxis through the extracellular space[124,125].

The sprouting vessels are led by environment-sensing filopodia-equipped tip cells, which guides the migration supported by proliferating stalk cells that produces tight junctions (TJ) to build and stabilize the new vessel (**see Figure 1**). A functional ratio of the two cell types is ensured by the Delta-like 4–Notch1 signalling pathway[126,127]. When the sprouting vessel has reached its goal, the proliferating endothelial cells differentiate, form a lumen and the sprout matures into a functional blood vessel[128]. In addition to sprouting, the vasculature will be dynamically sculpted through a combination of vessel-fusion, splitting of vessels via intussusception, and regression and pruning of unnecessary vascular connections[123], leading to a complex vascular network with larger arteries and veins as well as smaller capillaries and venules to support the organism.

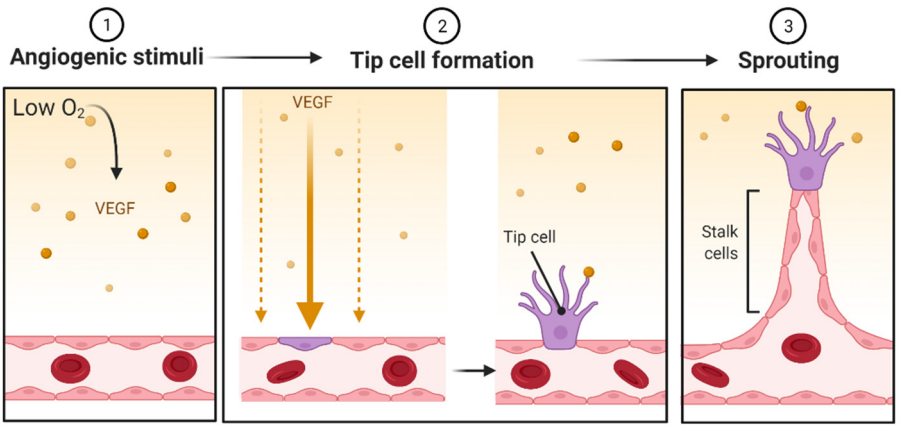

Figure 1. A simplified overview of VEGF-mediated angiogenesis as a consequence of hypoxia.

1.3.2 Development, structure, and regulation of the neonatal cerebrovasculature

The foundation for the human cerebral vasculature is laid down around gestational day 24. At this stage, precursor vessels start to grow from the base of the brain, a process that eventually forms the internal carotid artery and facilitates perfusion of the cortex around week six/seven, before the vessel density begins to stabilise in the third postnatal period[129]. Like in the rest of the body, the early precursor cerebral capillary networks are formed by vasculogenesis and are used as the base from which the cerebral vasculature is further formed via angiogenesis remodelling[130]. The brain has an immense metabolic activity but is unable to store energy for later use and is very susceptible to HI. The main

function of the brain vasculature is thus to regulate the CBF to ensure that the different brain regions metabolic needs are fulfilled[131]. The neurovascular unit (NVU), i.e. the cross talk between neurons, glial cells, pericytes, and endothelial cells that couples the vasculature and the nervous system, mediates this regulation through mechanisms that are not yet fully defined.

In summary, haemodynamic changes are initiated by neuronal signals in response to altered metabolic activity and oxygen requirements which are transmitted to astrocytes, either directly by the neuron or through connecting interneurons[132]. The astrocytes, in turn, propagates the signal to the vessel via their endfeet connected directly to endothelial cells, pericytes, or myocytes to induce vessel constriction or dilation to alter the CBF based on the current energy utilisation[133,134]. The multidimensional mechanism of neurovascular coupling is a complex machinery wherein cells of the NVU simultaneously communicates through many different mediators, the balance of which will decide the resulting vessel tone[131,135]. The NVU is established in part through the processes of neuroangiogenesis, the coordinated growth of neurons and blood vessels via shared mechanisms. Neuroangiogenic growth factors have stimulating effects on both neuronal and vessel growth and proliferation, and the guiding neuronal growth cones and endothelial tip cells share morphological features such as filopodia-projections[136].

1.3.3 Development, function, and pathology of the neonatal blood/brain barrier

An important component of the developing (and mature) brain cerebrovasculature is the protective BBB, a specialized function of the NVU that separates the brain from the rest of the body to maintain the microenvironment of the brain and stop entry of potentially harmful compounds to the central nervous system. The BBB is established early in development. Studies in human tissue have shown that barrier components and transporters are in place already at gestational week 12 and that systemic albumin and tryphan blue dye are denied access to the embryonal brain at this stage[137-139]. Despite the efforts made by researchers in the field, it is clear that a belief in an "immature" or even absent neonatal BBB still persists among the wider research community[140,141]. The BBB develops through neurovascular coupling between endothelial cells and pericytes. The structure is comprised of non-fenestrated endothelial cells of the cerebral capillaries that are

bound together by tight-junctions (TJ) and adherens junctions (AJ), pericytes, and astrocytic end-feet[142] (see **Figure 2**).

The BBB is a semipermeable barrier that mostly allows free diffusion of lipophilic molecules and gases while essential larger water-soluble substances, like many nutrients and insulin, gain entry via a range of different carrier- and receptor-mediated transportation systems[143]. The composition of these transporters appears to change during development to accommodate the nutritional needs of the brain at different stages of maturation. For example, amino acids are transported into the neonatal brain at higher rates than in the adult[141,144]. The BBB is also equipped with efflux transporters to eject potentially harmful compounds. These transporters are necessary to protect the brain from toxic substances and are in place early during development to, for example, hinder the influx of glutamate in the foetal circulation[145,146]. Apart from foreign toxins, these mechanisms also restrict the migration of immune cells into the brain during healthy conditions[141,147]. As mentioned before (and discussed in more detail in the introduction to **Paper III**) the BBB is compromised early in the development of HI brain injuries. The opening of the BBB is closely linked to the region and size of the brain injury and further exacerbates the damage by oedema formation and an increased access of inflammatory and toxic compounds to the brain[38]. The cerebrovasculature is thus an attractive potential target for limiting the damage seen after neonatal HIE.

1.3.4 Production and circulation of cerebrospinal fluid

CSF is formed from blood plasma, but has reduced levels of proteins and a different ion-composition. The fluid is mainly secreted by the ependymal cells of the choroid plexus, a tissue present in all cerebral ventricles, from where it flows through the brain ventricles and spinal cord centre canal to mechanistically protect the brain and support homeostasis[148]. The formation of CSF involves both passive diffusion due to osmotic pressure as well as active transport of water and ions through membrane transporters such as aquaporins and Na^+/K^+-ATPases [149,150]. It has been estimated that around 80% of all CSF is secreted by the choroid plexus while the remaining volumes comes from the interstitial fluid produced via transportation across the BBB[151]. The choroid plexus is equipped with a blood-CSF barrier (BCSFB) to deny CSF entry to the brain that, unlike the BBB, is made up of epithelial cells with underlying fenestrated capillaries to facilitate CSF production[152]. These structural differences means that the BBB and BCSFB have different criteria to allow

compounds through and the BCSFB is the primary route of entry for leukocytes and several different bacteria to the brain[153]. As discussed earlier, the CSF circulation plays a major role in the development of neonatal hydrocephalus[100], and is of great interest for studies investigating biomarkers of neonatal brain pathologies[49].

1.3.5 A short note on tight-junctions

The main physical barrier-function of the BBB comes from the TJs, complexes of claudins, occludin, and zonula occludens-1 (ZO-1), anchored to AJs. Proteins from the claudin family constitute a major part of TJs. Claudins are transmembrane proteins that seal the paracellular space and allow endothelial cells to adhere. While the main claudin protein in the cererovasculature is claudin-5, several other claudins are also present in the brain capillaries[154]. Transgenic mice with no claudin-5 forms macroscopically normal vessels with TJs based on claudin-12, but the junctions have a size-selective loosening, meaning that the BBB loses the ability to block the passage of molecules smaller than 800 Da[155]. The importance of claudin-5 was made abundantly clear as the authors also reported that all claudin-5 deficient mice died within hours after birth.

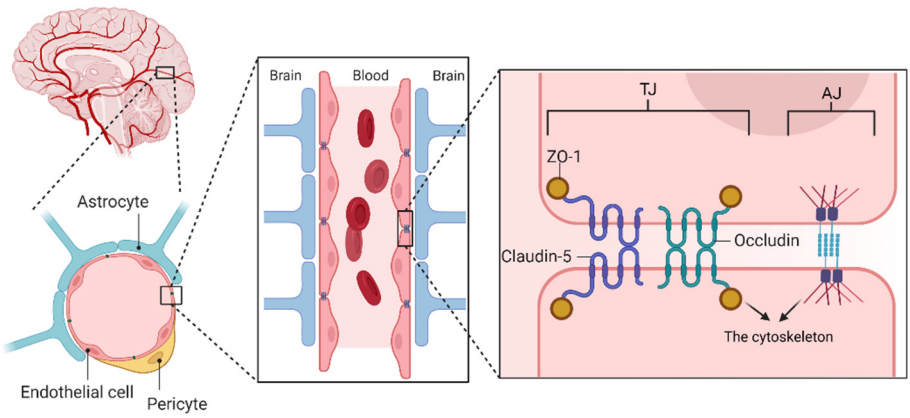

Figure 2. The blood/brain barrier with adherens junctions (AJ) linking endothelial cells together and tight junctions (TJ) sealing the gap tightly.

Occludin, another transmembrane protein, does not seem to be as directly involved in the mechanical sealing of TJs, but rather play a regulatory role and can alter the permeability of the barrier. Interestingly, occludin-depleted mice forms structurally normal TJs[156]. When occludin is overexpressed in cell lines, the trans-epithelial electrical barrier of the cells becomes more sensitive and easily disturbed

by proinflammatory signals[157]. Occludin is also sensitive to oxidative stress and will relocate to the cytosol during HI, which contributes to HI-induced breakdown of the BBB [158]. The transcellular parts of TJs are fixed in the cytosol by ZO-1, which acts as the scaffold to which claudins and occludin binds and works to reduce the tension in the junction and link it to the cytoskeleton[159,160]. ZO-1 also functions as the connection between the TJ complex and the anchoring AJs, the latter links cells together while the former seals the gap between them. TJs are formed when the TJ proteins aggregates into strands which pairs with TJ strands on adjacent cells and closes the intercellular gap, the amount of TJ-strands varies between tissues and correlates with the adhesive strength of the junction[161]. The architecture of the TJs at the BBB ranks among the most complex and restrictive sealing junctions in the body[154].

2 AIMS

2.1 GENERAL AIMS

The different pathologies underlying neonatal brain injury are often multifactorial and involve many different types of cells and injurious mechanisms. The cerebrovasculature is affected early and throughout the entire injury process. Thus, uncovering and understanding vascular mechanisms involved in neonatal brain injury would have important clinical ramifications. The years of research behind this thesis were aimed at investigating several different cerebrovascular aspects involved in the pathogenesis, evaluation, and potential treatment of neonatal hypoxia/ischemia and germinal matrix haemorrhage brain injuries in different animal models.

2.2 SPECIFIC AIMS

The specific aims in the different projects were as follows:

- Assesses the potential of circulating tight junction proteins as biomarkers for blood/brain barrier breakdown following different neonatal brain injuries.
- Quantitatively and qualitatively investigate the blood/brain barrier dysfunction following neonatal brain injury in a newly established model of germinal matrix haemorrhage.
- Investigate the effects on brain angiogenesis in the early stages following neonatal hypoxia/ischemia.
- Test RNAse A as a potential treatment for neonatal brain injury with cerebrovascular involvement.

3 MATERIALS AND METHODS

This section contains a generalized discussion of the methodological considerations behind the most important experiments presented in this thesis. Detailed information and specifics are included in the different papers and manuscripts.

3.1 ANIMALS EXPERIMENTS

3.1.1 Using rodents as models for neonatal pathologies

All work in this thesis was based on rodent models of neonatal brain injury, including rat hypoxia/ischemia (HI) (**Paper I**), rat germinal matrix-intraventricular haemorrhage (GM-IVH) (**Paper II**), and mouse HI (**Papers III-IV**). Rodent models are widely used to mimic human disease conditions in controlled environments and while there are many obvious differences between rodents and humans, from the molecular to the organ level, results from laboratory animals can still give valuable information that guides researchers towards new clinical applications. While there exist models using larger mammals, which have features more similar to human physiology and anatomy (as described below), rodents have several important characteristics that still makes them advantageous for research uses. The shorter lifespan of a rodent means that neurodevelopmental processes that takes years in humans are fast-tracked and occurs in the first weeks of a mouse or rat´s life. Rodents also have short gestation periods and generally births many pups per litter.

These features, coupled with their comparably small size that makes them easier to house in larger numbers and handle during experimental procedures, make rodents cost- and time-effective in an *in vivo* research context. Although not touched upon in this thesis, the relative ease of generating genetically modified mice and the abundance of different mutant strains available have proven valuable in many different research areas. The main limitations of rodents as models of human disease pertaining to this thesis are related to their neurophysiology and neuroanatomy. Rodents have a smooth brain and a different ratio of white/grey matter, rate of regional brain development, and cerebral blood flow regulation compared to humans[162]. The small size of rodents, albeit practical when it comes to housing and handling, also presents a

drawback in studies of neonatal rodents as it makes non-terminal/repeated sampling difficult.

Two different rodent species were used for the studies within this thesis. **Papers I-II** were based on albino Wistar rats. For **Paper I**, this was mainly due to the larger size of a rat compared to mice, which allowed for collection of greater volumes of blood and CSF. The model used in **Paper II** is only adapted for rats. **Papers III-IV** used C57BL/6 mice, the most used laboratory animal globally[163], the second-ever mammal to have its entire genome sequenced[164], and well characterized for the HI model in our laboratory. Due to the aforementioned neurodevelopmental differences between rodents and humans, it is difficult to correlate the level of nervous system maturation directly between neonatal rodents and humans. Numerous studies have been dedicated to elucidating the timing of brain development events and equating these between the species. Examples of such events include the development and maturation of neural cells, glial cells, and the immune system, establishment of the blood/brain barrier (BBB) and so on[162]. These findings have been summarized in a comprehensive review by Semple et al. that equates post-natal day (PND) 7-10 in rodents to a human infant at weeks 36-40 of gestation (i.e. term infants)[165]. It has however also been suggested that PND7 in rats is more similar to human gestation week 34-35[166]. PND5 rats were used to model preterm injury[167,168] as this age is comparable to the neurodevelopment of humans born around week 26-32 based on studies on white matter maturation[169] and the development of the cortex[170]. **Paper I** used PND7 rats to mimic near-term HI, the rats in **Paper II** were PND5 to mimic preterm GM-IVH, and **Papers III-IV** used mice at PND9 to mimic term HI.

3.1.2 Rodent models of neonatal hypoxia/ischemia

The HI models used in **Paper I, III, & IV** are based on the Levine preparation, a technique to induce unilateral HI injuries in adult rats[171]. The model was later modified for neonatal animals when Rice & Vannucci developed it in PND7 rats[172]. Finally, Ditelberg et al. adapted it for neonatal mice[173]. Many variations of the Rice-Vannucci-model, as it is commonly called, have been developed over the years, but the general procedure is similar. One of the common carotid arteries is permanently ligated (in our studies, it is always the left one) and the animal is subsequently subjected to a period of hypoxia, which induces unilateral brain injury in the hemisphere on the same side as the ligated artery. The brain hemisphere that is injured is termed the ipsilateral hemisphere

while the other hemisphere is termed the contralateral throughout this thesis and in all attached papers. The injury in the ipsilateral hemisphere is characterized by neuropathological damage to the white and grey matter in brain regions such as the hippocampus, cortex, and the striatum/thalamus[174]. The two-hit approach, including ligation of the artery and hypoxia, is necessary as the two events separately are not enough to produce an injury. The compensatory collateral blood flow through the circle of Willis ensures that the flow to the ligated hemisphere is unaffected by ligation only[175].

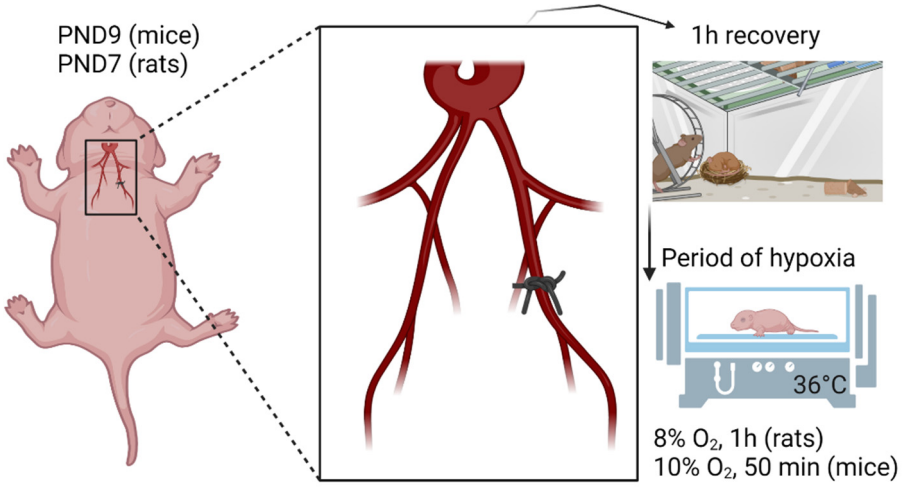

Figure 3. Inducing a unilateral hypoxic/ischemic brain injury in PND7-9 rodents.

The hypoxia itself does not produce major neuropathological tissue loss or BBB breakdown (see **Paper I**). Thus, the contralateral hemisphere can act as an internal control in some experimental settings. This allows for a reduction of the number of animals necessary for certain experiments, such as some neuropathological assessments (for example, see **Paper IV**). The use of the contralateral hemisphere as a general internal control in all experiments are not necessarily appropriate and the suitability of this approach must be thoroughly tested for every experimental setting. Reading the literature, it is evident that the parameters surrounding the execution of the model (e.g. the mode of artery ligation, degree and duration of hypoxia etc.) varies between different research groups. This is generally not a cause for concern as the model is dependent on external factors, such as the rodent species and strain used and the barometric pressure in the facility[176]. It is therefore more important to consider the extent of the injury when comparing different studies.

In the projects presented within this thesis, animals were anesthetized with isoflurane (3.5-5% in a 50/50 oxygen/nitrogen mixture) and the left common carotid artery were permanently ligated with a suture, a process that took three to five minutes per animal. Animals were kept on a 35 °C heating pad while outside their cages. The operated animals were brought back to their mothers to recover for one hour before they were placed in a 36 °C chamber. The chamber was perfused with air for 10 minutes, followed by a period of hypoxia through an air/nitrogen mixture, and ten additional minutes of air. PND9 mice were subjected to 10% oxygen for 50 minutes (**Papers III-IV**) while PND7 rats, being younger and less vulnerable to hypoxia, were exposed to 8% oxygen for one hour (**Paper I**), see **Figure 3**. Throughout the studies, we noticed that mice generally obtained a more pronounced injury in the hippocampus compared to the cerebral cortex while the opposite was observed in rats. Like all models, HI has several drawbacks that are important to consider. These include the variability in the produced injury[177] and the fact that hypoxia/ischemia in human infants can result in multifactorial pathology that can affect multiple organs that are not generally caused by a carotid artery blockage[178].

3.1.3 Preterm rat germinal matrix haemorrhage model

Several different models of germinal matrix haemorrhage (GMH) has been developed for use in rodents and the physiology of their germinal matrix regions have been well characterized[179]. GM-IVH does not occur spontaneously in rodents, so these models are based on injections of different lesion-inducing compounds into the brain or the use of transgenic rodents. Examples of the latter are mice with mutations in integrin, procollagen, or VEGF genes. As these genes are essential for survival, transgenic GM-IVH models are plagued with shortened lifespans and increased mortality[180]. The two most common approaches to manually induce GM-IVH like symptoms in rodents are injections with autologous blood or collagenase. Injections of autologous blood into the brain will effectively simulate a naturally progressing haemorrhage with the accompanying coagulation processes and induce behavioural changes[181], but the model does not directly damage or rupture the brain blood vessels[182]. **Paper II** was based on a model of preterm GM-IVH that mimics the clinical progression of a severe (i.e. grade III-IV) injury based on intracranial injections of bacterial collagenase (specifically from *clostridium histolyticum*) in PND5 rats developed by our group[167].

The basis of the model is that collagenase will degrade the basal lamina of brain blood vessels and cause bleeding into the brain parenchyma[183]. Rats are anesthetized with isoflurane and injected with two µl collagenase VII (0.3 units/mL) via a Hamilton syringe connected to an infusion pump with a flow rate of one µl/min (see **Figure 4**). The injections are performed free hand with an in-house designed guiding frame into the medial striatum in proximity to the germinal matrix (four mm lateral of the midline and one mm rostral of bregma) to a depth of 3.5 mm. The entire procedure takes less than five minutes per animal. This method causes an near immediate bleed and leads to grey and white matter tissue loss, striatal atrophy, ventricular dilation, and local activation of microglia and astrocytes as well as behavioural abnormalities[167]. This model is similar to a collagenase induced bleeding model in PND7 rats developed by Lekic et al[184]. However, as discussed earlier this age is deemed closer to near-term human infants, i.e. a developmental stage where the germinal matrix has withered and GMH is a rare event[185]. The adaptation to PND5 thus gives the model more clinical relevance. The main disadvantages of this model lies in the nature of the bacterial collagenase that in itself may directly damage different cell types and act as a neutrophil chemoattractant, thus potentially causing a more intensive inflammatory response compared to the more spontaneous blood vessel rupture seen in the human situation[186,187].

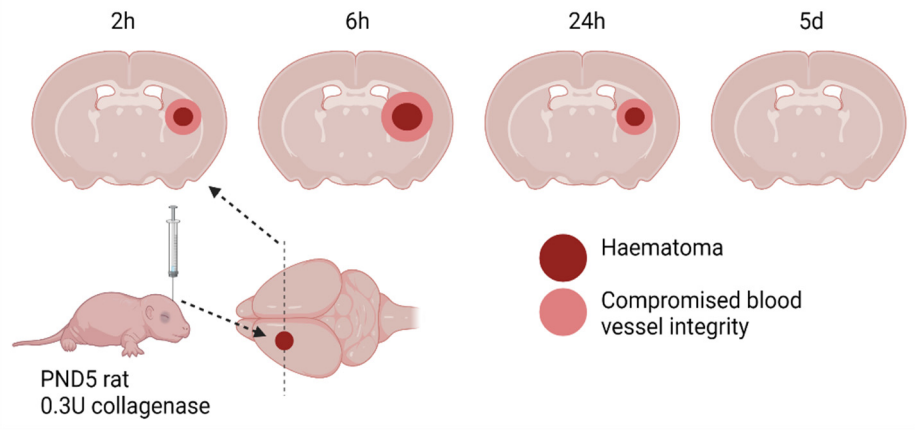

Figure 4. Induction and progression of GM-IVH in PND5 rats. All figures in the thesis frame were created with Biorender.com

3.1.4 In vivo BrdU-labelling

Bromodeoxyuridine (BrdU) is a synthetic analogue to the nucleoside thymidine that can be incorporated into newly synthesized DNA *in vivo* and thus be used to label proliferating cells[188]. BrdU injections was used in **Paper III** together with a marker for endothelial cells to quantify brain endothelial cell proliferation after HI through the use of immunofluorescence with antibodies directed against BrdU (see section 3.3.1). While BrdU is widely used to study cell proliferation, concerns have been raised regarding the toxicity and mutagenic properties of the compound, when administered repeatedly and/or over longer periods of time[189,190]. BrdU was given as a single dose only 3h prior to sacrifice in **Paper III.** This treatment regimen does not seem to confer any toxic effects[191] while still giving more than enough time for BrdU to incorporate into dividing brain cells[192].

3.1.5 RNAse A treatment

Active human ribonuclease type 1 (RNAse 1) is expressed by blood vessel endothelial cells, with the highest expression found in human umbilical cord endothelial cells[193]. As extracellular ribonucleic acid (RNA) released after tissue damage, such as ribosomal RNA, has been shown to be proinflammatory and have deleterious effects on blood coagulation and endothelial barrier function it has been proposed that endothelial RNAse 1 plays an important role in protecting blood vessels and promoting the integrity of the endothelium [194-196]. Based on this, treatment with exogenous RNAse, in the form of the RNAse 1 homologue RNAse A from bovine pancreas, have been tested in adult animal models for pathologies with vascular components and protective effects have been reported in several different settings and organs. Examples include cardiac and hepatic ischemia/reperfusion injuries[197,198] as well as cardiac arrest[199]. While the definitive mechanisms behind RNAse-mediated protection remains to be elucidated, it has been reported that the treatment reduces RNA-induced overexpression of proinflammatory tumour necrosis factor-**α**[198], decreases brain and heart oedema formation after stroke and myocardial infarction[200,201], and inhibits brain neutrophil activity after subarachnoid haemorrhage[202]. Due to these findings, RNAse A was tested as a neuroprotective treatment after neonatal HI in **Paper IV**. Intraperitoneal injections (i.p.) of RNAse were given in three doses; 30 min before HI followed by 30 min and 2h after HI.

3.1.6 Large animal models of neonatal brain injury

While not employed in the studies presented within this thesis, it is still important to briefly mention some examples of models of neonatal brain injury in larger animals, which may approximate the human condition better than rodents. Larger animal models employed in the research field today commonly use rabbits, pigs, and sheep. Rabbits have been used to model both cerebral palsy and GM-IVH, the former by subjecting foetal rabbits to intrauterine hypoxia[203]. To model GM-IVH, rabbits are delivered preterm by caesarean section and injected i.p. with glycerol which induces intracranial hypotension followed by a reperfusion that ruptures the germinal matrix blood vessels, closely mimicking spontaneous human GM-IVH[204,205]. Post-partum HI brain injury has been modelled in new-born piglets by exposure to hypoxia with[206] or without[207] carotid artery ligation. Ischemic brain injury in sheep is more commonly surgically induced in the foetus at different stages of gestation. Methods include bilateral occlusion of the foetal carotid arteries[208,209] or induction of whole body foetal asphyxia by occluding the umbilical cord[210]. While the obvious advantage of these animals are their closer similarities to human physiology, their disadvantages are more or less diametrically opposed to the advantages of rodent models discussed earlier. The size and gestational periods of these animals coupled with the more labour-intensive husbandry and surgical procedures necessary makes them very costly and time-consuming to work with[162]. This makes rodents more suited to investigate the fundamental biology behind diseases and physiological events while large animals functions as a stepping-stone towards translating eventual findings to clinical applications.

3.2 ETHICAL CONSIDERATIONS

The Gothenburg committee of animal ethics approved all animal experiments presented within this thesis after guidelines set by the Swedish Board of Agriculture, the Animal Welfare Body at the University of Gothenburg were informed of the studies. All personnel that handled animals within the projects had completed the Laboratory Animal Science training-course offered by the Laboratory for Experimental Biomedicine, the facility where all animals were housed and experiments conducted. The principles behind the 3Rs were followed and care was taken to get the maximum amount of information from the smallest number of animals possible. Studies were designed and reported in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines[211]. The ethical permits for rat and mouse

hypoxia/ischemia (**Papers I, III, & IV**) are filed under application no. 663/2018 and the permits for rat germinal matrix haemorrhage (**Paper II**) under no. 825-2017. All animals used in the studies were housed with their mothers in ventilated (mice) or open (rats) cages under a 12h light/dark cycle with free access to food and water.

3.3 IMMUNOASSAYS

3.3.1 Immunohistochemistry (IHC) & immunofluorescence (IF)

Immunohistochemistry (IHC) and immunofluorescence (IF) methods are in many ways the backbone of this thesis, and perhaps of all molecular biology. In its simplest form, an antibody directed towards the protein of interests binds to the specific antigen, and a detectable molecule conjugated to the antibody is visualized. The techniques combined with some form of microscopy enables us to study the expression of one or several target proteins directly in tissue samples. IHC and/or IF are used in all four papers to visualize different protein targets in brain sections. Applications in this thesis include quantification of protein or labelled tissues (e.g. blood vessels) and assessment of whether two different markers are co-expressed, all studies involved a primary detection antibody and a secondary antibody directed towards the primary while being conjugated to biotin or a fluorophore. The methodological principles behind the different immunostainings were similar in all papers regardless of the target protein(s). Preservation of the protein targets and structural integrity of the brain were achieved by whole-body fixation through transcardial perfusion with cold 6% buffered formaldehyde followed by tissue immersion-fixation in the same solution for at least 24 h.

Thin sections (7 µm) were collected by microtome-cutting paraffin embedded brains (**Paper I-IV**); for thicker sections (100 µm) brains were embedded in 4% agarose and cut on a vibratome (**Paper I-III**). Formaldehyde reacts with molecules in the tissue during the fixation process and can introduce crosslinking bonds that reduces antigenicity by blocking the formation of the necessary antibody-antigen bond[212]. The effects of formaldehyde were thus reversed by boiling the sections in a citric buffer[213], thicker sections were also permeabilized with the detergent Triton X-100 to further increase the antigen availability[214]. For IHC endogenous peroxidases in the tissue were blocked with hydrogen peroxide to counter unspecific staining and the sections were incubated

with a primary antibody directed against the protein of interest followed by a secondary biotinylated antibody, sections were incubated in an appropriate blocking solution to block unspecific binding of the secondary antibody. The staining was enhanced with the avidin-biotin complex method wherein avidin and biotinylated horseradish peroxidase (HRP) are added to the sections to form complexes with the biotin conjugated to the secondary antibodies[215]. A working solution of 3,3'-diaminobenzidine (DAB) was then added to react with the HRP, a process that is enhanced by the addition of nickel sulfate, and develop the staining[216]. As IF does not rely on the DAB-reaction or peroxidases the process is simpler and the antigen(s) are visualised via the fluorophore(s) conjugated to the secondary antibody/ies. Another strength of IF is the possibility to label more than one antigen on the same section by using several different fluorophores whose emission spectrums do not overlap significantly. The thicker vibratome-sections were stored free-floating and thus incubated with the various reagents for a longer duration compared to paraffin-sections to enable better penetration through the tissue.

The main sources of error when using IHC/IF are unspecific binding of the antibodies to the wrong antigens, as well as cross-reactions between different antibodies in a multi-staining. In all papers, the former was controlled for by blocking the sections with different blocking solutions and the latter by performing separate single-stainings with the antibodies used in a multi-staining and comparing the staining profiles with the one from the multi-staining. Secondary controls, where the primary antibody was omitted, was also performed to ensure that the fluorophores alone did not cause any background signalling in IF. Several different markers were targeted with antibody-based IHC and IF throughout the studies. Cluster of differentiation 31 (CD31), also known as Platelet endothelial cell adhesion molecule (PECAM-1), is a transmembrane protein that is expressed on the surface of several different types of circulating blood cells and in the intercellular junctions between connected endothelial cells. The protein is a reliable marker for endothelial cells and is commonly used to visualize blood vessels in both human[217] and murine tissues[218], CD31-IF was used in **Paper I & III**.

During the course of the studies, we found that CD31 did not adequately stain the entire area of sections from rat brains. The tight-junction protein claudin-5, which showed a similar, but more consistent, staining-pattern was thus used as the main vessel-marker in rat brain tissue (**Paper I-II**). Loss of grey and white matter was assessed by IHC of

microtubule-associated protein 2 (MAP2, **Paper I, III-IV**) and myelin basic protein (MBP, **Paper IV**). MAP2 is a dendrosomatic protein that is affected early after ischemia and thus a sensitive marker for grey matter tissue loss and infarction[219,220]. MBP is expressed in the myelin sheath of axons that makes up the white matter and is, like MAP2, lost after ischemia[221]. 4′,6-diamidino-2-phenylindole (DAPI) were generally used in the mounting media during IF to visualize cells and help localisation and navigation through the different brain regions. Details for how these sections were imaged and how the images were processed and analysed can be found in section 3.6 and 3.7, respectively.

3.3.2 Enzyme-linked immunosorbent assay (ELISA)

The science behind ELISA dates back to the 1960s and today the technique is a routine method employed in research- and hospital-laboratories globally[222]. ELISA was employed in **Paper I-II** to measure the concentration of tight-junction proteins claudin-5, occludin, and zonula occludens-1 in blood plasma and cerebrospinal fluid (CSF). All ELISA-kits were purchased from different vendors based on the tight-junction target and were of the sandwich-type pre-coated with the capture antibody. The capture antibody was allowed to bind the antigens in the sample before a biotinylated secondary antibody was added. Horseradish peroxidase conjugated to avidin is added and forms a complex (or, well, sandwich) with the other components. Finally, a substrate (3,3',5,5'-tetramethylbenzidine) is oxidized by the HRP to create a colour shift, which was measured as the optical density (OD) by a fluorometer. The concentration of the specific TJ protein could then be determined via the standard curve. As Kragstrup el al[223] has pointed out, validation steps should be planned when setting up ELISA-experiments to prevent false or misleading results. In the current studies, the samples, standard curve, and blanks were run in duplicates. Titration-tests were run to find the optimal sample dilution (CSF 1/10; plasma 1/20) and check that the measured OD scaled in proportion to the dilution titrations. Several plates were run for each protein in both studies and four samples were chosen as plate-controls and were included on all plates. The reproducibility between plates were adequate (samples with lower or higher concentrations were consistently lower or higher in each successive plate), but a plate/batch-effect was noticed and all results were thus normalized to the median of the time-matched controls ran on each plate.

3.3.3 Other fluorometric assays

Two non-antibody-dependent fluorometric assays were employed to measure the total protein concentration (**Paper I & IV**) and the activity of apoptosis-effector protease caspase-3 (**Paper I**) in homogenized and sonicated brain tissues. While the methodology differed between the assays, the result is a colour shift measured as an OD for each sample that is used to calculate a concentration from the standard curve. The bicinchoninic acid assay (BCA) were first described by Smith et al. in 1985[224] and is based on two reactions occurring under alkaline conditions. The first reaction (termed the biuret reaction) happens when copper sulphate is added to the brain samples and the peptide-bonds in the brain proteins reduces the Cu^{2+} to Cu^{1+}, this reaction is proportional to the total protein concentration in the samples. The Cu^{1+}-ions then bonds to bicinchoninic acid in the second reaction and forms a stable complex with a measurable purple colour, which is quantified and used to calculate the total protein concentration of the samples. The BCA was used in conjunction with the caspase-3 activity assay and the multiplex immunoassay (see 3.3.4) to set the results of those assays in context. The caspase-3 assay was used to measure the activity of caspase-3 in brain tissue[225], which is especially relevant in the current experiments as caspase-3 activation plays a large role in cell death after HI in young animals[33]. The assay is based on the fluorescent probe 7-amino-4-methylcoumarin (AMC) whose fluorescence is quenched when conjugated to a peptide substrate. The quenched form of AMC, as Ac-Asp-Glu-Val-Asp-AMC, is added to the homogenized brains and the peptide-bond is specifically cleaved by the protease activity of caspase-3[226], generating free and fluorescing AMC in proportion to the enzymatic activity of caspase-3.

3.3.4 Multiplex

Multiplex assays are a further development of traditional ELISAs that enables detection of several different proteins in a smaller sample volume compared to the single-target ELISAs that require more of the sample[227]. A bead-based/flow cytometric multiplex assay that bears many similarities to a sandwich ELISA was used in in **Paper IV** to measure the levels of eight different inflammation-related cytokines in homogenized brain tissue. Samples were incubated with several groups of different magnetic beads, each group coated with antibodies directed against one of the specific cytokines investigated. The groups of beads also have a fluorescent dye with a distinct emission wavelength. As during an ELISA, a secondary biotinylated antibody as well as streptavidin conjugated to a

fluorescent reporter (in this case phycoerythrin) are added to form a complex (multiple ones, in fact) with the proteins of interest. This process allows a cytometer to quantify the concentration (via the fluorescent phycoerythrin) of all investigated proteins (distinguished by the specific fluorescence of the magnetic beads) in one run.

3.4 ASSESSMENT OF BLOOD/BRAIN BARRIER FUNCTION

A significant amount of work in this thesis was focused on investigating the loss of functionality/permeability of the BBB in the different animal models. Specifically, the permeability was quantified by extravasation of radioactive sucrose and by injections of differently sized tracers.

3.4.1 ^{14C}-sucrose permeability assay

Sucrose is a 342 Da molecule that has been used extensively to study permeability of organic barriers. It is not metabolised in mammals or subjected to transport and is thus a suitable low-molecular marker to determine passive permeability of interfaces such as the BBB. Conjugating sucrose molecules to the isotope ^{14}C is a practical method to measure the regional BBB permeability, as shown in a myriad of studies and different models[228-231] since 1953[232]. The ^{14}C-isotope has many traits that makes it relatively practical to work with, these include its >5000 year half-life which enables stable re-measuring of samples over time and its low-intense β-radiation which has a comparably low penetration. This technique was applied in **Paper I, II, & IV.** ^{14}C-sucrose was injected i.p. exactly 30 min before sacrifice; different regions of both brain hemispheres were dissected out, and the amount of ^{14}C-sucrose present in the regions were quantified by liquid scintillation counting as counts per minutes/the weight of the tissue.

Results were expressed as the brain tissue/plasma ratio after correction for residual vascular space[233]. Our group has already confirmed[38], and we again showed in **Paper I**, that the sucrose-permeability in the contralateral hemisphere of HI-animals does not differ from that of control animals, which enables a reduction in the number of animals used for these experiments. Like other methods that relies on functional blood vessels for transportation, this technique has a limited viability in severely damaged brain regions (such as the middle of the infarct produced in the GM-IVH model). Therefore, while ^{14}C-sucrose is a very sensitive method to measure global changes in BBB-permeability, smaller

and focal lesions are better studied with molecular tracers (see 3.4.2). As mentioned earlier, repeated sampling is difficult in neonatal animals, which is a roadblock to conducting in-depth studies of the ^{14}C metabolism kinetics in these animals. Some recent studies have also suggested that the use of ^{14}C-sucrose overestimates the permeability of the BBB and the authors proposed that ^{13}C-sucrose is a better alternative for BBB-permeability studies[234,235].

3.4.2 Molecular tracers

The BBB in the GM-IVH model (**Paper II**) were further examined by the use of molecular tracers in the spirit of Paul Erlich and his student Edwin Goldmann who first discovered and began to characterize the barrier through intravenous tracer experiments starting in 1885 (Ehrlich, P. 1885. Das Sauerstoff-Bedürfnis des Organismus: Eine farbenanalytiche Studie. Hirschwald, Berlin, 8, p. 167). One small, i.e. having a low molecular weight, and one large tracer were used in the experiments. These were biotin-ethylene-diamine (neurobiotin, 286 Da) and biotin-dextran (BDA, 10 000 Da). Such tracers can be injected directly into the brain to label neuronal cells[236,237]. Here, they were instead injected outside the BBB in control and experimental animals to study how the barrier is affected after GM-IVH, an approach that has been used to study the BBB both in injury models[238] and in developing animals[233]. Based on preliminary experiments, the optimal administration routes were found to be i.p. for neurobiotin and retro-orbital (under full anaesthesia) for BDA, an alternative to tail-vein injections in young animals[239]. As these tracers are conjugated with biotin, they were visualised in brain sections by IHC using the avidin-biotin complex and DAB-development (see section 3.3.1 for more details). The optimal way to assess BBB-function is the combined use of both molecular tracers and ^{14}C-sucrose to get both a quantitative and qualitative assessment of the permeability.

3.4.3 Evans blue

Evans blue (EB) is a dye that binds to albumin, which is not able to pass the BBB under normal conditions and is slowly cleared *in vivo*[240]. The dynamic properties combined with the inherent fluorescence of EB, which emits at 680 nm, has made EB-extravasation a historically useful tool to easily and economically study BBB-permeability after injuries[241]. The infiltration of EB into injured brains can even be seen macroscopically as a deep blue colour when high doses are administered[242]. In later years, EB has somewhat fallen out of favour due to the emergence of more advanced techniques and concerns

surrounding the toxicity and side-effects of the dye[243,244]. For example its ability to modify the junctional morphology and microstructure of endothelium[245]. EB can still be applied in low doses to visualize EB-extravasation in the brain parenchyma of injured animals as a result of the BBB being compromised following neonatal HI (**Paper I**).

3.5 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (RT-qPCR)

The polymerase chain reaction (PCR) started a new epoch in scientific research and healthcare and the number of laboratories that have some type of PCR machine running right now is innumerably. The method's ability to diagnose viral infections also crashed the three-letter abbreviation straight into the collective consciousness during the strange times of 2019-2022 in a way probably unprecedented for a scientific method since Röntgen's X-rays. In real-time quantitative PCR (RT-qPCR), the expression of one or several genes can be quantified on the mRNA-level by synthesizing complimentary DNA (cDNA) from the total RNA isolated from a sample. In **Paper IV** the cDNA was used in an amplification reaction together with primers directed towards the studied transcripts and the resulting products were labelled with the fluorescing nucleic stain SYBR Green[246].

The fluorescent signal scales in proportion to the generated product and the result is expressed as the threshold cycle (Ct) for each gene, i.e. the amount of amplification cycles necessary for the fluorescence to cross a pre-defined threshold[247]. This value has an inverse correlation to the relative expression levels of the investigated gene and was used to calculate the relative changes in gene expression via the ΔΔCt-method[248,249]. The melting temperature of different PCR products varies due to the ratio of nucleobases and length of the sequence, so a melting curve analysis was performed for every gene in each experiment to control that only one product had been produced per reaction[250]. A critical step in RT-qPCR is to ensure the use of a good reference/housekeeping gene, to which the results are normalized. The expression of the ideal reference gene should not be affected by the model/treatment and be uniform between control and experimental animals[251]. For the experiments within this thesis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as several previous RT-qPCR studies from the group have shown that the expression of GAPDH is unaffected by HI[252–255]. However, in hindsight it may have been more

appropriate to use at least one more reference gene throughout all experiments to reduce the risk for errors[256].

3.6 MICROSCOPY

DAB-stained sections were visualised and photographed on lower magnifications (1.25X, 4X, 10X, and 20X objectives) with an Olympus BX60 light microscope equipped with a TH4-200 light-source and the cellSens software in (**Papers I, II, & IV**). IF-sections were imaged with an inverted Axio Observer Z1 fluorescence microscope using a Colibri 7 light-source and the ZEN Blue software (**Papers I-III**). The entire range of available objectives were used depending on the application. In **Papers I & III**, a tiling/stitching protocol was applied by taking an overview image of the entire section (using the DAPI channel) on 10X magnification, this image was used to define the regions of interest which were imaged by then tiling and stitching at the 20X magnification. A LSM 800 confocal microscope with the ZEN Blue software was used in **Paper II-III** to acquire Z-stacks of blood vessels, around 30 images per stack was taken with a 40X oil-immersion objective, in **Paper II** stacks were also 3D-imaged.

3.7 IMAGE PROCESSING AND ANALYSIS

Several different approaches were used to process and analyse the images acquired throughout the different studies, mainly by using the Fiji-build[257] of ImageJ[258] and Zeiss own program ZEN Blue. In **Paper I**, the vascular density of the hippocampus and cortex in fluorescent images were measured with an in-house developed macro based on using the difference of Gaussians to reduce background noise and enhance the vessels to enable automated measurements of blood vessel area. The area of fluorescent EB-staining was quantified by segmenting the images via applying a threshold to only measure the stained area in binary images. Likewise, the loss of MAP2 and the immunopositive area of CLDN5 were quantified in DAB-stained sections with the use of thresholds. CLDN5-results were expressed as a percentage of CLDN5 positive vessels per the area of the brain region and the loss of grey matter/ MAP2 were calculated using the MAP2-area in both hemispheres with the following formula: $(MAP2_{contra} - MAP2_{ipsi})/ MAP2_{contra} \times 100$[259].

Examples of fluorescent blood vessels expressing CLDN5 were also shown. In **Paper II**, several characteristics of tracers that had passed the BBB

were quantified manually in DAB-developed images. These included the number of leaking blood vessels, the area of the haematoma, and the distance from the haematoma to the most distal leaking vessel. Injured and uninjured vessels were also 3D-imaged using a confocal microscope. Measurements of grey and white matter tissue loss were quantified in **Paper IV** by manually delineating MAP2- and MBP-positive areas in DAB-developed images and using the equation described above, the MAP2-results were further confirmed with the threshold technique used in **Paper I**. All measurements done in **Paper III** relied on fluorescence images in which BrdU-positive endothelial cells and growing blood vessel tip cells were counted, the latter in Z-stacks acquired on a confocal microscope. All image-based analyses in all papers were done at several different levels of the brain for each animal and the results were averaged.

3.8 STATISTICS AND DATA VISUALIZATION

The statistical analyses presented within this thesis were performed using the programs GraphPad Prism (GraphPad Software, CA, USA) and Qlucore Omics explorer (Qlucore, Lund, Sweden). The latter was mainly used to generate heat-maps of data (**Papers III- IV**), perform principal component analyses (**Paper I**) and two-group comparisons for gender differences (**Papers I-II**). Several different statistical tests were performed on the results based on the experiment and group composition. Both paired (**Papers II & IV**) and unpaired (**Papers I-IV**) t-tests were done and the Benjamini-Hochberg method to control the false discovery-rate (FDR) was used when multiple t-tests were performed (**Papers I & IV**). One-way and two-way analysis of variance were commonly paired with a suitable *post-hoc* test to control the FDR[260], these include Dunnett's (to compare two or more groups with a single control group, **Papers I-III),** Holm-Šídák's (to compare selected pairs of means, **Papers II-III**), and Tukey's (when comparing all groups together, **Paper IV**) tests. Possible correlations in the results were tested by calculating the Pearson correlation coefficient (**Papers I & IV**).

4 RESULTS AND DISCUSSION

This section is comprised of summaries and short discussions of the main findings whereupon this thesis is based. The reader is directed to the four attached papers for more detailed descriptions, and comprehensive discussions, of the results.

4.1 BLOOD/BRAIN BARRIER DYSFUNCTION AFTER HYPOXIA/ISCHEMIA IN NEONATAL RATS

It is well established that neonatal HI disrupts the blood/brain barrier (BBB) in both rats and mice, but the available studies in neonatal rats have all used methods that are either qualitative or lacks the power to specifically investigate the disruption temporally or regionally. A common way of investigating BBB permeability is using immunohistochemistry to detect serum immunoglobulin G (Ig G) that infiltrates the brain parenchyma after BBB injury[261,262]. While this is evidence for impaired BBB function, the results from such studies only give information of how much Ig G has accumulated into the brain from inception of injury until end of experimentation, making it impossible to draw conclusions regarding changes of BBB function over time. It is even conceivable that BBB function is normalised at the time when the brain is studied since large molecules such as Ig G are cleared from the brain at slow rates. Other studies have injected the animals with observable compounds like Evans blue (EB) or sodium fluorescein, which accumulates in the injured brain and are visualized in sections or measured in homogenized brains with a fluorometer[263-265]. Similarly, the former approach has the same drawback as the Ig G method and the latter is unable to distinguish in which areas of the brain the barrier is broken.

We therefore elected to investigate the BBB permeability in the ipsilateral hemisphere after HI in neonatal rats using ^{14}C-sucrose extravasation to more precisely and accurately quantify the regional BBB disruption at different times after injury (**Paper I**) and get a better understanding of the injury induced barrier dysfunction. To our knowledge, this is the first such study in neonatal rats, although we have previously conducted similar experiments in neonatal mice[38]. We know from the studies in mice that the BBB permeability ratios of contralateral hemispheres from hypoxia/ischemia (HI) animals are not different from the ratios in control animals when this method is used. To start with, we tested this in rats and found that this was also true in the rat model; allowing a significant

reduction in the number of animals used in the experiments by letting us focus on investigating HI animals and comparing the ratios within each animal per region and time point. It must be emphasised that this approach is not necessarily suitable when studying other parameters as the physiology of the contralateral hemisphere is still affected by the HI (for example, see **Paper III**). The hippocampus, cortex, and striatum/thalamus were investigated 6h, 24h, and five days after HI, with the greatest barrier opening observed at 6h where all studied regions showed a compromised barrier. This was also evident when the permeability of the hemispheres, i.e. all regions combined, were assessed. The barrier being disrupted early after HI are in agreement with a study showing increased BBB permeability in adult rats immediately after one hour of hypoxia/reoxygenation[266].

The striatum/thalamus had regained barrier function by 24h while the other regions still showed a higher-than-normal permeability. As mentioned earlier, we generally see more injury in cortex compared to hippocampus in this model in rats; this was mirrored in the BBB results were the cortex showed the greatest BBB opening at all time-points. The most surprising results where the apparent decrease in BBB permeability five days after HI with several animals having a lower than baseline permeability across all investigated regions, with statistically significant differences in the cortex. This was also evident in the total hemisphere permeability ratios, and we do not have a fully satisfying answer to this outcome. Our initial hypothesis was that these animals had such extensive tissue and blood vessel damage that the sucrose could not be transported adequately. However, this was not supported by our results showing that the vascular density of remaining tissue at five days after HI was not different between control and HI animals. We have previously seen a similar effect, albeit in fewer animals, in the neonatal HI model in mice at seven, but not three, days after HI[38].

An upregulation of tight junction (TJ) protein expression after injury could potentially have a modulating effect on the barrier, but long-term studies on TJ proteins after injury are lacking. It has been shown that the cerebral blood flow (CBF) of the ipsilateral cortex is fluctuating after HI in this model with the initial increase reversing to a substantial decrease of CBF 48h after injury[220]. Potential later alterations in vascular physiology due to HI should therefore be looked into more closely at the five-day time point by e.g. measuring CBF and studying the functionality of the blood vessels in the ipsilateral hemisphere. A modulating effect by TJ proteins could theoretically have implications for the delivery of drugs

into the brain after HI brain injuries, but it needs to be further investigated before more decisive conclusions can be drawn. The ^{14}C-sucrose experiments were further complemented by an EB-based study that showed extensive dye extravasation into the brain parenchyma 6h post-HI, but not in control animals, meaning that the BBB was permeable even to large (66.5 kDa) serum albumin macromolecules at this stage.

4.2 GERMINAL MATRIX HAEMORRHAGE AND THE BLOOD/BRAIN BARRIER

As described in the introduction, the vessels of the germinal matrix (GM) in preterm infants are fragile and at risk for rupture, which could damage the BBB and lead to germinal matrix-intraventricular haemorrhage (GM-IVH). This fragility does not seem to stem from a reduction of TJ proteins in the GM as brain samples from GM-IVH infants showed no difference in TJ protein expression in this area compared to other brain regions[267]. The importance of the BBB in GM-IVH has been demonstrated in a rabbit-model of GM-IVH wherein haemoglobin was shown to have a damaging effect on the epithelium of the choroid plexus[268]. In **Paper II** we, for the first time, quantitatively and qualitatively assessed the BBB permeability in our recently developed GM-IVH model in post-natal day 5 rats[167] via ^{14}C-sucrose and molecular tracers. Injections of biotinylated tracers, which passed through the damaged BBB allowed us to visualize and measure the bleeding in the ipsilateral hemisphere as well as the number of leaking vessels.

No tracers were seen in the brain of control animals or in uninjured areas of GM-IVH animals. The haematoma had formed after 2h and reached its largest size 6h after GM-IVH, at 24h it had started to decrease in size and the bleeding was fully resolved five days after the collagenase injections. The largest amount and most widespread distribution of ruptured blood vessels were likewise found at 6h, suggesting that repair mechanisms were restoring vessel integrity and clearing the bleed between 6h and 24h. A group using a PND7 model of GM-IVH measured the amount of haemoglobin in the brain starting from 24h after GM-IVH[269]. While we could not macroscopically see bleeding five days after injury, they found residual brain haemoglobin still present in the brains of animals sacrificed seven days after injury (around an 80% reduction from animals at 24h). Confocal microscopy showed that peri-haematoma vessels leaked tracer molecules and had reduced immunoreactivity of TJ protein claduin-5 compared to vessels in the other hemisphere 24h after GM-IVH.

This was similar to earlier findings showing loss of claduin-5 and laminin in ruptured vessels of the ipsilateral hemisphere after collagenase injection[167]. In our study, all vessels had restored integrity and normalised BBB function after five days.

We suspected that the accumulation of blood and lack of viable blood vessels in the haematoma would make ^{14}C-sucrose based experiments difficult in the GM-IVH model and thus designed the experiments with these issues in mind. We focused on the peri-haematoma areas where we could not see leaking blood vessels and divided these into two parts, the immediate area closer to the haematoma (P1) and the regions beyond (P2). An increase in BBB permeability was found in both P1 and P2 at 2h compared to control animals. When comparing the permeability of the two hemispheres within each animal, the barrier was dysfunctional in both regions up to 24h after GM-IVH, the time period in which levels of circulating TJ proteins also were increased. No permeability changes were detected in the uninjured posterior area of the brain at any time point. Our results show that GM-IVH injury compromises the BBB in the area surrounding the bleed and not only in the core of the haematoma where the bleeding originated, but the barrier damage is less severe and widespread than after HI (**Paper I**).

These results are important, as there is a relationship between BBB breakdown, oedema formation, and the severity of the resulting injury following GM-IVH. This is exemplified in studies using treatment with osteopontin, fingolimod, and insulin-like growth factor 1 that showed a reduction in BBB dysfunction, measured as EB extravasation, and attenuation of GM-IVH brain injury in a collagenase-based model using PND7 rats[118,270,271]. Detailed studies of BBB function and dynamics in the context of GM-IVH are thus necessary to both understand the pathophysiology of the disease and to evaluate the efficacy of potential treatments with vascular-strengthening mechanisms of action.

4.3 OCCLUDIN AND CLAUDIN-5 AS BIOMARKERS FOR BLOOD/BRAIN BARRIER BREAKDOWN

There are no widely used fluid markers to easily and efficiently assess the health of brain blood vessels and the loss of BBB integrity seen in conjunction with many different neonatal brain injuries. BBB dysfunction is, in part, related to a loss of TJ function as hypoxia and focal ischemia has been shown to alter the morphology and reduce the sealing

capabilities of the junctions[272,273]. This reorganisation can be mediated by TJ-degradation via HI induced matrix metalloproteinases[274-276]. There is also evidence that TJ proteins can be internalized via endocytosis to either be degraded or recycled back to the plasma membrane, especially under inflammatory and/or ischemic conditions[273,277]. It has been hypothesized that TJ proteins can be released into the circulation after the junctions are disrupted, and several reports have shown that levels of circulating TJ proteins are elevated in human patients with stroke or intracranial haemorrhage and adult rat models for stroke[278-280]. This was the basis for our investigations into circulating TJ proteins as potential biomarkers in neonatal rat models of HI and GM-IVH. In **Paper I** we found different changes in claudin-5 (CLDN5) and occludin (OCLN) levels in blood plasma and cerebrospinal fluid (CSF) after HI.

The levels of these proteins had an inverse temporal relation with OCLN being elevated in CSF at 6h and plasma at 24h post-HI, while CLDN5 was very elevated in plasma already at 6h and in CSF at the 24h time point. Five days after HI, both proteins had returned to baseline in most animals. We also investigated the intracellular scaffolding protein zonula occludens-1 (ZO-1), but found no significant difference in the levels of circulating ZO-1. In fact, we were not even able to detect any circulating ZO-1 in several of the animals. We attributed this to the localisation and function of ZO-1, being the intracellular protein that holds TJs together, it seemed less likely to relocate into the circulation. Circulating ZO-1 is less studied that CLDN5 and OCLN, and the results are still inconclusive. Elevated levels of ZO-1 have been found in the serum, but not CSF, of patients with leukaemia that had metastasized into the central nervous system[281]. Circulating ZO-1 has also been investigated in patients with multiple sclerosis, where one study found elevated levels of the protein in blood serum[282] while the other reported no differences in circulating ZO-1 in plasma[283]. Based on our results showing no change in ZO-1, we instead focused on CLDN5 and OCLN. We analysed levels of OCLN and CLDN5 in CSF and blood from the same animals. Interestingly, the different groups were clearly separated and distinct from the control group based on time point when all measurements for each animal were combined into a principal component analysis up to 24h after HI.

The most important finding of this study was that the levels of CLDN5 in CSF correlates with the grey matter tissue loss after HI. This implies that the BBB dysfunction after HI, measured as circulating CLDN5, is proportional to the injury and that TJ-derived biomarkers has potential diagnostic value in the context of neonatal HI. The majority of measured

circulating TJ proteins has likely a cerebrovascular origin as studies measuring protein levels after middle cerebral artery occlusion in adult rats showed reduced levels of CLDN5 in brain microvessels[272] and whole-brain lysates[274]. The proportion of TJ proteins that reach the circulation after injury is likely only a fraction of all TJ proteins present in the brain, as we showed that the total CLDN5 immuoreactivity in brain blood vessels is not significantly altered after injury. Circulating CLDN5 showed a similar pattern after GM-IVH (**Paper II**) and HI, with an early increase in plasma and a later effect in CSF. OCLN levels in CSF after GM-IVH showed an even larger increase compared to controls than after HI and were increased up to 24h, while no significant effects were seen in the plasma from GM-IVH animals. The two proteins entered the circulation at different time points, an effect evident in both models. This timing variance could potentially be related to the differences in degradation pathways and linked to other intercellular proteins between CLDN5 and OCLN[284,285]. We found sex-differences in the levels of circulating TJ proteins after HI, with males having higher levels of plasma OCLN and CSF CLDN5 compared to females, but no such differences after GM-IVH. The sex-difference in HI animals were not fully unexpected as male animals generally suffer more severe injury in this model[286,287], potentially due to an augmented innate immune response in males[87].

4.4 NEONATAL BRAIN INJURIES AND ANGIOGENESIS

The role of blood vessels in injuries to the neonatal brain is a central theme in this thesis, especially in **Paper III** wherein we investigated angiogenesis in a mice model of term HI to shed some more light of the dysregulation of angiogenesis seen in both models and infants after neonatal hypoxic/ischemic encephalopathy (HIE). Our results showed no statistically significant changes in the density of proliferating endothelial cells in the hippocampus or cortex in the ipsilateral hemisphere compared to control animals up to 72h after HI. The density was very similar in the HI group compared to controls at 6h, but several HI animals showed a reduction in density at 24h. Half the animals had a reduced density in the ipsilateral hippocampus 72h post-HI while the other half showed normal density. We found reduced density of proliferating endothelial cells in both regions of the contralateral hemisphere at the 24h time point. The HI had a more profound effect on growing endothelial tip cells which were practically absent in the ipsilateral hippocampus, and heavily reduced in the cortex, up to 24h after the

injury, this effect was also evident in the contralateral hemisphere where a reduction of tip cells was seen at 24h. By the 72h time point, the tip cell density had restored to control levels in the ipsilateral hemisphere. The tip cell density was significantly higher in the contralateral hippocampus of HI animals by this time point, consistent with earlier results from our group that showed an increased brain blood vessel density following hypoxic preconditioning in near-term rats[288].

The temporal differences in angiogenic processes seems to be linked with time dependent changes in angiogenesis gene expression profiles. We found three gene clusters that were differentially expressed after 2h, 8h, and 24h in the ipsilateral hemispheres of HI animals. These different clusters likely represent different aspects of the cerebrovascular response to HI during the injury process from the onset of injury to the start of the reparative processes that restore the tip cell density and re-establish vasculature development. We also found changes in the expression of angiogenesis-related genes in the contralateral hemisphere early after HI, similar to the alterations of such genes after hypoxic preconditioning[288]. The regional rupture of brain blood vessels and accompanying BBB breakdown, shown as parenchymal extravasation of ^{14}C-sucrose, EB, and molecular tracers (**Papers I, II, & IV**) combined with an inhibition of cerebrovascular regrowth in the first 24h after (**Paper III**) thus contributes to the development of brain injuries after neonatal HI. On the other hand, as shown by our newly developed image analysis macro to quantify the cerebrovascular area in images of entire brain hemispheres, there was no difference in blood vessel area in the peri-infarct area after HI (**Paper I**). This combined with the seemingly normal endothelial cell proliferation and restoration of endothelial tip cells 72h after the insult implies that angiogenesis processes are slowly restored after their initial dysregulation in the early stages of the injury. This is also evident from the normalisation of BBB function and sharp reduction of circulating TJ proteins five days after induction of brain injuries (**Papers I-II**).

4.5 RNASE A AND NEONATAL HYPOXIC/ISCHEMIC BRAIN INJURIES

As RNAse A has shown promise as a protective treatment in different models of conditions with vascular attributes, we investigated potential neuroprotective effects of the compound in the mice model of neonatal HI (**Paper IV**). The treatment gave a marked reduction of both grey and

white matter tissue loss in all investigated regions throughout the entire brain as assessed one week after HI compared to non-treated animals. RNAse A treatment has been shown to reduce brain oedema in a stroke-model[200] and microRNA-210, a potential substrate for RNAse A, increases BBB permeability in neonatal rat HI[261]. We therefore hypothesised that the neuroprotective effect we found in part could be due to a reduction of BBB disruption. However, we did not find evidence for an RNAse A-mediated reduction of BBB permeability, measured with ^{14}C-sucrose, 6h after HI in the hippocampus, cortex, or striatum/thalamus. The permeability ratios of both treated and untreated HI animals were similar to what we have previously measured in this model[38] and what we saw in neonatal rats at the same time point (**Paper I**).

We further investigated the inflammatory response as RNAse A has been shown to reduce inflammation in different animal models[289]. However, we found no evidence that the treatment alleviated neuroinflammation after HI as RNAse A did not reduce either the protein levels or gene expression of the eight investigated cytokines. These results appear robust as we could show that the mRNA and protein levels of these cytokines were positively correlated. Studies in models of adult cardiac conditions have shown significant reductions in the expression of cytokine-panels, similar to the one we employed, but this effect does not seem to occur in the neonatal brain[198,199]. We can therefore not yet draw any conclusions regarding the mechanism behind the beneficial effects of RNAse A treatment after HI. One limitation of this study is that we only investigated neuroinflammation in brain tissue and it is possible that RNAse A instead mainly acts more peripherally in the body. As many different microRNAs have been shown to be increased in models and infants after neonatal HI, one hypothesis is that RNAse A mediated degradation interrupts possible deleterious pathways in which these microRNAs are involved[290,291].

5 SUMMARY AND FUTURE PERSPECTIVES

Neonatal brain injury is a significant cause of child mortality and lifelong disability and many aspects of the mechanisms underlying the development and course of these conditions are yet to be elucidated. This thesis investigated the role of the cerebrovasculature and blood/brain barrier in models of neonatal hypoxia/ischemia and germinal matrix haemorrhage. We found that OCLN and CLDN5 are released into the circulation after neonatal HI in a time- and sex-dependent manner. Especially CLDN5 showed promise as a biomarker for brain vascular health as the levels of the protein in CSF correlated with the severity of the brain injury. These markers were also temporally elevated in the blood and CSF, albeit not in a sex-dependent manner, of animals after experimental GM-IVH. To further the field, future studies should investigate the specificity and sensitivity of these identified potential biomarkers and optimize the assays used, especially in human samples[292]. These proteins may have clinical relevance as biomarkers for neonatal brain injury, but they will likely be part of a battery including other markers for diagnosis and monitoring of the injury to allow for an efficient assessment of several injury-related parameters at once.

We have also detailed how the permeability over the BBB is temporally and regionally affected after HI and GM-IVH, obtaining results that will help guide future studies of e.g. the pathophysiology behind these conditions and pharmacokinetics of possible future treatments. We further characterized vascular aspects and the nature of the bleeding in our newly developed GM-IVH model, which is currently being used by the research group for preclinical investigations into GM-IVH related pathophysiological mechanisms. When investigating how cerebral blood vessels and angiogenesis is affected by HI, we found a profound dysregulation of endothelial tip cells with accompanying changes in expression of angiogenesis-related genes, both in the hypoxic/ischemic and hypoxic hemisphere of these animals. Uncovering the pathways behind the disruption of angiogenesis after neonatal HI would allow for the development of pro-angiogenic treatments as a means to reduce the injury and promote recovery. A natural follow-up to these results is proteomics-based studies of isolated cerebral microvessels and blood to find other potential biomarkers for vascular dysregulation and novel targets for angiogenesis-enhancing therapies. This project is, at the time of writing, ongoing and will encompass studies in animals subjected to

hypoxia only as well as more in-depth investigations of cerebrovascular area and the expression of angiogenesis-related genes.

For the first time, we showed that treatment with RNAse A is neuroprotective after neonatal HI. This effect does not seem to stem from an altered inflammatory response or a protection of the BBB up to 6h after HI as we initially hypothesized. Thus the protective effect of RNAse A, and especially the mechanisms mediating it, needs to be further characterized. Several studies have reported a reduction in oedema formation and the accompanying deleterious mass effect as a result of RNAse A treatment[200,201], this could further be investigated in the neonatal HI model by magnetic resonance imaging or by quantifying the wet/dry brain weight[293]. It would also be interesting to investigate the effects which administration of RNAse A likely has on circulating microRNAs, possibly by using RT-qPCR.

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