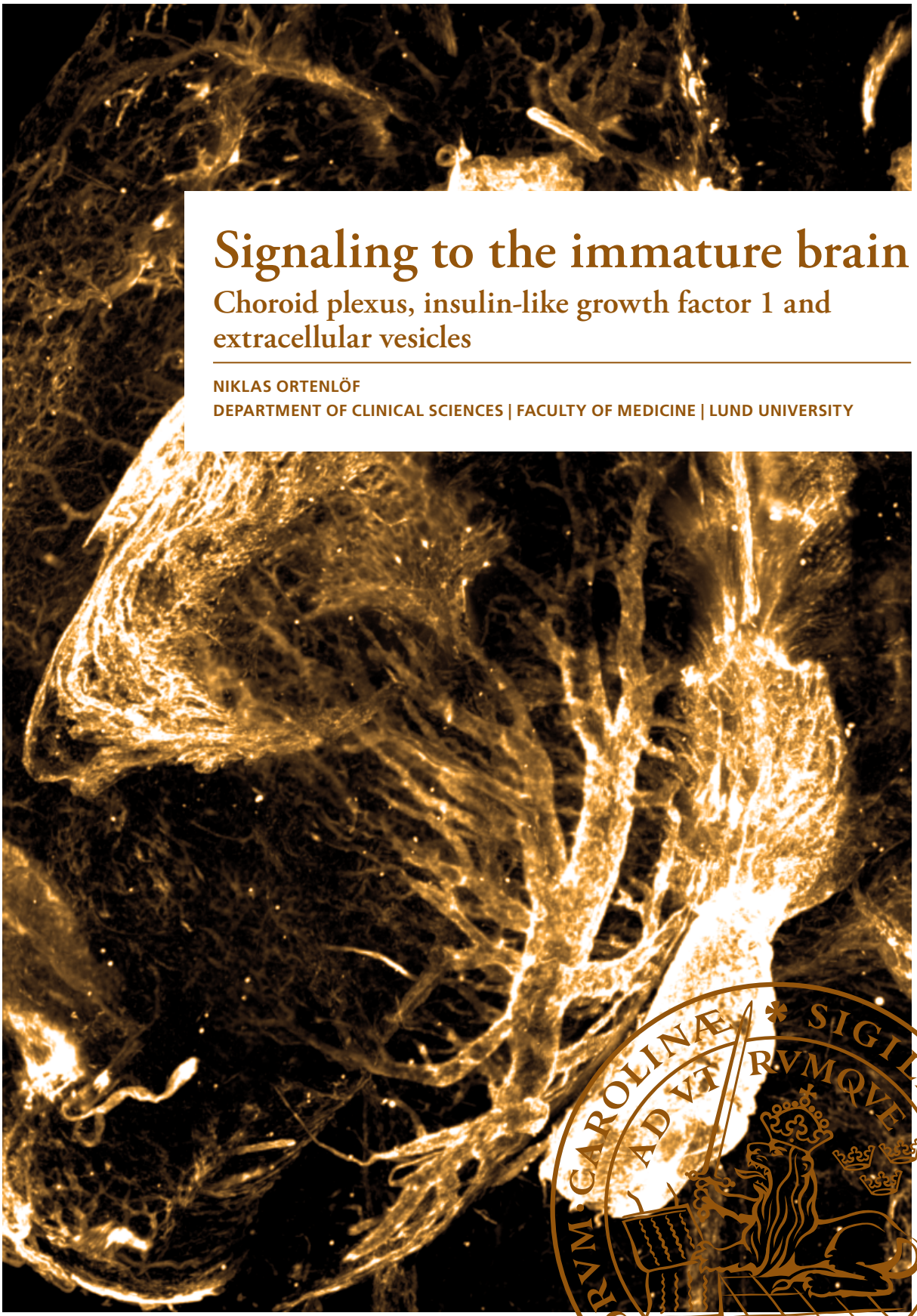
Signaling to the immature brain - Choroid plexus, insulin-like growth factor 1 and extracellular vesicles

Ortenlöf, Niklas

2023

Document Version:
Publisher's PDF, also known as Version of record



Signaling to the immature brain

Choroid plexus, insulin-like growth factor 1 and extracellular vesicles

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Signaling to the immature brain

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Niklas Ortenlöf

LUND UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden. To be defended at Segerfalksalen, BMC A10, Lund, Friday 8th of December 2023 at 13:00

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Organization: LUND UNIVERSITY

Document name: Doctoral dissertation **Date of issue:** December 8, 2023

Author(s): Niklas Ortenlöf **Sponsoring organization:**

Title and subtitle: Signaling to the immature brain - Choroid plexus, insulin-like growth factor 1 and extracellular vesicles

Abstract: Extremely preterm infants (i.e., born below 28 gestational weeks), are at high risk of developing brain morbidities including intraventricular hemorrhage (IVH) and neurodevelopmental impairment. Despite over 50 years of research, there is currently no effective therapy available for preventing IVH.

Insulin-like growth factor 1 (IGF-1) plays a pivotal role in perinatal development. Extremely preterm infants exhibit reduced levels of circulatory IGF-1, and low levels of IGF-1 during perinatal development are associated with poor weight gain, reduced brain volumes and impaired cognitive development.

While clinical data suggest that supplementary treatment with IGF-1, aimed at restoring physiological levels, could potentially enhance neurodevelopment, the precise molecular mechanisms underlying the protective effects remain incompletely understood.

One potential transport mechanism of blood-borne IGF-1 to reach the central nervous system is via the choroid plexus (ChP). All ventricles in the brain contain this tissue, which is the main producer of cerebrospinal fluid (CSF). By studying the onset of IVH using our experimental preterm rabbit pup model, we have discovered that ChP is the predominant site of bleeding in IVH in our model.

In this thesis work, we have investigated i) the interaction of blood-borne IGF-1 on the ChP in the immature brain, and ii) the impact of IGF-1 on the occurrence of IVH in an experimental model of IVH. Our findings reveal that blood-borne IGF-1 accumulates in the ChP following systemic administration of IGF-1, leading to an upregulation of genes associated with vascular maturation in the ChP. Furthermore, we observed that uptake of administrated IGF-1 from the circulation and CSF was dependent on postnatal age.

Through a combination of an in vitro cell culture system and a preterm rabbit pup model, we provide evidence for an extracellular vesicle (EV)-mediated transport of blood-borne IGF-1 through the ChP, and into the CSF. Ultimately, the IGF-1 carrying vesicles reach the deep layers of the hippocampus in the immature brain. Finally, we evaluated the potential of IGF-1 to prevent IVH in a preterm rabbit pup model.

Altogether, the results presented in this thesis highlight the importance of the ChP and IGF-1 in the developing brain. More research is needed to fully understand the interaction between IGF-1 and the ChP, to disclose the EV-mediated signaling via the ChP, and to clarify the potential preventive effects of IGF-1 on development of IVH.

Key words: IGF-1, ChP, preterm infants, immature brain, extracellular vesicles, CSF

Classification system and/or index terms (if any) Supplementary bibliographical information

Language: English **ISSN and key title**: 1652-8220

ISBN: 978-91-8021-490-2

Recipient's notes **Number of pages**: 89

Price Security classification

Signature Date 2023-10-26

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Coverphoto by Niklas Ortenlöf. A light-sheet image zoomed in on the choroid plexus in the preterm rabbit pup brain. The fluorescence is from IGF-1 conjugated with Alexa Fluor 647.

Faculty of Medicine

Department of Clinical Sciences, Division of Pediatrics

ISBN 978-91-8021-490-2

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University

Lund 2023

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To my family and friends

" There will be always something
new to discover in the field of neuroscience"

Nicholas N. Foster (1st of March 2022, Driver seat talk)
Assistant adjunct professor in neurobiology

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Preface

Now the day is here, and it is time to write my thesis. Well, is it not required to extend a thesis with a preface, but it is my view that a book should have a preface describing the initial thoughts of the writer.

The story of this thesis is a long story. For the sake of my readers, I have shortened it down here. I am trained in bioengineering and started to pursue, or at least started to consider a PhD during my internship at University of California in San Diego, USA, back in 2013. At that point, the focus was all about programming, but following a string of inconsistent circumstances, I am now a developmental neuroscientist.

Over the years, I have delved into entirely new fields (programming, microbiology and now neuroscience) to pursue and, I hope that these experiences have helped me to complete this PhD, Today, I am deeply grateful to be working in the field of developmental neuroscience, with a specific focus on the preterm infant.

Significant time, effort and money have been invested in thesis. I believe it has been worthwhile since my findings provide an increased understanding of what may help improve the lives of preterm infants in the future.

Thank you for joining me on this journey.

Niklas Ortenlöf

Abstract

Extremely preterm infants (i.e., born below 28 gestational weeks), are at high risk of developing brain morbidities including intraventricular hemorrhage (IVH) and neurodevelopmental impairment. Despite over 50 years of research, there is currently no effective therapy available for preventing IVH.

Insulin-like growth factor 1 (IGF-1) plays a pivotal role in perinatal development. Extremely preterm infants exhibit reduced levels of circulatory IGF-1, and low levels of IGF-1 during perinatal development are associated with poor weight gain, reduced brain volumes and impaired cognitive development.

While clinical data suggest that supplementary treatment with IGF-1, aimed at restoring physiological levels, could potentially enhance neurodevelopment, the precise molecular mechanisms underlying the protective effects remain incompletely understood.

One potential transport mechanism of blood-borne IGF-1 to reach the central nervous system is via the choroid plexus (ChP). All ventricles in the brain contain this tissue, which is the main producer of cerebrospinal fluid (CSF). By studying the onset of IVH using our experimental preterm rabbit pup model, we have discovered that ChP is the predominant site of bleeding in IVH in our model.

In this thesis work, we have investigated i) the interaction of blood-borne IGF-1 on the ChP in the immature brain, and ii) the impact of IGF-1 on the occurrence of IVH in an experimental model of IVH. Our findings reveal that blood-borne IGF-1 accumulates in the ChP following systemic administration of IGF-1, leading to an upregulation of genes associated with vascular maturation in the ChP. Furthermore, we observed that uptake of administrated IGF-1 from the circulation and CSF was dependent on postnatal age.

Through a combination of an in vitro cell culture system and a preterm rabbit pup model, we provide evidence for an extracellular vesicle (EV)-mediated transport of blood-borne IGF-1 through the ChP, and into the CSF. Ultimately, the IGF-1 carrying vesicles reach the deep layers of the hippocampus in the immature brain. Finally, we evaluated the potential of IGF-1 to prevent IVH in a preterm rabbit pup model.

Altogether, the results presented in this thesis highlight the importance of the ChP and IGF-1 in the developing brain. More research is needed to fully understand the interaction between IGF-1 and the ChP, to disclose the EV-mediated signaling via the ChP, and to clarify the potential preventive effects of IGF-1 on development of IVH.

Populärvetenskaplig sammanfattning

I Sverige överlever idag uppemot 80% av de barnen som är födda extremt för tidigt (födda innan graviditetsvecka 28). Dock medföljer en hög sjuklighet, där uppemot 20% av barnen utvecklar olika sjukdomstillstånd såsom blödning i hjärnas hålrum, även kallat intra-ventrikulär hjärnblödning (IVH). Dessutom löper de extremt för tidigt födda barnen en ökad risk att utveckla en intellektuell och neurologisk utvecklingsförsening med bl.a. cerebral pares, kognitiv reduktion och neuropsykiatriska avvikelser som följd. Trots stora framsteg inom vården och i forskningen, finns det idag ännu inga specifika behandlingar för att förebygga dessa funktionsnedsättningar hos det växande barnet.

En möjlig behandlingskandidat är insulinliknande tillväxtfaktor-1 (IGF-1). IGF-1 spelar en central roll för att understödja fostrets utveckling under utvecklingen i livmodern. Studier har visat att extremt för tidigt födda barn har låga blodnivåer av IGF-1 efter födelsen, vilket har kopplats till nedsatt viktökning, minskade hjärnvolymer och nedsatt intellektuell utveckling.

Kliniska studier har visat att en tillförsel av IGF-1, som syftar till att återställa normala blodnivåer, potentiellt skulle kunna förbättra hjärnutvecklingen hos extremt för tidigt födda barn och minska risken för att utveckla IVH. De exakta molekylära mekanismerna som ligger bakom dessa effekter är inte kartlagda.

En möjlig transportmekanism för IGF-1 från blod för att nå hjärnan är via en vävnadstruktur som kallas för plexus choroideus (ChP). Alla hålrum (ventriklar) i hjärnan innehåller denna vävnad som är en huvudproducent av ryggmärgsvätskan, den vätska som vår hjärna badar i. Genom att studera uppkomsten av IVH med hjälp av vår experimentella försöksdjursmodell, har vi visat att ChP är viktig i samband med att blod kommer ut i ventriklarna vid en IVH.

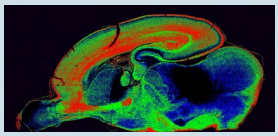
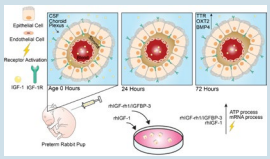
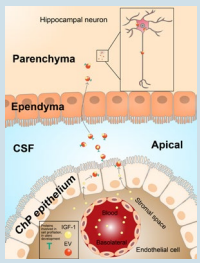
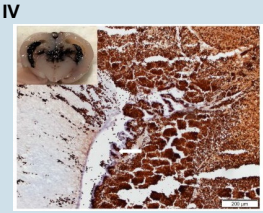
Syftet är med denna doktorsavhandling är undersöka effekten av IGF-1 på ChP i den omogna hjärnan, samt att utvärdera den skyddande effekten av IGF-1 på uppkomsten av IVH i en förtidigt född kanin IVH-modell.

Ett viktigt fynd i avhandlingen är att tillfört IGF-1 ansamlas i ChP, vilket leder till ett ökat uttryck av gener som är kopplade till utmognad och stabilisering av blodkärl. Dessutom observerade vi att upptaget av tillfört IGF-1 i både blodet och ryggmärgsvätskan är beroende av ålder efter födelse, vilket betyder att desto tidigare som IGF-1 tillförs efter förlossning, desto större blir upptaget. Genom att kombinera

cellodlingsstudier med studier i vår för tidigt födda kaninmodell upptäckte vi att IGF-1 transporteras med hjälp av s.k. extracellulära vesiklar (nanostora strukturer som celler utsöndrar) genom ChP ut i ryggmärgsvätskan. Dessa IGF-1 bärande vesiklar når slutligen de djupa lagren av hjärnan (hippocampus, ett mycket viktigt område i hjärnan för minne) hos våra försöksdjur. I avhandlingen har vi även undersökt möjligheten för IGF1 att förhindra uppkomsten av IVH i vår experimentella försöksdjursmodell.

Sammantaget belyser resultaten som presenteras i denna avhandling vikten av ytterligare forskning om IGF-1 och samspelet med ChP i den omogna hjärnan. Mer forskning behövs för att förstå effekten av ålder på samspelet mellan IGF-1 och ChP, samt att förstå hur ChP signalerar till hjärnan med hjälp av extracellulära vesiklar. Slutligen behövs ytterligare studier för att kartlägga hur behandling med IGF-1 kan förebygga risken för att utveckla IVH.

Thesis at a glance

Paper	Aims	Methods	Key results	Conclusions
I 	To characterize the cerebrovascular maturation and IGF-1R density in the brain of the preterm rabbit pup following administration of IGF-1.	Immunofluorescence microscopy of the IGF-1R density in preterm rabbit pups. Gene expression analysis of the ChP upon administration of IGF-1.	Genes related to angiogenesis were upregulated in preterm rabbit pups at 24 hours postnatal age. IGF-1R density was significantly reduced at 96 hours postnatal age.	IGF-1R density correlated with upregulated gene expression of angiogenesis in the ChP following IGF-1 administration.
II 	To investigate the temporal uptake of systemic IGF-1 across the ChP to the CSF in the preterm rabbit pup following administration of IGF-1.	ELISA-analysis of IGF-1 in CSF. Light-sheet and confocal microscopy analysis of the uptake of IGF-1 to the immature brain following IGF-1 administration in preterm rabbit pups. Western blot analysis of the IGF-1 receptor activation.	Uptake of IGF-1 in serum and CSF is dependent upon postnatal age. IGF-1 accumulated in the ChP and perivascular space following administration.	Uptake of IGF-1 in both CSF and serum in preterm rabbit pups is age-dependent and correlates with the activation of IGF-1R in the ChP.
III 	To investigate the effect of IGF-1 on the release of EVs from the ChP and their parenchymal destination.	Immunofluorescence microscopy of stained EVs injected i.c.v. in the preterm rabbit pup. Electron microscopy of IGF-1 presence in the ChP. MS proteomics of ChP derived EVs.	IGF-1 was present in intra- and extracellular vesicles of the ChP. EVs were observed in the hippocampus following i.c.v injection.	This study has provided evidence suggesting an EV-mediated transport of systemic IGF-1 from the ChP to the hippocampus of the immature brain.
IV 	To investigate if exogenous administration of IGF-1 prevents IVH in preterm rabbit pups	Immunohistochemistry, and high frequency ultrasound for determining IVH in preterm rabbit pups. ELISA to determine IGF-1 concentrations in serum.	The incidence of IVH following IGF-1 administration was not statistically different compared to vehicle treated animals.	Additional studies are needed to further characterize and establish the potential of IGF-1 in reducing the prevalence of IVH in the preterm rabbit pup.
Abbreviations	IGF-1, insulin-like growth factor 1; IGF-1R, insulin-like growth factor 1 receptor; ChP, choroid plexus; CSF, cerebrospinal fluid; MS, mass spectrometry; IVH, intraventricular hemorrhage; ELISA, enzyme-linked immunosorbent assay; i.c.v., intracerebroventricular; EV, extracellular vesicle.			

Original papers

I

Gram, M., Ekström, C., Holmqvist, B., Carey, G., Wang, X., Vallius, S., Hellström, W., **Ortenlöf, N**., Agyemang, A. A., Smith, L. E. H., Hellström, A., Mangili, A., Barton, N. and Ley, D. (2021). Developmental Neuroscience. 43, 281-295.

Insulin-like growth factor 1 in the preterm rabbit pup: Characterization of cerebrovascular maturation following administration of recombinant human insulin-like growth factor 1/insulin-like growth factor 1-binding protein 3

II

Ortenlöf, N., Vallius, S., Karlsson, H., Ekström, C., Kristiansson, A., Holmqvist, B., Göransson, O., Vaváková, M., Rydén, M., Carey, G., Barton, N., Ley, D. and Gram, M. (2023). Fluids and Barriers of the CNS. 20, 59.

Characterization of choroid plexus in the preterm rabbit pup following subcutaneous administration of recombinant human IGF-1/IGFBP-3

III

Ortenlöf, N., Vallius., Karlsson, H., Ekström, C., Kristiansson, A., Holmqvist, B., Pankratova, S., Barton, N., Ley, D. and Gram, M. (2023). Under review in The Proceedings of the National Academy of Sciences.

Choroid plexus extracellular vesicle transport of blood-borne insulin-like growth factor 1 to the hippocampus of the immature brain

IV

Ekström, C., **Ortenlöf, N**., Holmqvist, B., Jungner, Å., Vallius, S., Wang, X., Hellström, A., Barton, N., Carey, G., Gram, M. and Ley, D. (2023). Under review in Scientific Reports.

Evaluation of recombinant human insulin-like growth factor 1/insulin-like growth factor binding protein 3 on intraventricular hemorrhage prevention and survival in the preterm rabbit pup model

Additional papers not included in the thesis

Agyemang, A.A., Kvist, S.V., Brinkman, N., Gentinetta, T., Illa, M., **Ortenlöf, N**., Holmqvist, B., Ley, D., and Gram, M. (2021). Journal of Neuroinflammation. 18, 42.

Cell-free oxidized hemoglobin drives reactive oxygen species production and pro-inflammation in an immature primary rat mixed glial cell culture.

Kanagarajan, S., Carlsson, M. L. R., Chakane, S., Kettisen, K., Smeds, E., Kumar, R., **Ortenlöf, N**., Gram, M., Åkerström, B., Bülow, L. and Zhu, L. H. (2021). International Journal of Biological Macromolecules. 184, 955-966.

Production of functional human fetal hemoglobin in Nicotiana benthamiana for development of hemoglobin-based oxygen carriers.

Abbreviations

ChP	Choroid plexus
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGFBP-3	Insulin-like growth factor binding protein-3
PI3K	Canonical phosphatidylinositol 3-kinase
ERK	Extracellular signal-regulated kinase
IVH	Intraventricular hemorrhage
GW	Gestational weeks
GM	Germinal matrix
CNS	Central nervous system
CSF	Cerebrospinal fluid
SAS	Subarachnoid space
EV	Extracellular vesicle
IF	Immunofluorescence
IHC	Immunohistochemistry
TEM	Transmission electron microscopy
PHVD	Posthemorrhagic ventricular dilatation
HFU	High-frequency ultrasound
ZO-1	Zonula occludiens-1
TTR	Transthyretin
TEER	Transepithelial electric resistance
MS	Mass spectroscopy
FITC	Fluorescein isothiocyanate
i.c.v.	Intracerebroventricular
ELISA	Enzyme-linked immunosorbent assay
s.c.	Subcutaneous
Tmed2	Transmembrane P24 Trafficking Protein 2
m/z	Mass-to-charge ratio
i.p.	Intraperitoneal

Chapter 1: The immature brain of the preterm infant

"Only a life lived for others is a life worthwhile"
Albert Einstein (1879 - 1955)
Physicist

In essence, my thesis revolves around the preterm infant (born under 37 gestational weeks (GW), a normal pregnancy is 40) or more specifically, extremely preterm infants, born under 28 GW. This is a large patient group as from a global perspective, 15 million infants are born as preterm infants, and is the main contributor to neonatal morbidity and mortality[1]. Unfortunately, approximately 40% of all deaths in children under 5 years old are linked to preterm birth[2]. In Sweden, national guidelines advocate active care of extremely preterm infants, resulting in a uniform care strategy. These acts have resulted in an unprecedented high rate of survival (81%) of extremely preterm infants in Sweden[3]. However, it is not surprising that with this high survival rate comes a significant risk of severe morbidities (61%)[3], affecting various organs of the infant's body, including the lungs, eyes and brain[4]. Hereafter, this thesis will exclusively focus on different brain morbidities and how the findings presented in this thesis may be of assistance.

Before continuing, I would like to express my gratitude for the opportunity to contribute to the body of knowledge presented in this thesis, which perchance can make a positive difference for this highly vulnerable group. Unlike many patients suffering with non-communicable diseases who rely on scientific advancements to develop drugs and treatments, but may have means to manage their health themselves[5], this patient group lacks any means of self-help and depends on the efforts of healthcare professionals, the pharmaceutical industry, political decisions,

and the research community. I want to clarify that I am not implying that other patient groups, regardless of age, do not deserve the best possible treatments and support. However, from an ethical standpoint, this is the patient group whom my empathy truly belongs.

The immature brain

The brain is by no doubt the most complex organ in the human body. Analogous to a long-distant runner who paces themselves throughout a race and then sprints, the brain undergoes a gradual development during the first and second trimesters, followed by a period of rapid growth and increasing complexity during the third trimester[4]. This period is marked by extensive cortical, dendritic, and axonal branching, glial cell proliferation, programmed cell death (apoptosis), and differentiation[4,6]. Synaptogenesis and myelination are starting to develop at GW 18 and 28, respectively[6]. Myelination, in fact, continues to adulthood[6]. This combination of neurodevelopmental processes leads to an increase three- to fourfold increase in cortical and white matter volume during the third trimester[7], and the cerebellum undergoes similar proliferation, growth, and granule cell migration[8,9]. At 30 GW, only half of the total brain mass is established compared to full-term development[10]. Additionally, blood vessels develop in parallel with the parenchyma[4]. A summary of human brain development is provided in figure 1.

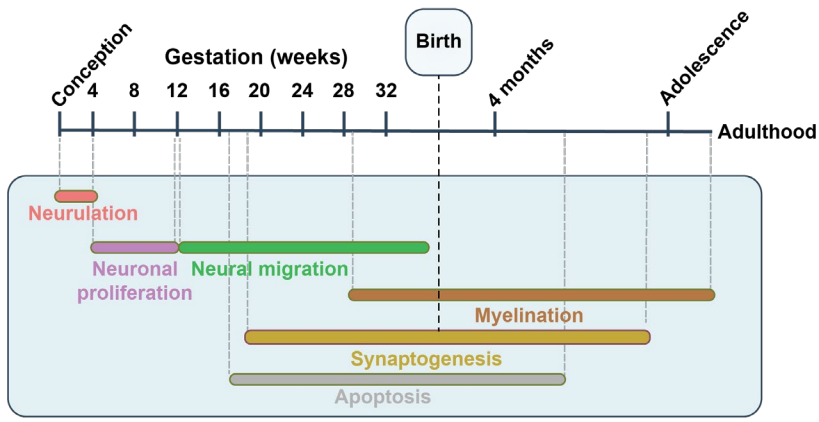


Figure 1. Neurodevelopment of the human brain.
See the main text for details. The artwork was created by Niklas Ortenlöf using Adobe Illustrator with inspiration from Tau et al[6].

Considering the above, extremely preterm infants are born with a significantly immature brain which consequently places them at high risk of various brain morbidities.

Neurodevelopmental disorders

A recent large registry-based study (383 infants based in Sweden), of extremely preterm infants born between 22 and 24 GW showed that 75% of Swedish children born before 24 GW displayed some sort of neurodevelopmental disorder. This included intellectual disabilities (40%), attention-deficit/hyperactivity disorder (30%), autism spectrum disorders (24%), speech disorders (52%), cerebral palsy (17%), epilepsy (10%), visual impairment (22%), and hearing impairment (5%). Moreover, 55% of the infants required habilitation services[11]. The rate of diagnosed infants with neurodevelopmental disorders increased as the children grew older, 71% in 2-5 years versus 82% in 10-13 years old children[11]. As a consequence of this study, it becomes evident that the vulnerable immature brain of the extremely preterm infant frequently leads to various forms of neurodevelopmental disorders. Thus, research is needed to help this vulnerable group.

Intraventricular cerebral hemorrhage

One of the focus areas in our lab is the morbidity intraventricular hemorrhage (IVH), diagnosed with a bleeding into the ventricles. Extremely preterm infants face high risk of developing IVH and approximately one-third of the surviving newborns are diagnosed with any grading of IVH[12] (see table 1 for details) and sever IVH (diagnosed as ≥ grade III) occurs at 20% of the diagnosed infants[13-15]. However, despite the severity of IVH, there is an increased risk of adverse neurodevelopmental outcomes resulting in altered behaviour[16-18]. In addition, 60% of infants with IVH grade IV face a substantial higher risk of developing motor and cognitive impairment impairment[13,19]. Despite >50 years of IVH investigation in extremely preterm infants, there is currently no therapy to prevent IVH or reduce resulting neurodevelopmental impairment.

The primary cause of IVH in the preterm infant is the rupture of the highly vascularized germinal matrix (GM), which is located between the ependymal lining of lateral ventricles and the caudate nucleus. The GM develops through embryogenesis to GW 35 and reaches its peak size at GW 24. From here, neurons and glia cells migrate peripherally to construct the grey matter of the developing brain. The origin of GM hemorrhage is multifactorial. IVH mainly occurs in

extremely preterm infants (GW < 28) due to factors such as increased cerebral venous pressure, fluctuations in cerebral blood flow, and hypoxia induced by preterm birth, all of which can lead to the rupture of the fragile blood vessels within the GM[20-23]. In addition, choroid plexus (ChP) hemorrhage leading to ventricular dilation also occurs in preterm infants, however less frequently[24]. This condition can be challenging to diagnose because clots resulting from GM bleeding can cause inflammation of the ChP making it difficult to determine the origin of the bleed[24].

Table 1. Intraventricular hemorrhage grading[12]

Grades	Classification
I	Bleeding limited to subependymal germinal matrix
II	Bleeding extends to <50% of the ventricular volume
III	Bleeding extends to >50% of the ventricular volume = ventricular dilatation
IV	Bleeding extends to the parenchyma = posthemorrhagic ventricular dilatation

Insulin-like growth factor 1

One prominent candidate for the treatment of neurodevelopmental disorders including IVH as described above, is the mitogenic hormone insulin-like growth factor 1 (IGF-1). IGF-1 is important for a multitude of processes including, proliferation, gene expression, inhibition of apoptosis, oxidative stress and protein translation[25-27]. IGF-1 is also a mediator of neuronal growth, angiogenesis, and is essential for fetal brain development[28].

Synthesis of IGF-1 occurs primarily in the liver, however, nearly all cells produce IGF-1 including cells of the brain, where production of IGF-1 declines rapidly with age[29]. IGF-1 affects recipient cells by binding to the IGF-1 receptor (IGF-1R), which is abundantly expressed throughout the brain, especially neuron-rich areas such as the granulate cell layers of the dentate gyrus and cerebral cortex[25,29]. Importantly, all neuroepithelial cells express IGF-1R[30].

Upon the IGF-1 binding to the receptor, an autophosphorylation of the intracellular part of the receptor occurs, followed by phosphorylation of the insulin receptor substrate-1 protein at multiple tyrosine sites. These activated phosphotyrosine sites in turn activate two signaling cascades, the canonical phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/extracellular signal-regulated kinase (ERK) signaling pathways, which induce cell metabolism and proliferation respectively[31,32] (figure 2).

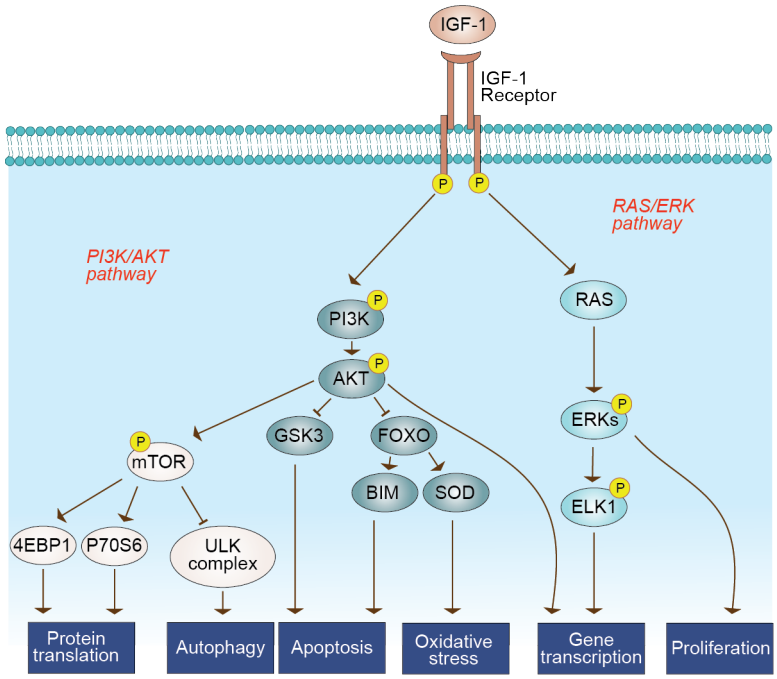


Figure 2. Classical IGF-1 signaling pathways.
Ligand binding induces receptor auto-phosphorylation. This activated state subsequently initiates two major downstream signaling pathways: the canonical phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/extracellular signal-regulated kinase (ERK). These pathways ultimately govern essential biological processes, including protein translation, autophagy, apoptosis oxidative stress, gene transcription, and proliferation. BIM, BCL-2 interacting mediator of cell death; ELK, ETS-like transcription factor; FOXO, forkhead box protein O; GSK3, glycogen synthase kinase 3; mTOR, mammalian target of rapamycin; SOD, superoxide dismutase; ULK, uncoordinated-like kinase. The artwork was created by Niklas Ortenlöf using Adobe Illustrator with inspiration from Fernandez et al[25].

The bioavailability of circulatory IGF-1 is modulated by IGF binding proteins (IGFBP), ranging from 1–6 which bind to IGF-1 with high affinity, even higher than the affinity to the IGF-1R. IGFBP-3 is most abundant, comprising 60- 70% of the circulatory IGFBPs[26,33]. Therefore, the IGF-1/IGFBP-3 complex must be cleaved

before the ligand-receptor interaction can occur. Several mechanisms regulate the release of IGFs from IGFBPs, which one prominent being protease-mediated shedding[25]. Of great importance for the focus of this thesis, i.e. the development of the immature brain, blood-borne IGF-1 has been describe to cross both the blood brain barrier and the blood-cerebrospinal fluid (CSF) barrier, ultimately reaching the CSF[25,34-36].

IGF-1 has been described to have a central role in extremely preterm infants. Throughout gestation, IGF-1 stimulates the placenta to transfer nutrients from the mother to the developing fetus. Fetal circularity concentration of IGF-1 is increased during gestation and reaching its maximum at term birth. At this stage, the amniotic fluid has a higher concentration of IGF-1 than the fetal circulatory and is swallowed by the infant. This boosted IGF-1 concentration at the later stage of gestation and at term is not accessible for extremely preterm infants[28,37,38]. In fact, preterm infants, especially those born at less than 28 weeks of gestation, have reduced circulating levels of IGF-1 at birth compared to the fetus remaining in utero at the corresponding gestational age[28]. Of central relevance to this thesis, low IGF-1 levels in preterm infants correlate with neurodevelopment impairment, lower brain volumes and poor weight gain[39,40]. Hence, supplementation of IGF-1 to increase the circulatory levels of preterm infants could be a potential treatment to reduce the morbidities described above, including IVH. In fact, a phase 2 randomized clinical study, aimed to achieve the circulatory levels corresponding to the healthy fetus in extremely preterm infants, resulted in a trend towards reduced occurrence of IVH[41].

Box: Aim of study I

To characterize the cerebrovascular maturation and IGF-1R density in the brain of the preterm rabbit pup following administration of IGF-1.

Furthermore, in the preterm piglet, supplementation with IGF-1 increased neurogenesis in the hippocampus and improved balance in behavior tests[42,43]. It could be speculated that the mechanism behind this putative protection results from a mixture of the biological effects exerted by IGF-1 (as outlined in figure 2) to the vasculature in the preterm infant. It could also be speculated that other morbidities

of the preterm infants born before 24 weeks, including intellectual disabilities, could be remedied by IGF-1. Altogether this warrants further investigation.

Box: Aim of study IV

To investigate if exogenous administration of IGF-1 prevents IVH in preterm rabbit pups.

Chapter 2: The ocean within us

"What's real can't die"
Gregg Alexander (1998)
Songwriter and frontman in New Radicals

The title might confuse people, I understand, but hear me out. Our central nervous system (CNS) bathes in a constantly renewable fluid, known as the cerebrospinal fluid (CSF). I would like to propose, wearing my philosopher's hat, that we never truly left the ocean, considering that the CSF system is conserved throughout evolution, dating back to the classification of Deuterostomia[44]. In this chapter, I will describe the major component of my thesis, the ChP, and its function in the ventricular system. The ocean within us.

The ventricular system

The CNS floats in CSF. There are series of fours connected cavities, holes or ventricles in the brain, filled with CSF. The ventricles are strategically positioned in the brain and claimed by Emerich et al[45] to be perfectly positioned as they are adjacent to several important subcortical structures[46]. These structures are summarized in table 2.

There are four ventricles present in the human brain, the two lateral (located in the telencephalon) one third (located in the diencephalon) and the fourth (located in the rhombencephalon)[47]. The two lateral ventricles are large c-shaped structures which

are located in each hemisphere, projecting out to the frontal, temporal and occipital lobes in the brain, termed horns. The roof of the frontal horn is formed by the corpus callosum, as it sits underneath.

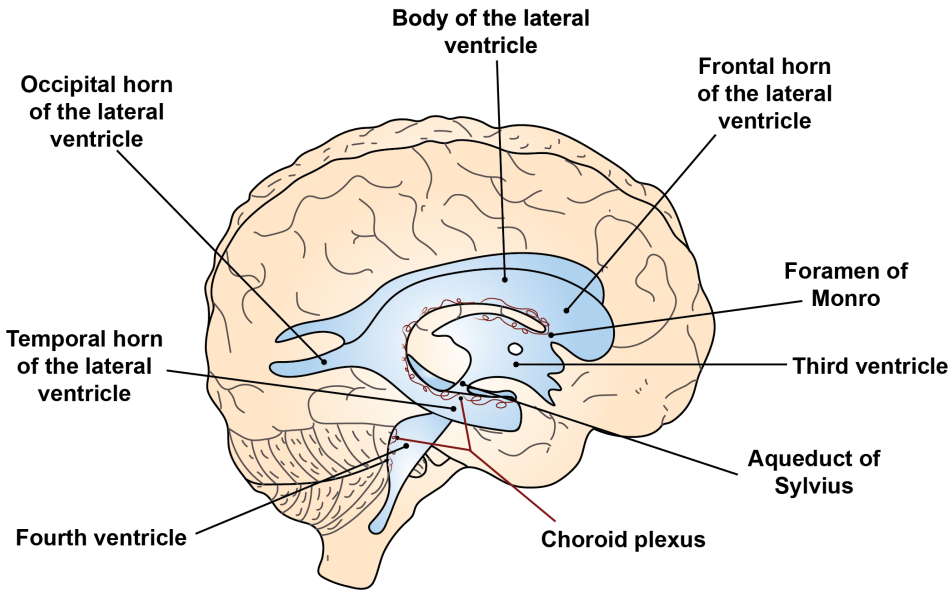


Figure 3. Diagram of the ventricles in the brain
The artwork was created by Niklas Ortenlöf using Adobe Illustrator.

The head of the caudate nucleus forms the lateral wall, and columns of the fornix forms the inferior parts of medial wall. The temporal horn is the largest parts of the ventricles, stretching from thalamus and ends at the amygdala. Below, the hippocampus is located. The occipital horn projects posteriorly to the occipital lobe and is surrounded by the optic radiations[48]. The CSF flows through the ventricles by assisted in part of synchronized beating cilia present on ependyma cells. These cells constitute a layer on the CSF side throughout the ventricular system[49].

The lateral ventricles communicate with the third ventricle through the foramen of Monro. The third ventricle constitutes a narrow passage in the diencephalon, laterally bounded by the medial aspect of each thalamus, hovering over the hypothalamus. From the third ventricle, the CSF flows through the aqueduct of Sylvius into the fourth ventricle, located in the brain stem adjacent to the cerebellum[50]. A simplified illustration of the ventricular system can be seen in figure 3. From the fourth ventricle, CSF may travel one out of two pathways via the medial

(of Magendie) or the lateral (of Luschka) apertures. It can either continue its journey down to the spinal cord via the central canal, or travel at the cisterna magna to the subarachnoid space (SAS), surrounding and bathing the brain[51]. In the classical model of CSF dynamics, the CSF moving through the SAS is cleared into the dural venous sinuses via the arachnoid granulation[52], see figure 4 for a schematic illustration. An alternative possibility, is the penetration of CSF from the SAS into the parenchyma, referred to as the glymphatic system, where the CSF travels down to the perivascular space[53]. Here, the CSF is combined with interstitial fluid and parenchymal solutes, finally exiting down via the venous paravascular spaces[53]. Of interest, the primary focus of my thesis, i.e. the brain of the preterm infant, it is unclear if the glymphatic system is developed[54]. Consequently, this system is not the focus of the thesis.

Table 2: Important subcortical structures adjacent to ventricles and their function

Important structures adjacent to each ventricle	Lateral ventricles	Third ventricle	Fourth ventricle	Function
Caudate nucleus	x			Planning the execution of movement, learning, memory[55]
Corpus callusum	x	x		Needed for efficient executive function[56,57]
Thalamus	x	x		Important for sensory and cognitive functions[58]
Amygdala	x			Key functions in attention[59]
Hippocampus	x	x		Critical function in memory, navigation, and cognition[60]
Optic radiation	x			Posterior part of the central visual pathway[61]
Optic chiasm		x		Where two optic nerves fuse[62]
Hypothalamus		x		Regulate fundamental aspects of physiological homeostasis and behavior[63]
Circumventricular organs		x	x	Regulate exchange of molecules between the bloodstream and the brain[64]
Cerebellum			x	Important for motor skills[65]

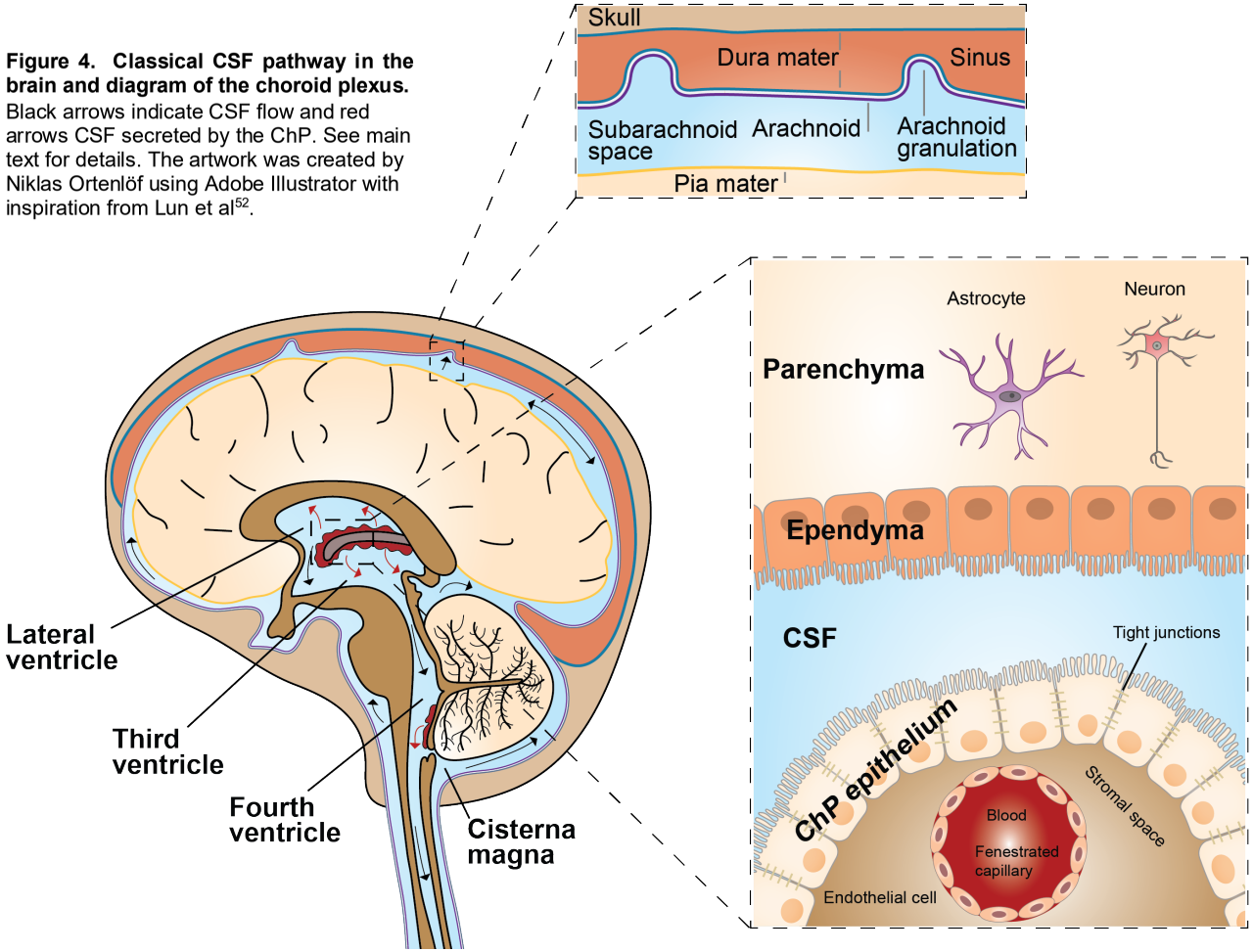
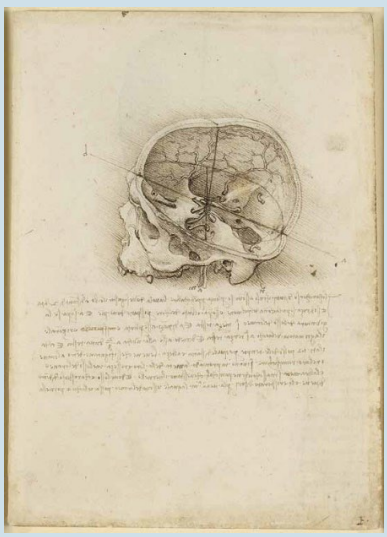


Figure 4. Classical CSF pathway in the brain and diagram of the choroid plexus. Black arrows indicate CSF flow and red arrows CSF secreted by the ChP. See main text for details. The artwork was created by Niklas Ortenlöf using Adobe Illustrator with inspiration from Lun et al[52].

Box: Leonardo Da Vinci's search for the soul

The ventricles have fascinated scholars throughout history. Beginning with Herophilus around 290 B.C., they were considered the source of the soul, memory, and vital spirits of the body[66,67]. Centuries later, in 1489, the Renaissance universal genius Leonardo Da Vinci embarked on a personal quest to locate the "senso comune", the soul[66]. His unique opportunity to examine the human brain and body ex vivo allowed him to create highly detailed and remarkably realistic drawings, a standard not surpassed until the 1800s. As a painter and scientist, Da Vinci was particularly intrigued by the optic tract. He postulated, based on the prevailing belief that the soul resided in the ventricles, that "the cavity of the eye socket and the cavity in the bone supporting the cheek, nose, and mouth are of equal depth and terminate in a perpendicular line below the senso comune". With our modern understanding of the ventricular system, we now know that he was referring to a location in the third ventricle, just dorsal to the optic chiasm. In an attempt to further visualize the ventricles, he injected molten wax into the lateral ventricles of an ox, removed the surrounding brain tissue, and painted, for the first time, a beautiful and fairly accurate drawing of the ventricular system.



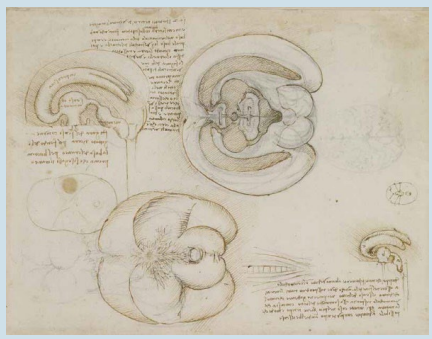


Figure 5. Ventricular location of the soul. Sketches by Leonardo da Vinci part of the Royal Collection Trust/© Her Majesty Queen Elizabeth illustrating da Vinci's location of the soul, dorsal to the optic chiasm (left) and the ventricular system followed by a detailed diagram after performing wax injection.

The choroid plexus

Historically, the CSF have been viewed primarily and a cushion medium of the brain, protecting it from impacts and supplying adequate pressure[47,67,68]. Currently, the interest of CSF research has expanded significantly, and is likely to expand further in the future[47]. CSF is produced by a specialized tissue called the choroid plexus (ChP), located in each of the ventricles[52,69], see figure 3 and 4. The ChP has two different embryological origins, where its epithelium is derived from neuroepithelial cells and its stromal compartment from head mesenchymal cells.

The production of CSF components arises from two phenomena involving the ChP. First, a filtration of blood through the ChP, which contributes for the water content of the CSF. Second, the ChP itself produces of a wide range of molecules found in the CSF[69]. Indeed, most of the proteins found in the adult CSF originates from the ChP[70]. In addition to CSF production, the ChP constitutes the blood-CSF barrier, providing the brain's first line of defense[71]. The barrier function is made possible by tight-junction proteins, such as E-cadherin and zonula occludens 1[72]. Blood-borne molecules can flow freely through the stroma of the ChP, but they are restricted at the epithelium[47]. The ChP has numerous functions of central importance for the brain, including immune surveillance[73], chemical surveillance[74,75], neurogenesis[76] and mediator of the circadian clock[71,77,78]. Additionally, the thyroid hormone distributor protein transthyretin fundamental for brain development, is primarily secreted in the brain by the ChP[79].

The ChP, with one situated in each ventricle, have distinct developmental patterns, appearing first in the fourth ventricle, followed by the lateral ChP, and finally the third ventricle. For example, in rats, the fourth ChP appears at embryonic day (E) 12, the lateral ChP at E13, and the third ChP at E16. Furthermore, ChP development is divided into four distinct stages[80].

Stage I of ChP development in the human fetus occurs between GW 7 to 9. During this stage, epithelial cells are tall, with central nuclei, and have limited glycogen content. The ChP size is relatively small in relation to the ventricles. Stage II develops between GW 9 to 17, features low columnar cells with apical nuclei and abundant glycogen. The ChP is notably larger compared to the ventricles at this stage. Stage III, which progresses from GW 17 to 29, involves the development of characteristic cuboidal cells, the presence of villi, and a transition of nuclei from

apical to the central compartment within the cells. The ChP becomes smaller in size and has reduced glycogen content compared to stage II. The III stage develops from GW 17 to 29 17th[52,80]. As such, the transition of ChP development from stage II and III in occurs during the period where a preterm birth will be referred to as extremely preterm. In stage IV, the ChP has its established morphology, with basal nuclei, a complex villi structure with numerous fronds, an absence of glycogen, and smaller tissue in relation to the ventricles. This morphology continues throughout adult life[52,80].

For the reader to fully grasp the complexity and beauty of the ChP, I have included a transmission electron microscope image of the ChP extracted from a preterm rabbit pup (see figure 6). The rationale for using the rabbit ChP will be further explained in chapter 4. This image nicely displays several key features of the ChP, including the tight junctions between the epithelial cells, the squeezed erythrocytes in the fenestrated capillaries, the numerous fronds of the villi, and the stromal compartment.

Box: Aim of study II

To investigate the temporal uptake of systemic IGF-1 across the ChP to the CSF in the preterm rabbit pup following administration of IGF-1.

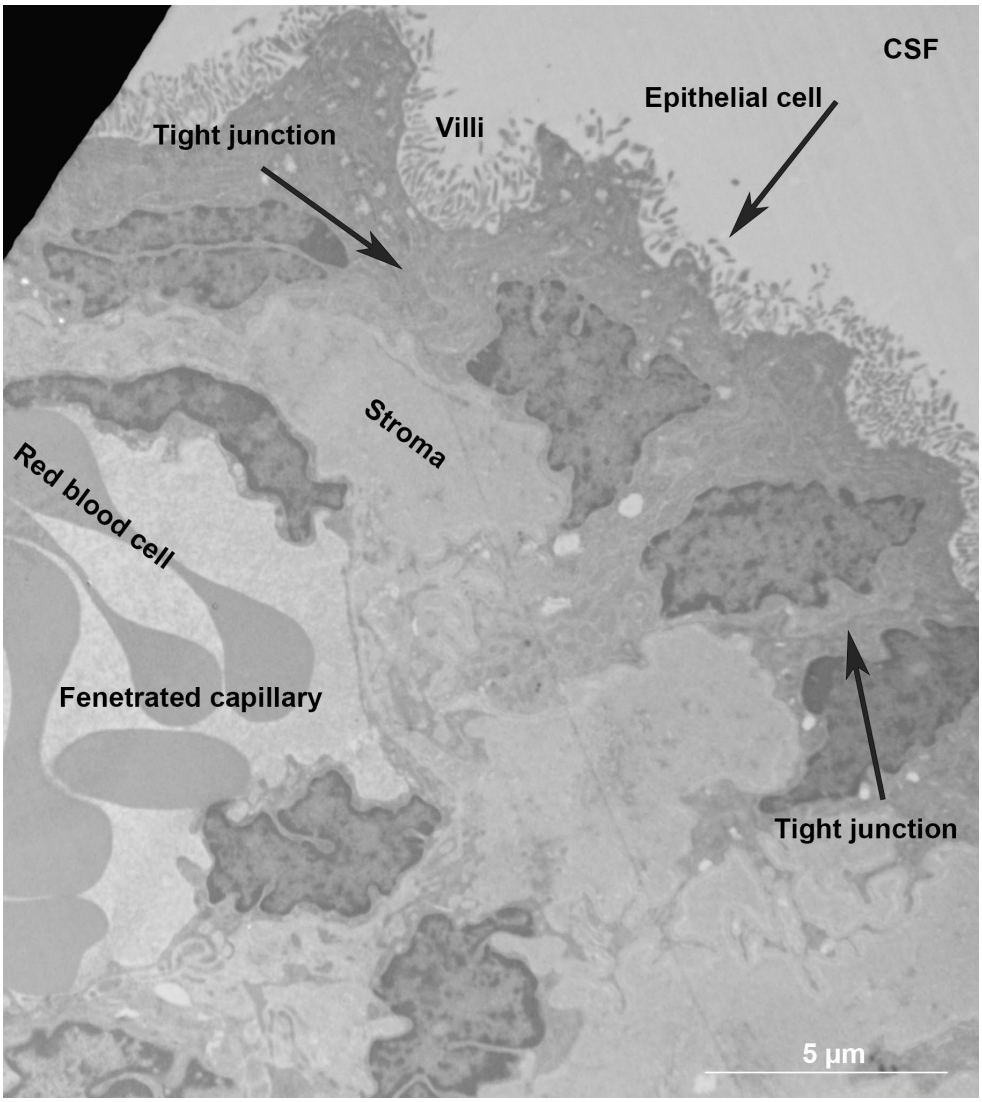


Figure 6. Transmission electron microscopy image of preterm rabbit pup ChP.
Representative image of the ChP derived from a preterm rabbit pup delivered by cesarian-section 3 days before term age with the postnatal age of 2 days. Photograph by Niklas Ortenlöf.

Chapter 3: The public transport of biomolecules

"If you seek tranquility, do less things better"
Marcus Aurelius (121 – 180)
Emperor of Rome

Can free molecules in the CSF or the blood travel to reach their recipient cells? Well, in some cases, yes. However, similar to a person walking down Rome's busy streets, heavy with traffic and pollution, that person could clearly benefit from being transported in a fast and safe way. My mind turns to public transport, such as buses, for example. Biomolecules seem to have the same "mindset", thus favoring some form of "public transport" when traveling in biological fluids. There are several ways for biomolecules to be transported. One way is for biomolecules to be packaged in nanosized membrane encapsulated cell like structures called extracellular vesicles (EVs). In this chapter, I will describe EV transport.

Extracellular vesicles

Historically, intercellular communication has been proposed to be based on secretion of solvable components, or direct interaction between cells[81]. However, after the discovery of EVs, which can facilitate both short and long distant vesicle mediated signaling, this transport mechanism has received significant attention[82-84]. EVs are nanosized vesicles encapsulated in a lipid bilayer membrane which surrounds a cargo of biomolecules. The content of EVs is a mixture of proteins,

lipids, and nucleotides (where RNA and small non-coding RNAs are most prominent), reflecting their parental cell origin and potentially the intended recipient cell[85]. While the transportation of proteins to be transported by EVs has been known since the 1980s[86], it took over than 20 years to make the groundbreaking discovery of mRNA and miRNA transportation in EVs and their delivery to recipient cells[87]. Multiple cell types secreted EVs during physiological and pathological conditions, including brain cells. Consequently, EVs are present in various biological fluids such as saliva, urine, blood, CSF, amniotic fluid and milk[83]. Interesting, the secretion of EVs is conserved across multiple species, allowing EVs from donor species to mediate cellular biological effects in another recipient species[88,89]. For instance, cow milk derived EVs can induce multiple benefits in human infants and mice[83,88,90,91].

Types of extracellular vesicles

EVs are typically divided into three categories, exosomes, microvesicles and apoptotic bodies[92]. Moving on, I will not describe apoptotic bodies (small parts of a dying cell) as they are not relevant for the present thesis. Exosomes and microvesicles are distinct in size, buoyant density and secretion process. Exosomes have a diameter between 30-150 nm (average of 100 nm) and microvesicles are considered larger ranging from 100 to 1000 nm[83]. The exosomal vesicles are formed through endocytosis (i.e. inward membrane budding), where they progressively accumulate into intraluminal vesicles with larger multivesicular bodies/late endosome, starting with the early/sorting endosome[83]. Intraluminal vesicles sequester specific proteins, nucleotides and cytosolic components and when released from the cell, are referred to as exosomes. Additional cargo to the multivesicular bodies is transferred from the trans-Golgi network and possible from the surrounding cytosol[92].

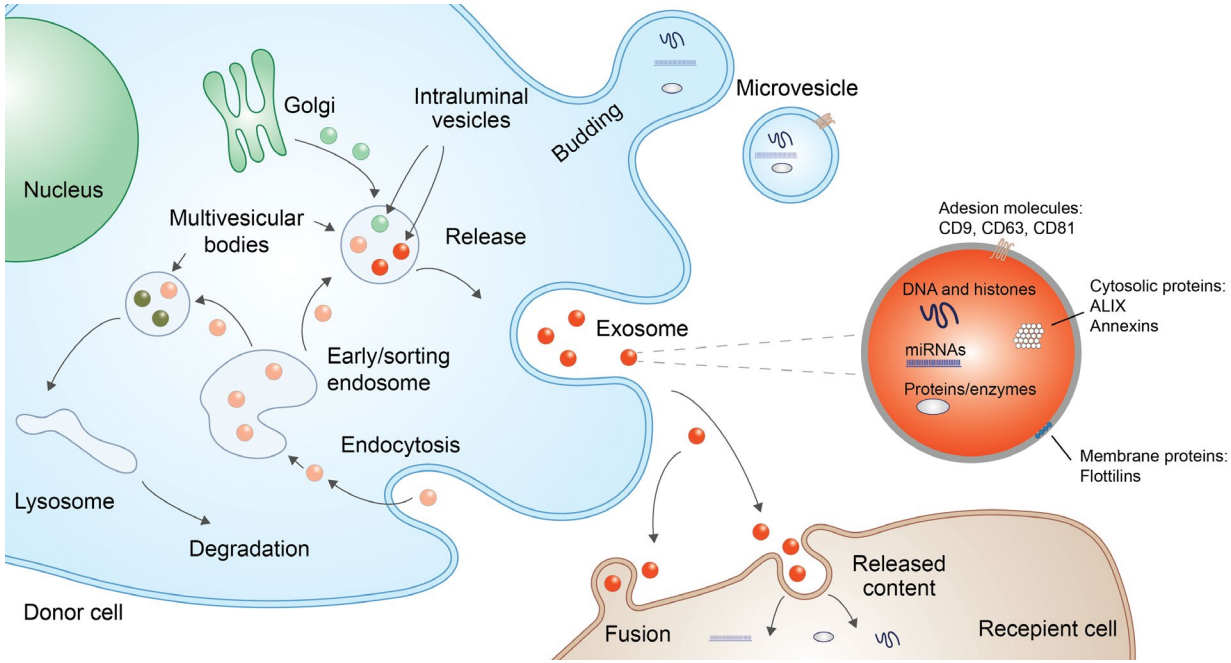


Figure 7. Extracellular vesicle secretion and uptake.
Exosomal vesicles are formed by endocytosis (beige shapes) and packed into early/sorting endosomes. The vesicles are then sorted into multivesicular bodies, where they can either be degraded by the lysosome (dark green shapes) or transitioned into intraluminal vesicles (red and light green shapes). Added cargo is provided by the trans-Golgi network (light green shapes). The multivesicular bodies then fuse with the plasma membrane, and the intraluminal vesicles are released as exosomes. Recipient cells uptake of exosome is primarily via fusion with the plasma membrane or endocytosis and the contents, including nucleotides and proteins are released to the cytoplasm. See the main text for additional details. The artwork was created by Niklas Ortenlöf using Adobe Illustrator with inspiration from Holm et al[85].

In the donor cell, the multivesicular bodies are transported to the plasma membrane, where they are fused with the cell surface and the transported intraluminal vesicles are secreted as exosomes[93]. A subset of multivesicular bodies follows a degradation pathway to the lysosome with subsequent degradation[94]. The main proteins integrated into the exosomes are members of the tetraspanin family (adhesion molecules), including proteins such as CD9, CD63 and CD81, endosomal sorting complex required for transport proteins (Alix, TSG101), as well as actin (cytoskeleton), heat shock proteins (stress protein), and flotillins (exosome biogenesis)[83,92,95]. Exosomes are subsequently taken up by recipient cells primarily via fusion with the plasma membrane where the content is later released into the cytoplasm[92,96,97]. In contrast to the biogenesis of exosomes, the shedding of microvesicles stems from the outward budding of the plasma membrane, generating microvesicles which are secreted directly into the extracellular environment[98]. A schematic description of the exosome and microvesicle pathway is displayed in figure 7.

Box: Aim of study III

To investigate the effect of IGF-1 on the release of EVs from the ChP and their parenchymal destination.

Choroid plexus and extracellular vesicles

In line with the growing research interest in the ChP in recent years, there has been a parallel increase in research focusing on EV transcytosis, ChP vesicle secretion, and vesicle trafficking in the CSF[99-103]. Studies have shown that ChP derived EVs can penetrate the ependymal layer of the brain, delivering micronutrients and miRNAs to distant parenchymal cells[101,102]. Additionally, viruses and systemic inflammation impact the ChP, leading to the secretion of EVs that subsequently infect parenchymal cells[102,103]. Interestingly, when ChP derived EVs are injected systemically into mice, they find the way back home to the brain[100].

The role of EV secretion by ChP is truly fascinating and more research is needed to better understand the vesicle mediated transport from the CSF to the surrounding brain. Among questions that arises are, can growth factors be transported in EVs secreted by the ChP? Is this transportation relevant in the immature brain? These

questions are to some extent addressed in the current thesis, and is investigated in study III.

Box: Injection of encapsulated choroid plexus to treat neurological disease

Vesicles and ChP go hand in hand. In fact, several studies have shown protective effects against neurological disease in rodents and primates with following implantation of ChP cells (epithelial ones) or whole encapsulated ChP tissue into the ventricles or the parenchyma. Matsumoto et al. illustrated beautifully how cultured ChP epithelial cells injected into the ventricles restored brain damage in ischemic rats[104]. With these results in mind, an Australian group started to use pig derived ChP and encapsulated them within a hydrogel coated capsule for better transport and immune protection (figure 8, left panel). In rodent models, neuroprotective effects have been shown following intracerebral injection of these encapsulated ChP against Huntington's disease[105] and stroke[106]. Additionally, in a Parkinson's rhesus monkey model, intracerebral injection of encapsulated ChP, improved neurological functions and increased tyrosine hydroxylase staining in the striatum[107] (figure 8, right panel). Currently, clinical trials have been initiated, however in a Phase IIb study, no reduction of symptoms was observed[108]. Thus, further clinical studies are needed in the future, and I look forward to following the development.

Figure 8. Encapsulated choroid plexus tissue to treat Parkinson's disease.
Encapsulated ChP tissue (left panel) with the average diameter of 600 µm. Intracerebral injected encapsulated ChP tissue (right panel) increases tyrosine hydroxylase staining in the striatum in Parkinson's rhesus monkey model compared to non-injected (Control). Modified from Luo et al[107].

Summary of thesis aims

As you perhaps have noted, the aims of the thesis have been displayed throughout the first three introduction chapters. In essence, my thesis revolves around the impact of IGF-1 on the ChP in the immature brain and the occurrence of IVH in our preterm rabbit pup model. I will provide an overview of the main methods used in this thesis in the following chapter.

Aim of study I

To characterize the cerebrovascular maturation and IGF-1R density in the brain of the preterm rabbit pup following administration of IGF-1.

Aim of study II

To investigate the temporal uptake of systemic IGF-1 across the ChP to the CSF in the preterm rabbit pup following administration of IGF-1.

Aim of study III

To investigate the effect of IGF-1 on the release of EVs from the ChP and their parenchymal destination.

Aim of study IV

To investigate if exogenous administration of IGF-1 prevents IVH in preterm rabbit pups.

Chapter 4: Methodological descriptions

"If you follow nature, you will never be poor. If you follow opinion, you will never be rich"

Seneca the younger (4 BC - AD 65)

Stoic philosopher, statesman and dramatist

In this chapter, the methods of using our quite unique animal model in concert with the work in the cell lab and other techniques will be described. Being granted the opportunity to receive extensive training in animal handling have truly transformed a bioengineer into a competent in vivo scientist.

The detailed methods are described in the original papers, presented at the end of the thesis. Here, the main methods are briefly described.

The preterm rabbit pup

Throughout all the studies included in this thesis, the preterm rabbit pup model has been utilized. While there are many models for simulating preterm brain development, the most frequently used are rodent models, but piglets, cats, and dogs have also been employed throughout the years. However, a significant limitation with these models is that they are mostly term-born[109]. For instance, in rodent models (mice and rats), postnatal days 1-3 correspond to GW 23-32 in humans, representing a developmental stage when humans are still securely enclosed in the uterus[109]. One of the main justifications for the use of rodent models is the similarities to humans in oligodendrocyte maturation[109,110]. Additionally, one

important discrepancy is that term animals, including rodents, have a mature systemic physiology compared to the preterm infant[109]. If we exclude the stress induced by preterm birth in the animal model, several crucial aspects of prematurity may be overlooked including, a deficit of trophic factors like IGF-1, respiratory instability, coagulation system deficiencies, and spontaneous hemorrhage in the immature brain[21,38,111-114].

No animal model perfectly resembles the human preterm condition, but the preterm rabbit pup model provides one of the more relevant small animal models available, particularly for IVH research[115-118]. The preterm rabbit pups are delivered on postconceptional day 29 (term 31–32 days) via cesarean section and are at the time of birth, displaying systemic physiology similarities to that of human preterm infants. Similarities includes tendency to develop necrotizing enterocolitis similar disease[119], renal immaturity disease, rudimentary alveolar structure[120] and deficiency of trophic factors such as IGF-1[121]. Importantly, the brain development of the preterm rabbit pup unlike to rodents develops throughout the perinatal period with a temporal white matter development corresponding to that of the human infant[111,118] as well as the brain growth velocity spurt immediately prenatally which continues during the first month of life[122]. Furthermore, an important aspect for the current thesis is that the morphology of the fetal rabbit ChP closely resembles that of the fetal human ChP[123]. Moreover, the rabbit has a more complex brain structure with a higher proportion of white matter compared to rodents and thus the rabbit can contribute to bridge the translational gap between humans and rodents[124].

Regarding animal models of IVH, both rodent and piglet IVH are typically induced by intracerebroventricularly (i.c.v.) injection of centrifuged blood with elevated hematocrit concentration[125-128] (age-matched whole blood has also been utilized[129]) or by intracerebral injection of collagenase[130,131]. In the preterm rabbit pup model, IVH is induced by a glycerol-induced vessel rupture in the GM or ChP[21,115,116]. Systemic dehydration, elevated serum osmolarity, and intracranial hypertension are induced via an intraperitoneal (i.p.) injection of glycerol. This elevated pressure on vessel walls causes ruptures in vessels within the GM[22,118,132]. Our studies have also shown prominent bleeding in the immature ChP[115-117,133]. The mechanism of IVH in preterm rabbit pups more closely mimics the hemorrhage observed in preterm infants.

Taken together, the preterm rabbit brain development at postconceptional day 29 arguably corresponds to brain development in humans at GW 24–28.

High-frequency ultrasound

Diagnostic of IVH and posthemorrhagic ventricular dilatation (PHVD), or IVH grade in the preterm rabbit pup is conducted in our lab by using high-frequency ultrasound (HFU) with a frequency of 40Mhz, see figure 9. Since the preterm rabbit pup has not yet developed fused scull bones, the fontanel provides an acoustic window[133]. One major advantage of this diagnostics is that it is performed without the need for sedation As a result, normal CSF flow can be continuously monitored[133]. The pups rest calmy in a prewarmed hand and the probe is gently directed to the fontanel with the other.

Intracerebroventricular injection

Another valuable application of HFU is the possibility of performing non-sedated i.c.v. injections. As seen in figure 9, IVH creates a large window in lateral ventricles for the needle injection. Also, although the window is small in control or non-IVH animals, reproducible i.c.v. injections have been feasible, as presented in study III. Injections are made on non-sedated animals that rest calmy on a heating pad while the ultrasound probe display the lateral ventricles. Injections up to 25µl directly posterior to the eye are subsequently performed. The procedure is illustrated in figure 10.

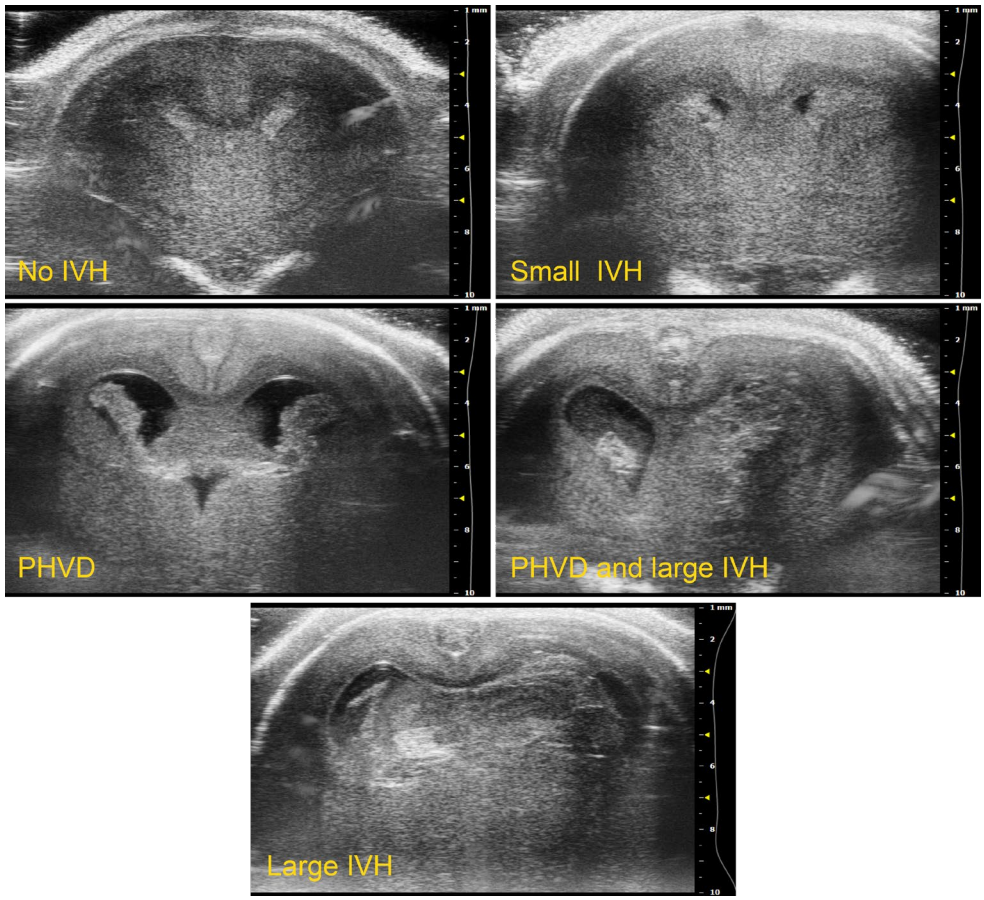


Figure 9. Diagnostic of IVH using high-frequency ultrasound.
Classification of IVH and PHVD in preterm rabbit pups using HFU: Imaging and assessment. Normal size of the lateral ventricles is displayed in upper left panel. Note the increased size of the lateral ventricles during a small IVH in the upper right panel. In PVHD, the ventricles are dilated and the ChP is enlarged and swollen as displayed in the middle-left panel. Middle-right and lower panels display large IVH. Here, the ventricles are dilated and filled with blood and blood cloths. Photographs by Niklas Ortenlöf.

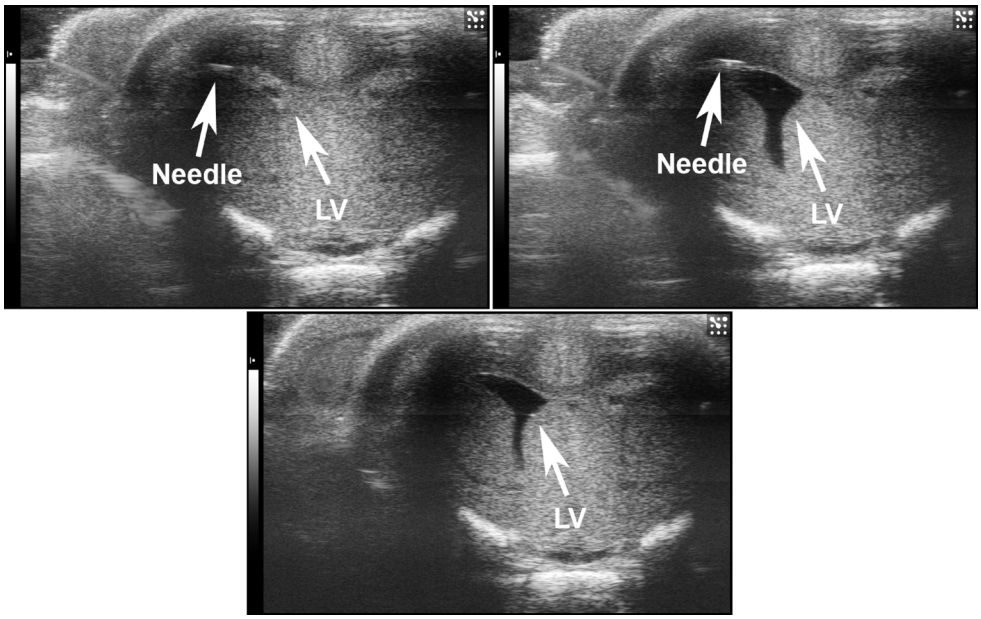


Figure 10. Ultrasound guided intracerebroventricular injection.
Ultrasound guided i.c.v. injection into the lateral ventricles of non-sedated preterm rabbit pups. Presence of injected needle in the lateral ventricle (upper left panel). Injection of fluid into the lateral ventricle (upper right panel). Needle withdrawal (bottom panel). LV, lateral ventricle. Adapted from Ortenlöf et al (unpublished results).

Extraction of choroid plexus

The main brain tissue investigated in this thesis is the ChP, and during my thesis work we have developed an extraction protocol from both preterm rabbit pups and neonatal mice. The procedure of extracting ChP from preterm rabbit pups is as follows. After decapitation, remove the skin. To remove the scull, use a small fine scissor to cut ventrally around the brain on both sides, from posterior to anterior, until reaching the eyes. Continue by cutting dorsally, from posterior to anterior, reaching the olfactory bulb. Next, cut laterally over the fontanel until reaching halfway on both hemispheres. Now by using fine forceps, it is possibly to gently open the skull, revealing the brain. Flip the skull upside down and scoop out the brain from the skull. Extracting the ChP from the lateral ventricles is straightforward with a lateral cut just dorsal to the ventricles. A light microscope is needed for visualizing the ChP. Grab the visible reddish ChP (in non-perfused animals, otherwise it is white/grayish) with one forceps while holding the anterior brain tissue up with another set of forceps. In study II, where the IGF-1R activation in the ChP

was investigated, ChP was extracted and frozen in liquid nitrogen as quickly as possible due to the phosphorylation of downstream proteins[134].

The extraction of the ChP from neonatal mice follows a similar process. However, due to the smaller size of the mouse brain, some additional steps are necessary. The mouse brain needs to be gently fixed in place using tiny needles on a gel matrix in a petri dish filled with cold Hanks' Balanced Salt Solution media and the brain should be kept on ice. Also, as mentioned above, a light microscope in needed for visualization the ChP. To increase the yield, the ChP in the fourth ventricle is also collected to from the neonatal mice. Use the small needles, carefully scrape through the cerebellum from dorsal to ventral side where the large and more developed ChP is located. Once the ChP is exposed, gently grab it with the forceps for extraction.

In vitro culture of choroid plexus epithelial cells

When the research focus is on obtaining precise mechanistic insights, in vitro cell culture is often a very useful option, and using rodent (in our studies, murine) cells for primary cell cultures has distinct advantages. This includes a more straightforward breeding of mice as compared to rabbits, as well as available antibodies for the specific target of interest. Moreover, for mass spectrometry (MS) proteomic analysis, rodents offer advantages because larger peptide databases are available. This allows for more extensive proteomic studies using rodents.

To simulate the blood-CSF barrier constituted by the ChP, we utilized transwell cultures[102]. In this setup, extracted murine ChP tissue was digested by pronase to dissolve the epithelial cells[135]. The cells were then cultured on porous membranes in transwell inserts suspended in a plastic compartment, see figure 11. This setup enables studies where compounds (such as IGF-1) can be added to the basolateral compartment (corresponding to the blood) and the apical compartment (corresponding to the CSF) can easily be collected. To verify that the epithelial cells create a tight barrier preventing compounds from the basolateral compartment from entering the apical compartment, we used immunofluorescence labeling and analyzed with confocal microscopy (as described below) to detect the presence of transthyretin (TTR, a ChP marker) and zonula occludiens-1 (ZO1, a tight junction protein), see figure 12. We also conducted transepithelial electric resistance (TEER) measurements to ensure a tight barrier. A probe inserted into the transwell system, providing an inverse presentation of the electrical conductance through a cellular layer. A high TEER value of the cellular layer points to an intact cellular monolayer

and therefore suggests restricted permeability of molecules and ions[136] (i.e., low conductance). Furthermore, as the establishment of a tight barrier was crucial for all the results presented in study III, we also performed a diffusion assay. In this assay, fluorescent dextran was added to the basolateral compartment, and the fluorescent signal was evaluated in the apical media.

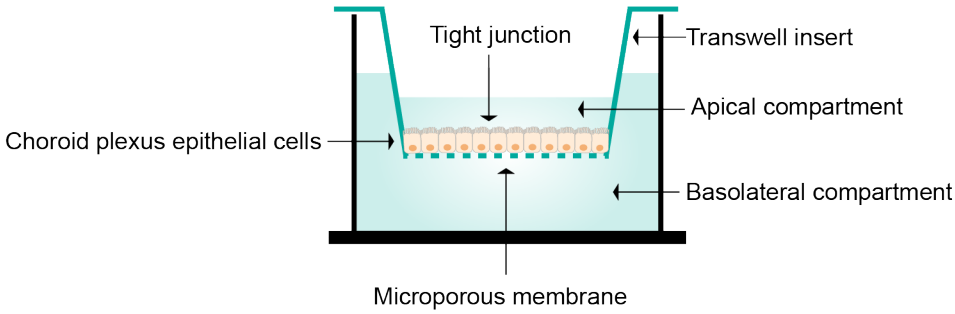


Figure 11. The transwell system.
Schematic illustration of the transwell system used in study III. The system simulates the in vivo situation by applying the basolateral compartment as the "blood" side and the apical compartment as the "CSF" side. Once the ChP epithelial cells, cultured on a microporous membrane, have matured and developed tight junctions, the experiment begins by adding compounds to the basal compartment. At the end of the experiment, the apical supernatant is collected. The artwork was created by Niklas Ortenlöf using Adobe Illustrator.

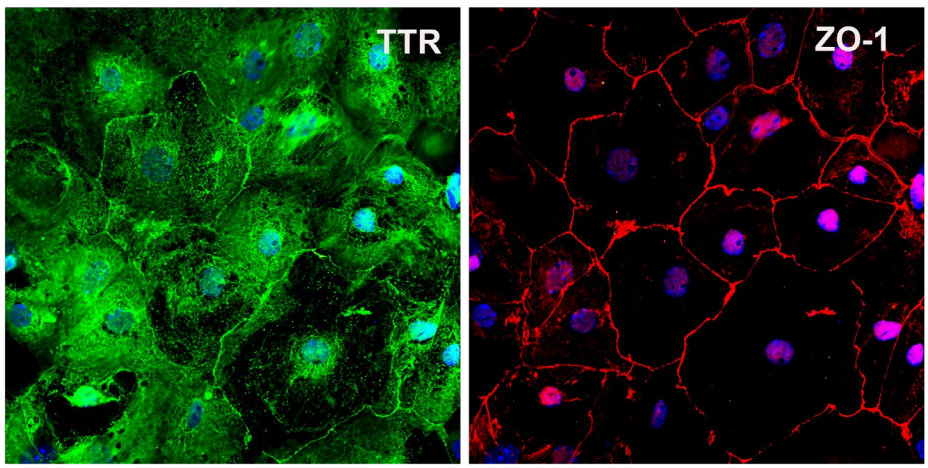


Figure 12. Verification of an intact ChP epithelial barrier by confocal microscopy analysis.
Representative confocal microscopy images of the murine ChP epithelial cells cultured in a transwell system. The presence of TTR (green, left panel) and ZO-1 (red, right panel) is illustrated. Modified from Ortenlöf et al (unpublished results).

Imaging techniques

Throughout all the studies I-IV, imaging techniques have been utilized.

Immunofluorescence microscopy

As the name suggests, immunofluorescence (IF) microscopy uses the fluorescence created from light emitted from a fluorophore conjugated to an antibody. Basically, there are two methods used and both are used in the thesis namely wide-field and confocal microscopy. Wide-field fluorescence microscopy employs epi-fluorescence illumination to illuminate the entire field of view, encompassing even the out-of-focus planes. All emitted light is routed back through the objective lens via dichroic mirrors and filters, allowing the detection of distinct fluorophores, typically emitted as blue, green, or red light[137] (see an example in figure 12 and a schematic describing the method in figure 13). The other method, confocal fluorescence microscopy uses pinholes to restrict excitation light, exclusively illuminating close to the focal point. A corresponding conjugate pinhole further confines emitted light to the same focal plane. This effectively eliminates out-of-focus fluorescence, resulting in enhanced depth resolution compared to a wide-field microscopy setup[137]. Confocal microscopy has been most utilized in the current thesis, as this can visualize the presence of proteins in cell organelles with high resolution.

To create the optical signal, the fluorophore is carried by a secondary antibody, which detects the primary antibody already bound to the antigen of a protein of interest. To ensure the presence of a protein in a sample, controls experiments are conducted, including incubation with only the secondary and primary antibodies and blank to collect the background fluorescence.

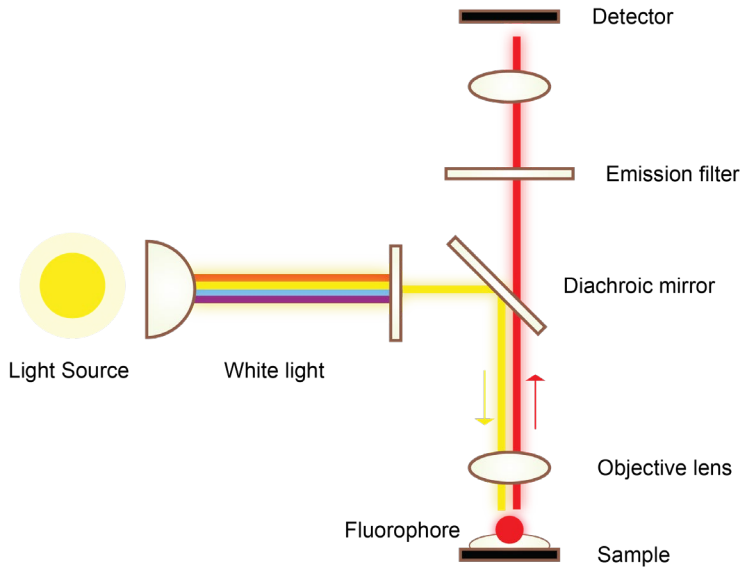


Figure 13. The principle of fluorescence microscopy.
A powerful light source emits light that is channeled through a condenser. This light then passes through a filter cube, selectively allowing specific wavelengths, like green (like in the current example), to pass through. A dichroic mirror behaves like a filter, reflecting light at particular angles while permitting light of different wavelengths to pass through. The directed light is aimed at the sample through the objective lens. Within the sample, a fluorophore, red in this case, such as Alexa 594 is stimulated by the green light and, in response, emits red light. The emitted red light retraces its path, passing back through the objective lens and the dichroic mirror. Here, any light at wavelengths other than red is filtered out using an emission filter. Finally, the red light reaches the detector, allowing for the observation and capture of the fluorescent signals. The artwork was created by Niklas Ortenlöf using Adobe Illustrator with inspiration from the thesis by Pontus Nordenfelt[138].

Electron microscopy

With the use of the beam of electrons rather than visible light, electron microscopy can visualize at the nanoscale level fine details is a sample with incredible resolution not achievable via the use of general optical microscopes. The fundamental principle is the interaction of generated electrons with the electrons in the sample and the detection of electrical signals to create images. In analogue with optical lenses described above, electromagnetic lenses are used to focus and control the electron beam to form the final image. There are two main types of electron microscopy namely transmission electron microscopy (TEM), where the electrons are transmitted through the sample and scanning electron microscopy, where electrons are scattered over the sample surface, creating a 3D images[139]. In the studies of my thesis, TEM has been used in studies II and III to analyze the translocation of IGF-1 through the ChP and the IGF-1 presence in intracellular and extracellular vesicles.

This was made possible by immunogold labelling secondary antibodies were conjugated with a nanosized gold particle resulting in black dots revealing the presence of a protein in the sample. An overview TEM image can be seen figure 6 in chapter 2.

Light-sheet microscopy

With this technology, a thin plane of laser light is used to selectively illuminate a single optical section (slice) of the sample. This illumination occurs perpendicular to the objective lens, generating a fluorescent signal from the sample labeled with a fluorophore, which reduces photodamage to regions above and below the focal plane. By rotating the sample during analysis, a 3D image is produced along with 2D images of slices of the (in the case of the current thesis) the brain. The sample must the cleared (making it transparent) prior to the analysis to ensure that the light sheet can pass through the sample (brain) with minimal scattering and distortion[140].

Protein analysis and extracellular vesicle preparation

Mass spectroscopy

In study II and III, discovery bottom-up proteomics was utilized using MS. The process begins by extracting proteins from the sample (ChP epithelial cells in study II and ChP and piglet CSF derived EVs in study III), followed by enzymatic digestion to convert them into peptides. These peptides are separated using liquid chromatography and then introduced into the MS instrument, where they undergo ionization and separation based on their mass-to-charge ratio (m/z). In the MS instrument, the most abundant precursor ions are selected for fragmentation and analyzed in a secondary MS run. The resulting fragmentation ions provide the basis for peptide sequencing and subsequent in-silico protein prediction and analysis. Quantification can be achieved through either label-free or labeled methods[141]. For protein identification and quantification, specialized software tools such as Proteome Discoverer were employed, along with custom R scripts (coding language).

Extracellular vesicle preparation

In study III, the investigation focused on the transport of IGF-1 in EVs from the ChP. One of the challenges in EV research is the preparation of EVs from different

biological fluids. The most commonly employed method, which is considered to be the "gold standard", is sequential ultracentrifugation[142]. In this process, the fluid is subjected to very high speeds (equivalent to 100,000 times the force of gravity) for several hours and repeatedly, to sediment the EVs. While ultracentrifugation can be a suitable option for less complex fluids like CSF and cell media, it is a time-consuming process that requires substantial sample volumes and expensive equipment. Also, this centrifugation process may co-centrifuge protein aggregates and lipoproteins from plasma samples[143]. An alternative to ultracentrifugation is acoustophoresis, a microfluidics method that utilizes acoustic forces to cluster EVs in a channel. This technology minimizes protein aggregation and lipoprotein co-sedimentation because it does not require high-speed centrifugation. In addition, a minimal sample volume is required, as little as 30 µl[144-147]. In study III, I have used precipitation techniques with commercial reagents. The exact components of the precipitation media are not disclosed, but polyethylene glycol is commonly used because it reduces the solubility of EVs, causing them to aggregate[148]. Once all the EVs have aggregated, the sample is centrifuged at 10,000 times the force of gravity to sediment the EVs, creating a pellet. Using these commercial kits has several advantages, including low sample volume requirements, minimal laboratory steps, and compatibility with a regular centrifuge[149]. However, it is important to note that the precipitation media may aggregate more particles than just EVs, including protein aggregates and lipoproteins, making this method unsuitable for plasma samples[150]. In study III, EVs have been prepared from less complex fluids like CSF and cell media, for which precipitation methods have shown to be suitable, as demonstrated by previous research[149,151,152] and the results presented in study III in chapter 5 below.

Chapter 5: Destiny of a growth factor

"Stay hungry. Stay foolish"
Steve Jobs (1955 - 2011)
Co-founder of Apple Inc

My years as a PhD-student have been exiting, interesting and dramatic. The outcomes of my thesis will be disclosed in this chapter. I truly encourage readers to read the original papers at the end of thesis to receive a full presentation of all the results. Key results are only presented and discussed here.

Study I

This story originated as a first step to gain a mechanistic insight of the results derived from a phase 2 randomized study which displayed a trend towards decreased IVH occurrence upon IGF-1 treatment in extremely preterm infants[41]. More specifically, the aim was to characterize the impact of cerebrovascular maturation and IGF-1 receptor density in the brain of the preterm rabbit pup following administration of IGF-1. We utilized our preterm rabbit pup model, conducted subcutaneous (s.c.) injections of IGF-1 at certain timepoints (starting at 3 hours of postnatal age and repeated every 12 hours until approximately 72 hours). The pups were terminated at timepoints ranging from 0 to 120 hours of age. Vaginally delivered term pups were also included in the experimental setup as comparison of normal development. The IGF-1 used in this study was conjugated with the IGF binding protein 3 (IGFBP-3) to increase the half-life of IGF-1 in the circulation[153], and was administered at a concentration of 8 mg/kg IGF-1/IGFBP-3 (used in study I, II and IV).

IGF-1R in the preterm brain

To ensure that IGF-1 can interact with its receptor, the IGF-1R, we investigated the IGF-1R density in the brain at different time points using immunohistochemistry (IHC) with subsequent heat-mapping of the signal. IGF-1R immunolabeling exhibited a widespread distribution throughout the brain at 4 hours postnatal age. Notably, areas of the brain with the highest IGF-1R densities included the ChP, subfornical organ, meninges, periventricular regions, and major fiber tracts, see figure 14A. This widespread presence of IGF-1R decreased with postnatal age was abolished at 96 hours postnatal age, except for the ChP and the meninges (figure 14B). The IGF-1R density in term animals showed the same time-dependency as the preterm pups (data not shown). IF analysis further revealed the abundance of IGF-1R in the ChP, particularly at the apical site of the epithelium (figure 14C).

The chance for IGF-1 to interact with IGF-1R is certainly time-dependent and these findings align closely with those reported in rodents, indicating a persistent expression of IGF-1R in the ChP and meninges in adult rats[154,155]. This contrasts with the widespread distribution observed during the embryonic period[154], implying that the shift to postnatal life has a more pronounced impact on cerebral IGF-1R distribution than maturation itself.

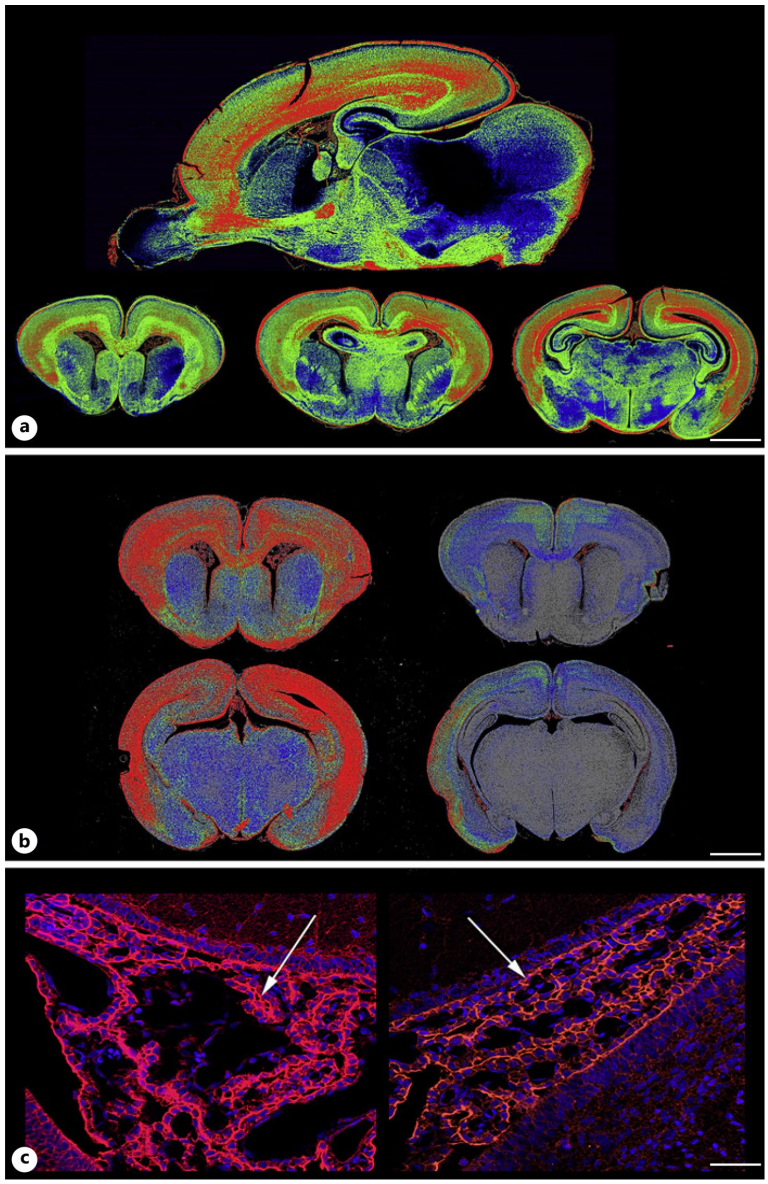


Figure 14. IGF-1R immunoreactivity in the preterm rabbit brain.
A Color-coded images display the varying densities of IGF1R immunoreactivity in whole brain representative sections from preterm rabbit pups. The IGF1R density is color-coded to represent high (red), medium (green), and low (blue) intensities. Scale bar represents 2 mm for the upper sagittal section and 4 mm for the lower coronal sections. **B** The images display representative coronal sections of the preterm rabbit brain at 0 hours of postnatal age (left panel) and after 96 hours (right panel). Scale bar represents 3 mm. **C** Confocal microscope images providing a detailed view of IGF1R localization within the ChP of a preterm rabbit pup with the postnatal age of 0 hours. Notably, there is prominent labeling of the epithelial cells, as indicated by arrows. Cell nuclei were counterstained with DAPI (blue). Scale bar represents 50 µm. DAPI, 4′,6-diamidino-2- phenylindole. Adapted from Gram et al[156].

Upregulation of IGF-1 and genes involved in angiogenesis and extracellular matrix in the ChP following exposure to IGF-1

As described previously, the ChP along with the GM, is the main origin of hemorrhage in the preterm rabbit pups. In study 1, ChP was therefore used for further IF analysis and gene expression analysis of genes involved in angiogenesis and extracellular matrix following IGF-1 administration in preterm rabbit pups. Interestingly, relatively higher intensities of IGF-1, along with IGF-1R immunofluorescence, were detected in animals exposed to IGF-1 at 4 hours postnatal age. In addition, co-localization of IGF-1 and IGF-1R was also detected in the ChP (figure 15A and B). Furthermore, gene expression analysis revealed an upregulation of genes involved in angiogenesis, including IGF-1 and angiopoietin-1, in 24-hour-old pups exposed to IGF-1. Upregulation of collagen type I alpha 1, important for the extracellular matrix establishment was also observed (figure 15C).

The conclusion of study I is that IGF-1R density is correlated with upregulated gene expression of angiogenesis and endogenous production of IGF-1 in the ChP following IGF-1 administration.

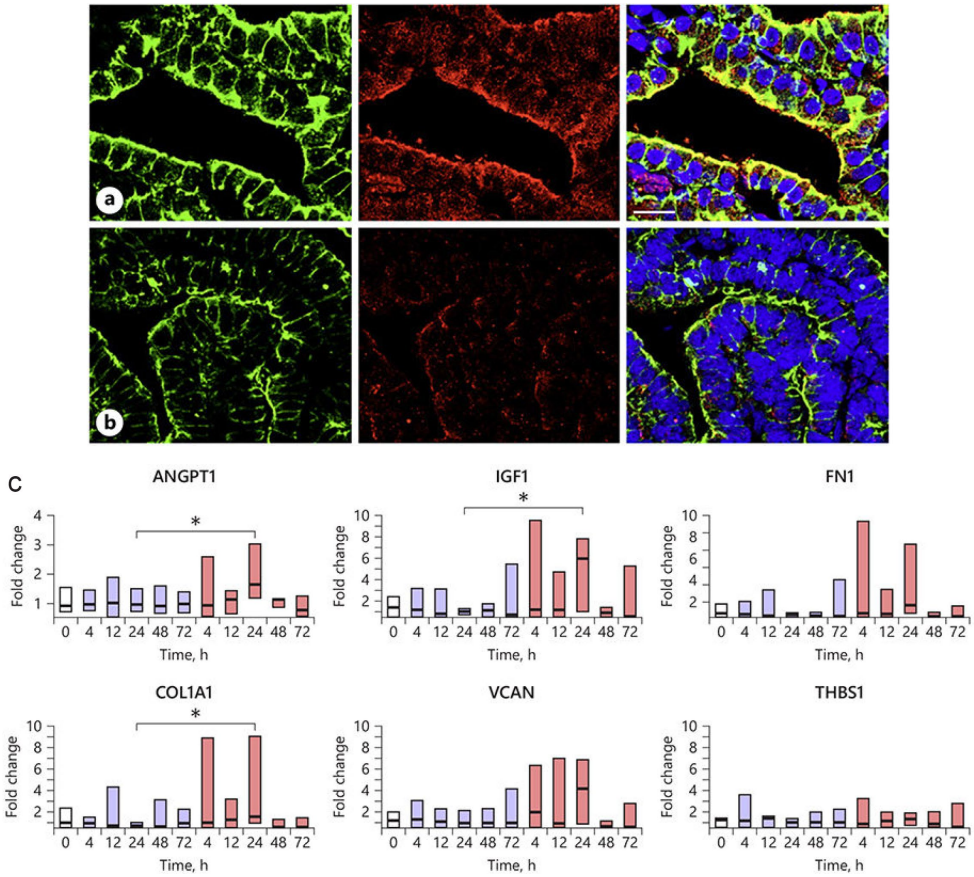


Figure 15. IGF-1 and genes involved angiogenesis and extracellular matrix analysis in the ChP. **A**-**B** Representative confocal images of IGF-1 (red) and IGF-1R (green) in the ChP of preterm rabbit pups at 4 hours post s.c. administration of IGF-1 (**A**) and vehicle-treated animals (**B**). Co-labeling of IGF-1 and IGF-1R is displayed in the right panel in yellow. Cell nuclei were counterstained with DAPI (blue). Scale bar represents 20 µm. **C** Preterm rabbit pups received either 8 IGF-1 (indicated by red bars) or vehicle (indicated by blue bars) starting at 3 hours of postnatal age, with subsequent doses administered every 12 hours until they reached 75 hours. ChP samples were collected at intervals of 0, 4, 12, 24, 48, and 72 hours after the initial dosing. The ChP samples (n = 3-8) were subjected to qPCR analysis. Fold changes were determined by first normalizing to GAPDH and then against the values for vehicle-treated animals at 0 hours (depicted as white bars). Discrepancies between IGF-1 and vehicle-treated animals were assessed using a one-way ANOVA with post hoc Sidak. *$p < 0.05$. DAPI, 4′,6-diamidino-2- phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANGPT1, angiopoietin-1; FN1, fibronectin-1; VCAN, versican; THBS1, thrombospondin-1; COL1A1, collagen type I alpha 1. Modified from Gram et al[156].

Study II

Originally, this study was aimed at investigating the IGF-1R activation, primarily in the ChP, upon IGF-1 administration in preterm rabbit pups. Over time, the research aim expanded to encompass an investigation of IGF-1 uptake in the CSF and the immature brain from the circulatory system. The experimental setup was similar to study I, with the exceptions that IGF-1 was administered already within the first hour of postnatal life, and that the injected IGF-1 was conjugated with either FITC, biotin or Alexa Flour (AF) 647 to enable a distinction between exogenous and endogenous IGF-1.

Prescence of systemic administrated IGF-1 in the preterm brain

We started to investigate the presence of AF647-labeled IGF-1in the preterm rabbit brain using light-sheet microscopy. The pups were s.c. injected with AF647-labeled IGF-1 at 24 hours postnatal age, and terminated 5 hours post-injection. We observed that IGF-1 was mainly located within the ChP of the lateral and third ventricles, the perivascular, and the subarachnoid space (see a dorsal view in figure 16A, depicted in white). It is worth mentioning that there was a comparatively lower presence of IGF-1 in the fourth ventricle. We further used confocal microscopy IF imaging, and discovered an accumulation of IGF-1 at the endothelial lining of the ChP (figure 16B). Using immunogold-labeled antibodies targeting FITC-conjugated IGF-1 and TEM analysis, we observed a translocation of IGF-1 across the ChP (figure 16C, white and black arrows).

The notion of IGF-1 transport through the ChP has been described before[36], however to the best of our knowledge this is the first description in the preterm rabbit brain. The lower presence of IGF-1 in the fourth ChP is interesting and could be due to the faster development of fourth ChP in comparison to the lateral and third ventricle. The decline in IGF-1R density during postnatal development could potentially extend to the fourth ChP. Neither study 1 described above nor studies in rats have specifically investigated the fourth ChP[155].

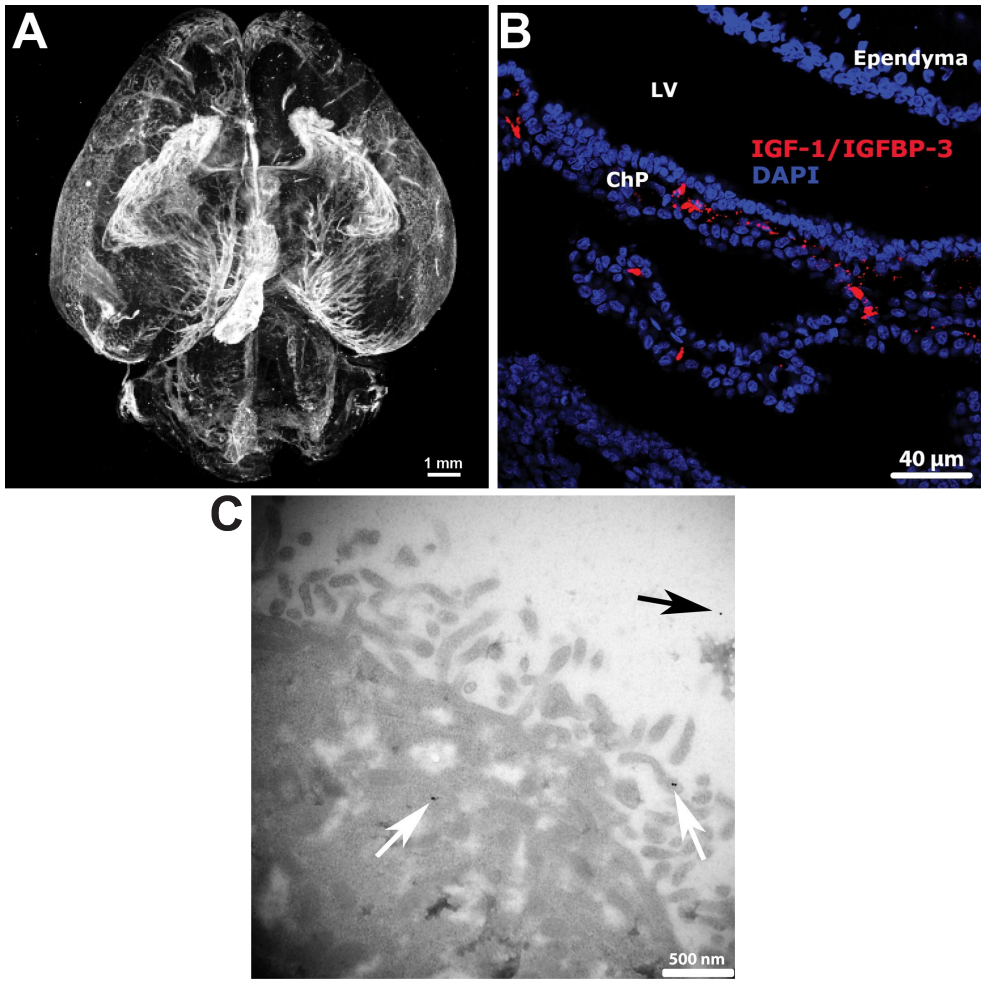


Figure 16. Presence of IGF-1 in the developing brain of the preterm rabbit pup.
A Dorsal view of a representative three-dimensional light-sheet microscopy image displaying the presence of AF647-labeled IGF-1 (depicted in white) in the ChP, perivascular space, and subarachnoid space. **B** Representative confocal microscopy image displaying biotin-labeled IGF-1within the ChP of the lateral ventricle, visualized using streptavidin-coupled AF647. Cell nuclei were counterstained with DAPI. Confocal images were collected from 3 independent experiments. Representative TEM images showcasing IGF-1 labeled with FITC (later visualized with anti-FITC gold conjugate) at the apical border of the ChP in the lateral ventricles. Gold-labeled IGF-1 is indicated by white and black arrows. TEM images were collected from 2 independent experiments. LV, lateral ventricle; DAPI, 4′,6-diamidino-2- phenylindole. Modified from Ortenlöf et al[157].

Time-dependent serum and CSF uptake of blood-borne IGF-1 in the preterm rabbit pup

We subsequently aimed to investigate if the uptake of IGF-1 to blood and CSF, following s.c. administration is dependent on postnatal age. Pups injected with IGF-1 at a postnatal age of less than 1-hour displayed higher IGF-1 levels both in serum and CSF 5 hours post-injection as compared to pups injected at a postnatal age of 24 or 72 hours (figure 17A-B). The ratio of IGF-1 concentration between CSF and serum remained consistent across postnatal age, regardless of exposure. However, using a linear regression analysis to explore the relationship between IGF-1 concentration in serum and CSF, we observed a positive correlation in pups receiving IGF-1 prior to 1 hour of life, see left panel of figure 17D.

Given that the translocation of IGF-1 from the circulatory compartment to the CSF appears to be influenced by the postnatal age of the preterm rabbit pup, we aimed further to explore whether IGF-1R activation exhibited a comparable pattern. Extracted ChP tissue was subjected to western blot analysis to assess the phosphorylation of the IGF-1R signaling pathway proteins, including ERK (pERK1/2) and PKB/Akt (pPKB), see figure 2 in chapter 1 for details. It was observed that both pERK1/2 and pPKB phosphorylation was increased at 5 hours postnatal age following administration of IGF-1, as compared to the vehicle-injected pups (figure 17E-F, upper panel). Interestingly, no difference in phosphorylation was observed at later time-points (figure 17E-F, middle and lower panel). Of note, only the ChP in the lateral ventricles were analyzed.

To conclude study II, the results presented here highlights the potential importance of early administration of IGF-1 with regards to uptake of IGF-1 in the serum and CSF, and activation of the IGF-1R in the ChP.

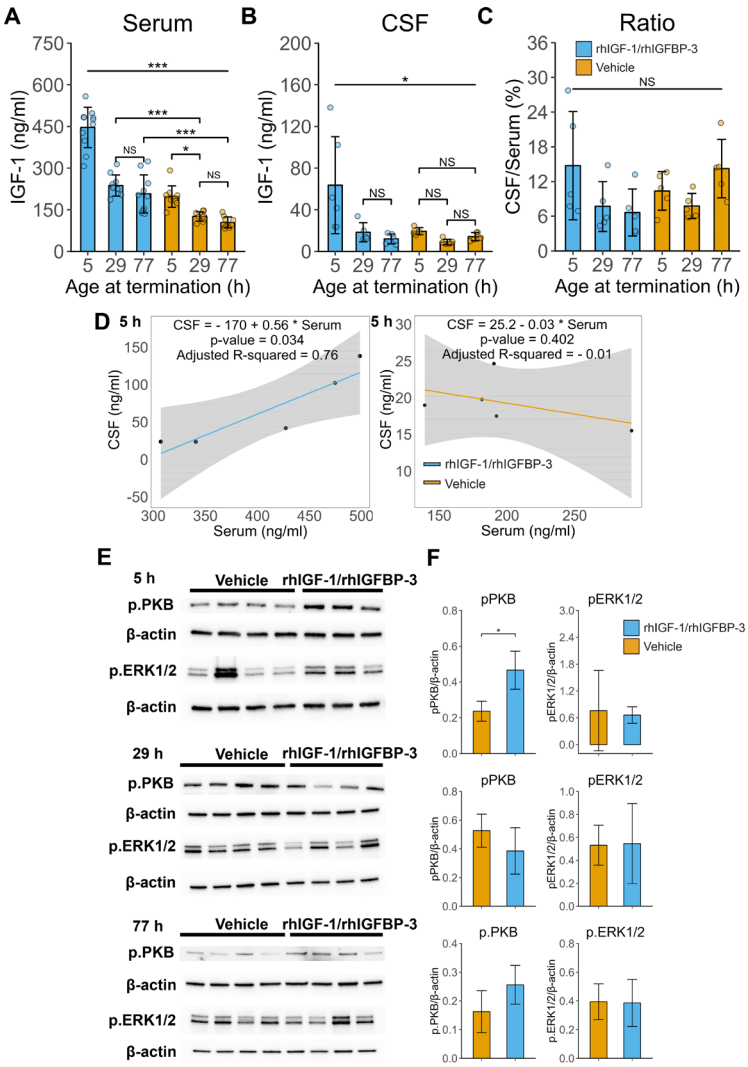


Figure 17. The influence of postnatal age on the IGF-1 levels in serum and CSF and the activation of IGF-1R in the ChP of the developing brain.

A-D. In (**A**) and (**B**), the levels of IGF-1 in serum and CSF of preterm rabbit pups were assessed at 5 hours following s.c. injections of IGF-1 or vehicle at 0 (5), 24 (29), or 72 (77) hours postnatal age (with corresponding time-points for termination in parentheses). The ratio of CSF IGF-1 to serum IGF-1 was calculated in (**C**). A linear model illustrating the relationship between IGF-1 levels in serum and CSF at 0 (5) hours postnatal age is presented in (**D**), with the dark grey area representing the 95% confidence level. The data is presented as means ± SD (N = 6–9), and differences in IGF-1 concentrations were assessed using one-way ANOVA with post hoc Tukey test for multiple comparisons of means, with *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 denoting significance. **E-F**. Western blot analysis of pERK1/2 and pPKB in the ChP from preterm rabbit pups at 5 hours after the s.c. administration of IGF-1 at 0 (5, upper), 24 (29, middle), or 72 hours (77, lower) postnatal age (with corresponding time-points for termination within parentheses). The data, derived from three different experiments, is presented as means ± SD (N = 3–4), and a representative blot is shown. Differences between groups were analyzed using Student's t-test, with *P ≤ 0.05, and NS indicating no significance. Modified from Ortenlöf et al[157].

Study III

In this study, my curiosity inspired me to investigate how IGF-1 is transported through the ChP and further in the CSF. IGF-1 is mainly bound to IGFBP-3 in the circulation and this complex must be cleaved prior to the IGF-1 interaction with the IGF-1R[25]. Micronutrients and inflammatory miRNAs are transported in ChP derived EVs to the CSF[101,102], thus it can be speculated that EV mediated transport also include growth factors, such as IGF-1. To investigate this, we established an in vitro cell culture system using a transwell setup with primary murine ChP epithelial cells from postnatal day 3-8, see figure 11 in chapter 4 for details. Human IGF-1 was added to the basolateral compartment and EVs purified from the apical supernatant along with the ChP epithelial cells were analyzed using IF, TEM, MS and nanotracking analysis 24 hours after exposure, see schematic illustration in figure 18A.

Localization of IGF-1 within intracellular vesicle in ChP epithelial cells

We observed the presence of IGF-1 within the cultured ChP epithelial cells using confocal light microscopy. Additionally, immunolabeling against IGF-1R exhibited positive signals within the ChP epithelial cells. Notably, co-labeling with the late endosomal marker CD63, which is characteristic of multivesicular bodies, displayed co-localization with IGF-1, IGF-1R, and TTR, indicating an intracellular vesicle location of IGF-1, its receptor, and TTR, see figure 18B. Furthermore, TEM immunogold labeling of the exosomal marker flotillin-2 revealed the presence of intracellular membrane-enclosed vesicles (figure 18C, shown by white arrows), indicating the production of EVs[158]. Additionally, immunogold labeling against IGF-1 displayed an accumulation within intracellular membrane-enclosed vesicles and in membrane budding in the ChP epithelial cells, suggesting the potential release of IGF-1-positive vesicles into the extracellular space (figure 18D-E, shown by white arrows).

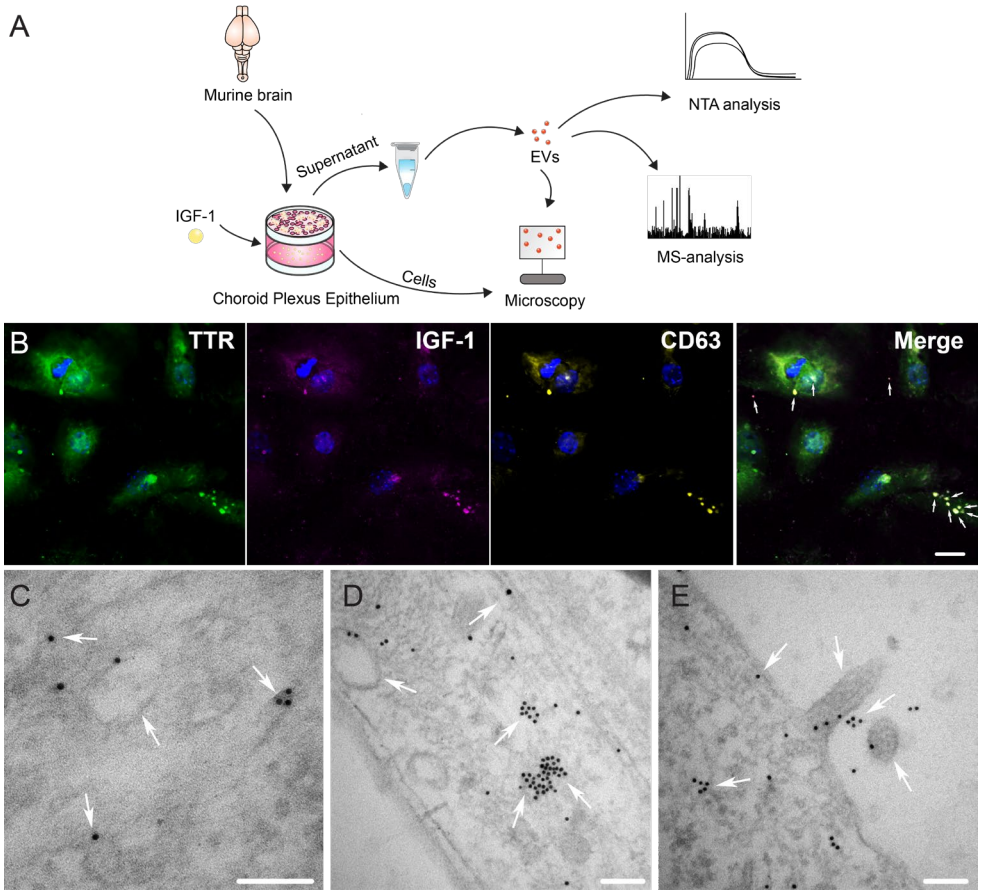


Figure 18. Localization of IGF-1 in neonatal primary murine ChP epithelial cells
A Schematic illustration of the in vitro study. **B** Representative confocal microscopy images displaying the labeling of TTR, (green, **B**, far left and far right), IGF-1 (magenta, **B**, middle-left and far right), and CD63 (yellow, **B** middle-right and far right) in murine ChP epithelial cells cultured a the transwell system. The co-localization of IGF-1 with the late endosomal marker CD63 is indicated by white arrows in the merged image (**B**, far right). Scale bar represents 20 µm. **C**-**E** Representative TEM images displaying immunogold labeling of flotillin-2 (**C**) and IGF-1 (**D**-**E**) in murine ChP epithelial cells cultured in a transwell system. White arrows highlight immunogold labeling in membrane-enclosed vesicles. Scale bars in **C**-**E** represent 100 nm. The confocal microscopy and TEM images were obtained from three independent experiments. Modified from Ortenlöf et al (unpublished results).

IGF-1 transport in EVs from ChP epithelial cells

To assess whether IGF-1 is transported from ChP epithelial cells within intracellular vesicles to the extracellular space, ChP epithelial cells were exposed to varying concentrations of IGF-1 (40, 100, and 250 ng/ml) on the basal compartment for 24 hours and the apical supernatant were subsequently collected for EV purification. The successful preparation of EVs was confirmed by TEM analysis, which demonstrated the presence of exosomes, and positive labeling for the exosomal marker flotillin-2 in the ChP epithelial cell-secreted supernatant (figure 19A). TEM analysis of IGF-1, using immunogold labeling, revealed the presence of IGF-1 on, or within the purified vesicles, including both larger and smaller vesicles (figure 19B). Worth noting is that it was not feasible to distinguish between administered (human) IGF-1 and endogenous (murine) IGF-1 since the antibody used detects both epitopes. Subsequently, we quantified the number of EVs released into the apical supernatant following exposure of the ChP epithelial cells to varying concentrations of IGF-1 (40-250 ng/ml). We observed that exposure to 40 ng/ml IGF-1, but not 100 or 250 ng/ml, resulted in a significant increase in ChP epithelial cell-released EVs compared to control cells (figure 19C-D). Consequently, 40 ng/ml IGF-1 was selected for subsequent experiments.

We further investigated whether the proteome of EVs isolated from the ChP epithelial cell supernatant, as determined through MS analysis, would exhibit any alterations following exposure to IGF-1. Upon analysis, IGF-1 appeared to induce only marginal effects, considering significance based on an adjusted p-value (Benjamini–Hochberg-corrected p-value ≤ 0.05) as the criteria. Interestingly, exogenous (human) IGF-1, along with Transmembrane P24 Trafficking Protein 2 (Tmed2), which plays a vital role in in utero embryonic development[159], showed significant enrichment (figure 19E, shown by yellow dots).

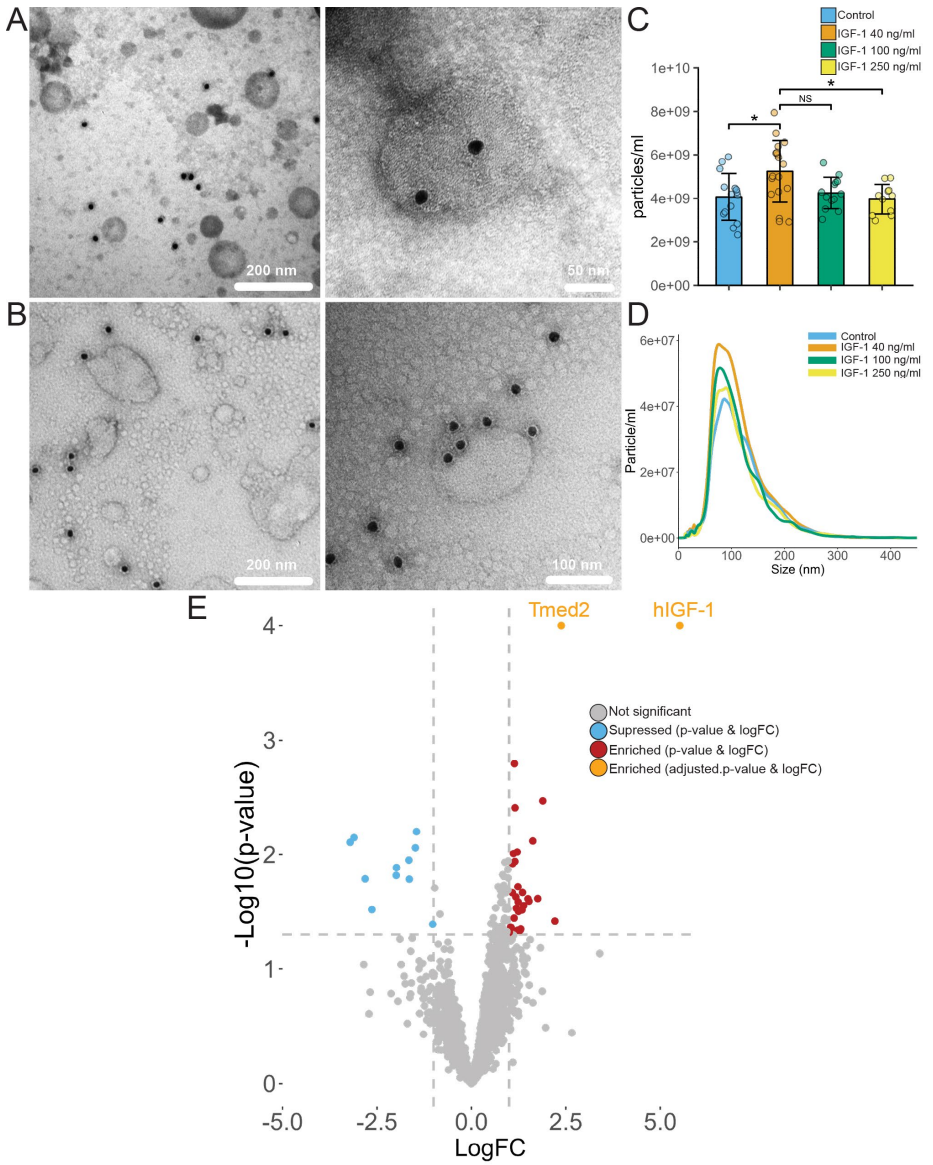


Figure 19. IGF-1 positive vesicles are released from ChP epithelial cells following hIGF-1 stimulation.

A-**B** Representative TEM images of murine ChP epithelial cell derived EVs immunogold labeled with flotillin-2 (**A**) and IGF-1 (**B**). **C**-**D** NTA quantification (**C**), and size distribution (**D**) of ChP epithelial cell derived EVs following exposure to hIGF-1 (40, 100 and 250 ng/ml) for 24 hours. Data are from four independent experiments (N=4-6). Data are presented as means ± SD. Differences between 40, 100 and 250 ng/ml hIGF-1 vs. Control was analyzed using one-way ANOVA with post hoc Tukey test for multiple comparisons of means, *P≤0.01. **E** Volcano plot depicting the proteome of EVs derived from ChP epithelial cells exposed to IGF-1 (40 ng/ml) in comparison to control cells. hIGF-1, human insulin-like growth factor 1. Modified from Ortenlöf et al (unpublished results).

Hippocampal uptake of ChP derived EVs in the preterm rabbit brain

We next aimed to determine whether ChP epithelial cell-derived EVs containing IGF-1 could be delivered to a specific subcortical structure. This was performed by injecting PKH26 (a general cell marker) stained ChP epithelial cell derived EVs into the lateral ventricles of non-sedated preterm rabbit pups using ultrasound guidance, see figure 10 in chapter 4 for details. After a period of 4.5 hour, pups were terminated, brains collected and cryosectioned. The PKH26 fluorescence signal was analyzed using confocal microscopy, as illustrated in figure 20A. The analysis revealed the presence of vesicle- or organelle-like structures, resembling EVs which were detected beyond the ependymal lining of the hippocampus (figure 20B-C) extending into the deeper layers of the hippocampus, reaching both the pyramidal and the molecular levels in the cornu ammonis (CA)2-CA3 region (figure 20D).

To gain further insights into the interaction between EVs derived from ChP epithelial cells and the hippocampus, we conducted an EV uptake experiment in vitro. Primary rat hippocampal neurons were incubated with purified EVs stained with the membrane marker PKH67. After a two-hour exposure, the neurons were fixed, fluorescence-labeled with an antibody targeting GAP-43 and examined using confocal microscopy (see figure 20A). We observed that EVs were readily taken up by the neurons, with localization both in the cell soma and in the neurites (as depicted in Figure 20E). Additionally, when we compared the exposure of neurons to EVs from IGF-1-stimulated ChP epithelial cells to EVs from unstimulated control cells, we noted a trend ($p = 0.07$) indicating an increased uptake following IGF-1 stimulation (as shown in figure 20F).

The key results presented in study III suggest an EV-encapsulated IGF-1 transport through the ChP to the CSF and finally the hippocampus. Data suggest that the ChP-derived EVs can penetrate to other sub-cortical structures adjacent to the ventricles, see table 2 in chapter 2 for details. While beyond the scope of this study, it would be of importance to conduct further research on this matter. A better understanding of EV-mediated communication between the ChP and important sub-cortical structures during preterm brain development may offer new therapeutic opportunities in promoting neurodevelopment following extremely preterm birth.

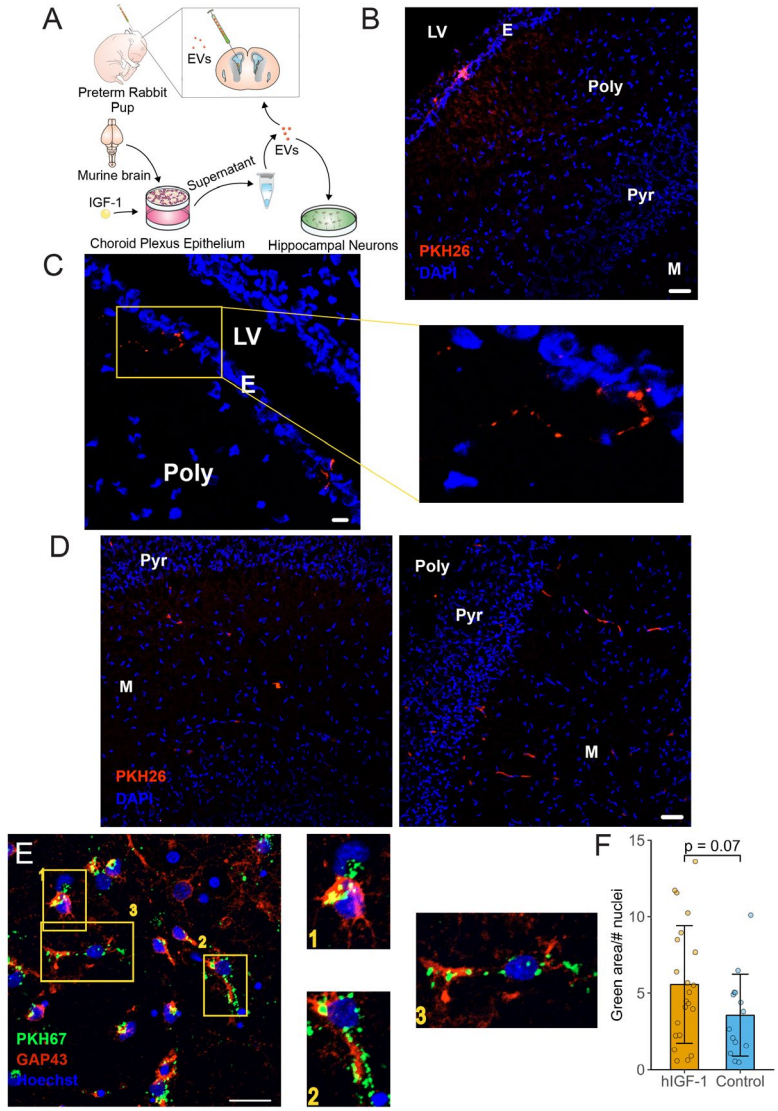


Figure 20. In vivo and in vitro hippocampal uptake of ChP epithelial cell-derived EVs.
A Schematic illustration outlining the experimental process. **B**-**D** Representative confocal microscopy images displaying the presence of EVs derived from ChP epithelial cells stained with the dye PKH26 (red) in the hippocampus of the preterm rabbit pup brain after i.c.v. injections. **B**-**C** Images depicting EVs located beyond the ependymal layer in the polymorph layer. **D** Images displaying EVs in deep structures of the CA2-CA3 regions of the hippocampus proper, in the pyramidal layer and further in the molecular layer. Representative images were collected from N = 8. Scale bars represent 50 µm. **E** GAP-43 labeled rat hippocampal neurons (red) incubated with ChP epithelial cell-derived EVs stained with PKH27 (green). Scale bar represents 25 µm. **F** Quantification of the green signal per number of nuclei in hippocampal neurons exposed to EVs derived from ChP epithelial cells stimulated with hIGF-1. The data is presented as means ± SD (N = 12), and differences between groups were analyzed using a Student's t-test. E, ependymal layer; Poly, polymorph layer; Pyr, pyramidal layer; M, molecular layer; LV, lateral ventricle. Modified from Ortenlöf et al (unpublished results).

Study IV

Studies I-III have explored the potential IGF-1 mechanisms involved in the preventive effects on IVH. In study IV, we aimed to investigate the potential of IGF-1 in preventing glycerol-induced IVH in preterm rabbit pups.

No prevention of IVH in the preterm rabbit pup by systemic administration of IGF-1

IVH is traditionally induced via an i.p. glycerol injection at approximately 2-3 hours postnatal age. Based on the results obtained in studies I-III, we can conclude that vascular protective effects of IGF-1 require some induction time. Thus, to extend the time for IGF-1 to induce these mechanisms, we selected the time-point of 6 hours postnatal age for the glycerol injection. A total of 77 animals were included in the study with a 6-hour window for glycerol injection, with 38 in the group receiving IGF-1 (at 3 hours of age, and subsequently every 12 hours for four consecutive time points, resulting in a total of 5 administrations) and 39 in the vehicle-administered group. Among these 77 animals, 19 developed IVH as determined by HFU (see figure 9 in chapter 4 for details) at 48 (54) hours post-glycerol administration, with the postnatal age indicated within brackets. The results indicate an average IVH incidence of 24% which is relatively low in historical context[115,116,133]. The incidence of IVH following IGF-1 administration was not statistically different compared to vehicle treated animals.

The possible reasons for the lack of prevention of the IVH incidents by IGF-1 are twofold. First, as specified above, the low incidence of IVH resulted in limited statistical power. Second, there might not be sufficient time for IGF-1 to induce the putative protective mechanism against IVH.

Chapter 6: Thesis summary and perspective

"There's no room for facts when our minds are occupied by fear"
Hans Rosling (1948 - 2017)
Professor of international health

Like all stories, an end will come, and this chapter will be the final chapter of this thesis. The results of the thesis are in, all that is left to do is to summarize and give a perspective, as the title suggests. Here, I also want to take the opportunity to thank all the readers who have made it this far through the thesis with stamina left to invest energy in reading this final chapter. Thank you!

Main findings

Novel aspects of IGF-1 involvement in the immature brain have been described throughout this thesis. The main findings are summarized in the list below and figure 21. The numbers displayed below are linked to the numbers featured in figure 21.

1. The presence of IGF-1R in the brain of preterm rabbit pups is initially high at birth but significantly decreases by 96 hours postnatal age, except for the ChP and the meninges.
2. Systemically administered IGF-1 accumulates in the ChP in preterm rabbit pups.

3. Exposure of ChP by systemic IGF-1 induces an increased expression of genes involved in angiogenesis, including IGF-1, in the preterm rabbit pup.
4. Early s.c. administration, before 1-hour postnatal age, of IGF-1 in preterm rabbit pups increases the uptake in serum and CSF compared to later administration, i.e. 24 and 72 hours postnatal age.
5. Blood-borne IGF-1 is transported from the ChP into the CSF via EVs.
6. ChP derived EVs penetrate the ependymal lining and subsequently infiltrate deep into the molecular level of the hippocampus.
7. Using the preterm rabbit pup model of glycerol-induced IVH, the incidence of IVH following IGF-1 administration was not statistically different compared to vehicle treated animals.

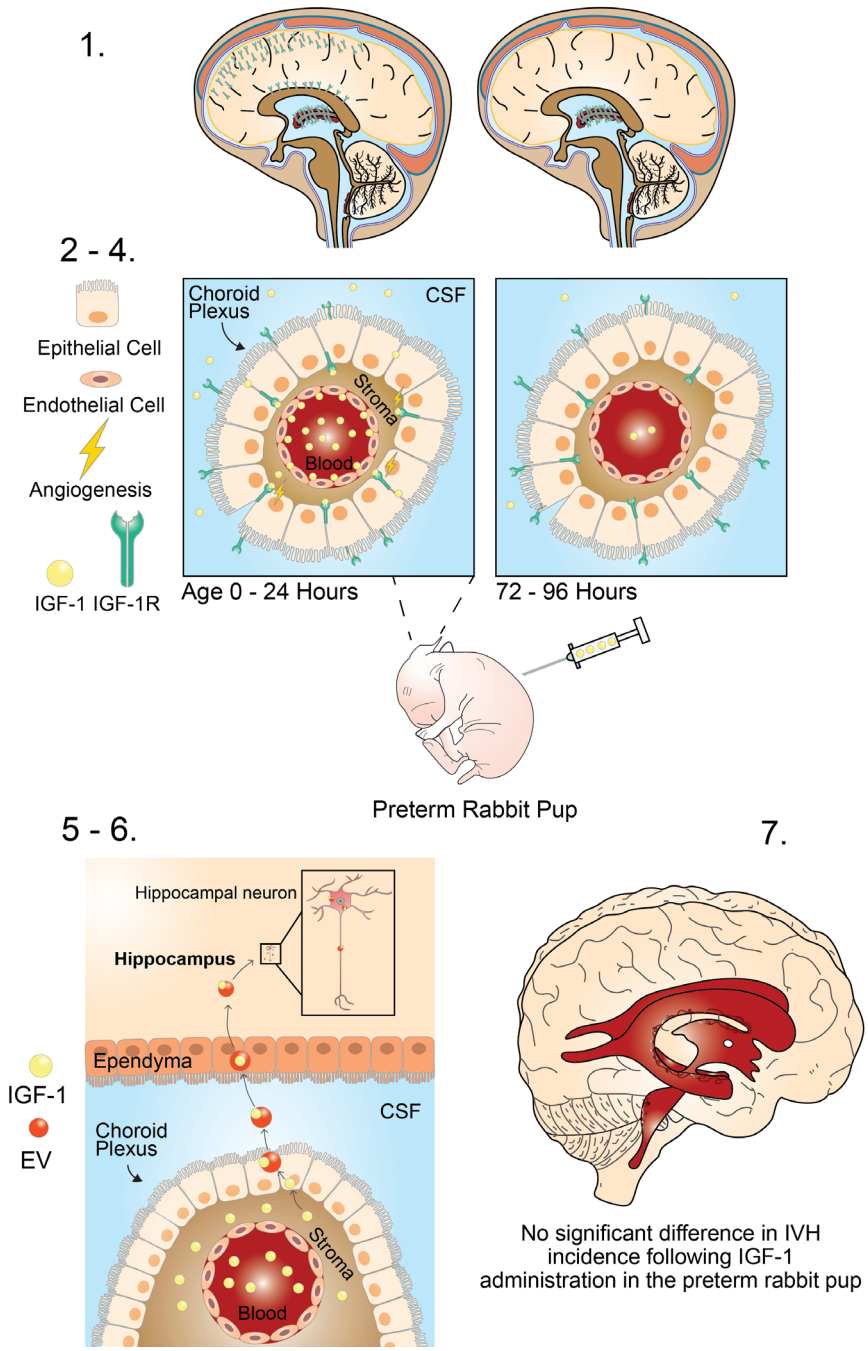


Figure 21. Main findings.
Schematic illustration of the main findings presented on page 69-70. The numbering is linked to the numbers described on page 69-70. The artwork was created by Niklas Ortenlöf using Adobe Illustrator.

Limitations and methodical considerations

What more or less all scientific papers have in common is experimental limitations. In this section, I will present some experimental limitations of my thesis.

In paper 1, one of the results were the upregulation of the IGF-1 gene in the ChP of preterm rabbit pups following systemic IGF-1 administration. This was determined by gene expression analysis with quantitative PCR array, individual primers and IF intensity. Concerning the IF measurements, the time was evaluated at a postnatal age of 4 hours, while the upregulation of the IGF-1 gene occurred at 24 hours postnatal age. As such, the results are not directly comparable. Furthermore, the IF measurements could not distinguish between exogenous and endogenous IGF-1, and therefore preventing an analysis of the increased abundance of IGF-1 protein in the ChP.

In paper 2, the postnatal-dependent uptake of IGF-1 in serum and CSF following s.c. IGF-1 administration in preterm rabbit pups was evaluated by ELISA measurements. As can be observed in figure 18B (chapter 5), the CSF IGF-1 concentrations in 2 pups impacted the data quite significantly. Furthermore, due to limited available CSF sample volumes, additional protein analysis with other methods such as MS or western blot were not feasible. These analyses could potentially have strengthened the robustness of the result.

Summary and future perspectives

Impact for the basic science concerning the ChP

I have a deep fascination for the ventricular system and the ChP. Even seemingly trivial details, like the distinct positions of the ChP in the ventricles, intrigue me. I encourage readers to refer back to figure 3 in chapter 2. While this figure simplifies the ChP positions in the ventricles, it raises questions: Why does the ChP stop at the roof of the ventricle? Why doesn't it continue through the third ventricle down to the fourth? Why is there no ChP in the occipital horn? Perhaps I should aim to explore and answer some aspects of these questions in my future academic career.

While IGF-1 has been thoroughly investigated in the past, especially in scientific fields related to aging and cancer, it appears that the postnatal age-dependent effects

of IGF-1 on neurodevelopment warrant further research. In this thesis, we have presented evidence of novel mechanistic interactions between blood-borne IGF-1 and the ChP in the immature brain. Blood-borne IGF-1 accumulates in the ChPs located in the lateral and third ventricles, as well as in the SAS and perivascular space. Subsequently, IGF-1 might be packaged in vesicles destined to exit the ChP and enter the CSF to reach the hippocampus. Altogether, the systemic signaling to immature brain via the ChP deserves more attention and personally I foresee this to occur in a near future.

The clinical impact for assisting extremely preterm infants

During my PhD, there has been a focus of using the preterm rabbit pups as a translation model to understand how systemic supplementation of IGF-1 affect the immature brain development and the prevalence of IVH. Of note, the IGF-1 compound used in study I, II, and IV will be further evaluated in clinical studies of extremely preterm infants[160]. One result presented in this thesis that might be of relevance to the clinical utility of IGF-1 is the uptake of IGF-1 in serum and CSF that appears to be dependent on the postnatal age of the preterm rabbit pup. Supplementation of IGF-1 (s.c. administration) before the postnatal age of 1 hour increased the uptake of IGF-1 in the serum and the CSF compared to the postnatal ages of 24 and 72 hours. This result suggests that there is a temporal window of IGF-1 supplementation, and the earlier it is supplemented, the more IGF-1 potentially reaches the brain. An aspect that perhaps should be considered in the clinical trial and future clinical practice. Furthermore, in our preterm rabbit pup model, the rupture of the ChP is one cause of IVH. As such, the undeveloped ChP during the transition between stage II and III (see chapter 2 for details) could be vulnerable to the impact of preterm birth and osmotic pressure induced by glycerol we create in our model. To date, this theory has not been investigated, and has not been a focus of this thesis, however, I will be happy if someone, after reading this thesis, aim to set up an investigation.

Other results presented in this thesis, such as the suggested transport of IGF-1 in EVs from the ChP to the hippocampus in the immature brain, could potentially be valuable for drug development targeting the hippocampus in the future. However, this is difficult to foresee. That is the beauty of basic science. I do not know, and I do not want to know. I want to be surprised.

Acknowledgement

This has been a journey like no other. I am truly grateful for the opportunity to write this thesis and for the support I have received from my colleagues, family, and friends.

I would like to begin by expressing my gratitude to my supervisors.

Very few people can answer emails as fast and polite as my main supervisor **Magnus**. Your guidance has helped me become more organized and focused on one task at a time. I am incredibly thankful for your belief in me and for facilitating my entry into this research group. Your kindness and understanding are always welcome. Thank you.

I am grateful for having **David** as my co-supervisor. Our discussions, either if they are scientific, music, pet, or philosophy related, are always inspiring and fruitful. I sometimes have the feeling that your intelligence is contagious and certainly you have given me tools to use in becoming a better scientist and a person. Also, thank you for believing in me and letting me in to this group.

Another inspiring person is my co-supervisor, **Thomas**. Your great problem-solving attitude towards projects and situations in general, have helped me to evolve my own project planning skills, in science and in life.

I would like to thank the current and past members of the Neonatal Neuroprotection group. Our lunchbreaks, adventures abroad, time in the in vivo, celebrations and friendship have made us to become a little extra family. I am grateful for having the lab queen **Suvi** in the group, as she shares to me her secrete knowledge of laboratory tricks and techniques. I thank **Helena** for the support, especially in animal handling. She also has astounding expertise and our time in the in vivo are always fun and pleasant. **Claes** is always easy to work with, everything comes with ease whether we are working in the vivo or jamming music. This is great so thank you. I thank **Åsa** for the great office atmosphere, and **Amanda** for the lively discussions. I know that we often disagree, but this is enjoyable.

I am thankful for that one part of my PhD journey has been in the Nanobiotechnology and Lab-on-a-chip group. You are a very nice set of people with whom I have shared many happy and lifeful moments with. Special thanks to my two partners in crime when operating the AcouTrap, namely **Andreas** and **Megan**. Both have been a joy to work with.

A big thank you to all my co-authors and technicians who have helped me during my PhD. Special thanks to **Stasja** for the scientific consultations and experimental help. Also, **Charlotte** and **Lina** deserve extra thanks for helping me conducting important experiments!

I would like to thank the people at ImaGene-iT. It has been a joy collaborating with you, and I am grateful for all the help. The mentorship I have received from **Bo** is priceless. Thank you for that.

I send many thanks to the current and past people at B14 and C14. You have created a pleasant, relaxed and sometimes very lively atmosphere. I have spent years both at B14 and C14 and therefore, you are too many to mention but I am sure that you know who you are.

I am grateful for the friendship and support I have received during my PhD from **Axel**, **Magdalena Johan**, **Teo**, **Lisa**, **Matilda** and **Simon**.

I am very grateful for the support from my family, especially the support from my mother **Christel**. You are always there for me.

Lastly, I would not been able to come through these years in one piece without the love and support from **Liv**. You are my everything!

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Printed by Media-Tryck, Lund 2023 NORDIC SWAN ECOLABEL 3041 0903

About the author

Niklas Ortenlöf is a trained biotechnologist who, due to a series of unpredictable circumstances, has evolved into a developmental neuroscientist. Niklas PhD-thesis revolves around the themes of the immature brain, choroid plexus, insulin-like growth factor 1 and preterm cerebral intraventricular hemorrhage.

LUND UNIVERSITY

FACULTY OF MEDICINE

Division of Pediatrics
Department of Clinical Sciences

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2023:148
ISBN 978-91-8021-490-2
ISSN 1652-8220