Perinatal brain damage - phagoptosis and neuroprotection

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Gothenburg 2024

Cover illustration: Nils Hillerström and Andrea Jonsdotter

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ISBN 978-91-8069-587-9 (PRINT)
ISBN 978-91-8069-588-6 (PDF)

Printed in Borås, Sweden 2024
Printed by Stema Specialtryck AB

Till farfar

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ABSTRACT

Background: Neonatal encephalopathy is a serious outcome in term infants affecting 1-2/1000 live births and is often caused by perinatal hypoxia-ischemia (HI). However, brain injury is multifactorial and infection in the mother during pregnancy could cause or aggravate brain damage. Cerebral palsy (CP) is a known complication due to brain damage and occurs in both term and preterm infants. In preterm infants the risk of CP is inversely proportional to gestational age.

Aim: To better understand mechanisms of brain damage in preterm and term infants and thereby develop new strategies for neuroprotection.

Material and methods: Paper I, III and IV are animal experiments on mice and rats. **Paper I** focuses on the neuroprotective effect of the glucagon-like peptide-1 (GLP-1) receptor agonist exendin-4 after term HI in neonatal mice, with or without therapeutic hypothermia. In **Paper IV** the neuroprotective effect of exendin-4 in a preterm model of cerebral GMH is addressed in rats. **Paper III** aims to evaluate the neuronal cell death in a mouse model of HI after gene deletion of the phagocytic receptor Mer-tyrokine kinase (Mer-TK). **Paper II** presents a clinical study where a bolus dose of magnesium sulfate ($MgSO_4$) is administered to pregnant women with imminent risk of preterm delivery to determine the concentration of serum magnesium (s-Mg) in both the mothers and the umbilical cords of the infants.

Results: Paper I: Exendin-4 treatment alone showed significant neuroprotection and it enhanced the cerebroprotective effect of hypothermia ($p < 0.0001$). Tissue infarction was significantly reduced after only one dose of exendin-4 injection (saline: 50% ± 6.9%) and (exendin-4: 17% ± 8.6%) ($p=0.03$) and adding the dose regime every 12 h over a 48h period reduced

brain injury further to 2% ± 1.8% (p=0.02). **Paper IV:** This study shows that exendin-4 reduced brain injury after GMH. The neuroprotective effect was detected as early as 48 h after GMH (p=0.05 in striatum and p=0.04 in hippocampus) and was sustained until adulthood (P40, p <0.0001). Exendin-4 improved motor skills significantly in different behavioral tests including rotarod (P20, p=0.003; P40, p <0.0001), eye opening (P14, p=0.05) and negative geotaxis (P8, p=0.05). **Paper III:** Genes related to phagoptosis including MerTK and Gas-6 were upregulated at 6-72h after HI in the brain. Brain injury was reduced by 48% in gray matter (p=0.002) in MerTK knock-out (KO) vs wild-type (WT) animals and in white matter by 32% (p=0.04). Immunostaining of neurons and microglia indicated less neuronal phagocytosis by microglia in MerTK KO vs WT animals, (p= 0.03). **Paper II:** A bolus dose of 6 g of $MgSO_4$ seems to be well tolerated by the women and no extra surveillance is needed. The target concentration of s-Mg was reached in the blood of most of the women and the concentrations were low (0.87 to 1.4 mmol/l) in the umbilical cord at birth unlikely to adversely affect the newborn infants.

Conclusion: This thesis shows that: 1. the GLP-1 receptor agonist exendin-4 provides strong neuroprotection in rodent neonatal models of HI and GMH, and has, suggestedly, potential for clinical implementation; 2. gene deletion of the microglial MerTK receptor reduces both microglial phagocytosis of neurons and brain injury implicating involvement of phagoptosis in HI; 3. a 6 g bolus of $MgSO_4$ is well tolerated in pregnant women and is not likely to adversely affect the newborn preterm infant. This regimen is now established and implemented in all Swedish hospitals that provide care for deliveries up to 32 weeks of gestation.

Keywords: hypoxia-ischemia, germinal matrix hemorrhage, exendin-4, Mer-TK, magnesium sulfate, preterm, cerebral palsy, hypothermia, phagocytosis, microglia.

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SAMMANFATTNING PÅ SVENSKA

Barn som föds för tidigt löper en ökad risk att utveckla hjärnskada (orsakat av t.ex. blödning eller infektion) jämfört med de som föds i fullgången tid. Detta i sin tur kan orsaka komplikationer i form av neurologiska funktionsbortfall, kognitiva svårigheter och i värsta fall dödsfall. Årligen föds globalt ca 15 miljoner barn för tidigt (innan graviditetsvecka 37), dessa barn har en större risk att utveckla neurologiska funktionsvariationer, såsom exempelvis cerebral pares. Cerebral pares är en vanlig komplikation till hjärnskada hos barn och ju lägre gestationsvecka desto större risk att drabbas av cerebral pares. Barn som föds i fullgången tid löper risk att exponeras för syrebrist under förlossningen. Kylbehandling är den behandlingen som just nu kan erbjudas vid svår asfyxi med akut hjärnpåverkan (=encefalopati) hos nyfödda i fullgången tid i de länder som har ekonomiska resurser att erbjuda neonatal intensivvård. Det är av största vikt att försöka hitta alternativa och kompletterande behandlingar till kylbehandling som kan användas även i länder med begränsade sjukvårdsresurser och som kan erbjudas även för tidigt födda barn som vi i nuläget inte kan erbjuda någon behandling alls. Kunskapen om skademekanismer och hur hjärnskadan utvecklas över tid i den prematura och fullgångna hjärnan är fortfarande begränsad. Vi behöver en större insikt i när och hur hjärnskadan uppstår för att kunna utveckla bättre behandlings-strategier.

Den här avhandlingen syftar till att försöka förstå de bakomliggande skademekanismerna i den omogna hjärnan. Vi har valt att studera den hjärnskyddande effekten av glukagon-liknande peptid-1 (GLP-1) receptor agonisten exendin-4, betydelsen av fagocytreceptorn tyrosinkinaset Mer-TK i en musmodell för syrebrist samt effekten av magnesiumsulfat ($MgSO_4$) hos kvinnor med hotande förtidsbörd. $MgSO_4$ har länge varit känt inom förlossningsvården men har inte i Sverige använts i neuroprotektivt syfte förrän 2021. Detta efter att vår studie genomförts där gravida mellan graviditetsvecka 23+0 – 31+6 erhållit 6 gram $MgSO_4$ som profylax mot senare utveckling av hjärnskada hos det nyfödda barnet. Denna behandling är nu implementerad nationellt på alla sjukhus som tar emot gravida med hotande förtidsbörd <32 gestationsveckor.

Brist på syre och blodtillförsel till hjärnan är en allvarlig orsak till hjärnskada hos barn födda i fullgången tid. Exendin-4 är en GLP-1 receptor agonist som används för att behandla diabetes typ II. I första delarbetet visar vi att exendin-

4 har en uttalad neuroprotektiv effekt efter en syrebristorsakad skada i hjärnan hos möss, och att behandlingen kan ges 2 timmar efter exponeringen för syrebrist. När exendin-4 kombinerades med kylbehandling förstärktes dess neuroprotektiva effekt.

Då hjärnblödning är en allvarlig orsak till hjärnskada hos barn födda extremt för tidigt, ville vi utvärdera effekten av exendin-4 även vid detta tillstånd. Efter att ha utvecklat en modell på råttor där en hjärnblödning inducerades genom att injicera kollagenas i germinalzonen i hjärnan, kunde vi utvärdera den potentiellt neuroprotektiva effekten av exendin-4. Exendin-4 givet efter hjärnblödningen minskade skadan signifikant när den utvärderades 2 dagar samt 7 veckor efter hjärnblödningen och behandlingen med exendin-4 förbättrade dessutom den neurologiska funktionen hos djuren.

Mer-TK (Mer tyrosin kinas) är ett protein som aktiveras vid en hjärnskada och uttrycks i mikrogliaceller och fungerar som en fagocytosreceptor som anses vara kritisk för att avlägsna skadade och döda celler. I en tidigare studie på vuxna djur har det visats att om genen för Mer-TK slås ut så kan död av funktionella men skadade neuron förhindras och på så sätt blir hjärnskadan betydligt mindre. Vi ville nu i delarbete III undersöka om fagocytos har betydelse även i den omogna hjärnan. Detta tillämpades i en etablerad modell på nyfödda möss där en hjärnskada inducerades genom ligering av karotisartären i kombination med inandning av gas med nedsatt syrehalt (=hypoxi). Vi jämförde utfallet hos möss där Mer-TK genen hade slagits ut med möss som hade en normal genotyp. Vi fann att de möss som saknade Mer-TK genen utvecklade 48% mindre hjärnskador efter syrebrist jämfört med djuren som hade intakt genotyp.

Sammanfattningsvis har vi visat på flera potentiellt verksamma neuroprotektiva strategier på djur och människa. Den hjärnskyddande effekten av exendin-4 som vi påvisat i djurmodeller för mus och råtta kommer att följas upp med prekliniska försök på nyfödd gris med målsättning att sedan utföra implementerande studier på nyfödda barn med svår asfyxi. Det faktum att deletion av MerTK receptorn minskade skadorna indikerar att mikroglia kan fagocytera även levande celler och därmed öka förlusten av nervceller efter syrebrist. Detta är ett viktigt fynd som måste beaktas vid utveckling av nya behandlingsstrategier. $MgSO_4$ är redan implementerat i kliniken och studier pågår för att utvärdera dess neuroprofylaktiska effekt i en svensk population. Vår förhoppning är att denna avhandling har bidragit till ny kunskap vad gäller potentiellt hjärnskyddande behandlingar för nyfödda barn i framtiden.

LIST OF PAPERS

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

I. Rocha-Ferreira E, Poupon L, Zelco A, Leverin A-L, Nair S, Jonsdotter A, Carlsson Y, Thornton C, Hagberg H*, Rahim A*. Neuroprotective exendin-4 enhances hypothermia therapy in a model of hypoxic-ischaemic encephalopathy. Brain 2018 Oct 1;141(10):2925-2942. doi: 10.1093/brain/awy220 *equal contribution

II. Jonsdotter A, Rocha-Ferreira E, Hagberg H, Carlsson Y. Maternal and fetal serum concentrations of magnesium after administration of a 6-g bolus dose of magnesium sulfate ($MgSO_4$) to women with imminent preterm delivery. Acta obstet Gynecol Scand. 2022 Aug;101(8):856-861. doi: 10.1111/aogs.14372. epub 2022 May 2

III. Jonsdotter A, Hagberg H, Leverin A-L, Joakim Ek, Kerstin Ebefors, Rocha-Ferreira E, Carlsson Y. Mer-TK and the role of phagoptosis in neonatal hypoxia-ischemia. *Manuscript*

IV. Jonsdotter A, Carlsson Y, Leverin A-L, Svedin P, Eberfors K, Ek J, Hagberg H*, Rocha-Ferreira E*. Exendin-4 improves neurodevelopmental outcome in a preterm rat model of germinal matrix hemorrhage. *Manuscript*

CONTENTS

ABBREVIATIONS

APGAR	Appearance Pulse Grimace Activity Respiration
BMI	Body mass index
C	Celsius
cAMP	Cyclic AMP
CNS	Central nervous system
CP	Cerebral paresis
EBM	Experimental Biomedicine
Ei3ja	Eukaryotic translation initiation factor 3 subunit a
EMA	European medicines agency
ERK	Extracellular signal-regulated kinase
Ex4	Exendin-4
FDA	Food and drug administration
GAS-6	Growth arrest specific gene-6
GLP-1	Glucagon like peptide-1
GMH	Germinal matrix hemorrhage
GO	Gene ontology
GW	Gestational week
h	Hour
HET	Heterozygote
HI	Hypoxia-ischemia

HIE	Hypoxic ischemic encephalopathy
IL-6	Interleukin-6
IP	Intraperitoneally
IV	Intravenously
IVH	Intraventricular hemorrhage
IUGR	Intrauterine growth restriction
KO	Knock-out
LPS	Lipopolysaccharide
s-Mg	Serum magnesium
MAP-2	Microtuble associated protein-2
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein-1
MerTK	Myeloid epithelial reproductive tyrosine kinase
MFG-E8	Milk fat globule-E8
$MgSO_4$	Magnesium sulfate
MMP-9	Matrix metalloproteinase -9
MPO	Myeloperoxidase
NEC	Necrotizing enterocolitis
PND	Postnatal day
PFA	Paraformaldehyde
PS	Phosphatidylserine

PVL	Periventricular leukomalacia
RCT	Randomized controlled trial
RT	Room temperature
SAL	Saline
STAT3	Signal tranducer and activator of transcription 3
SOCS3	Suppressors of cytokine signaling 3
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
VNR	Vitro nectin receptor
WHO	World health organization
WT	Wild type

INTRODUCTION

CLINICAL BACKGROUND AND PREVALENCE

When born preterm there is a risk of neurodevelopmental disabilities, such as cerebral palsy (CP). The rate of infants born preterm (< 37 weeks of gestation) globally is estimated around 11% (1). In Sweden, the approximate rate of preterm birth is around 5.8% (2), where most of the preterm birth occur between gestational week 32-37 (3). The infants who survive are at greater risk of developing neurological impairment, such as CP (4). Even though obstetric and neonatal care has improved the past years with a decrease in neonatal mortality as a result, more extremely preterm born infants survive which has resulted in an increased total number of CP among these infants (5). In addition, infants born at term after severe asphyxia are also at risk of neurological deficits including CP (5).

For both preterm and term born infants with brain injury there is a need to improve long term outcomes as well as to decrease mortality. For preterm born infants there is a need to develop treatment strategies since neuroprotective treatment is currently lacking, and preterm infants are not eligible for hypothermia treatment (6). For term born infants (from gestational week 36-37) with hypoxic ischemic encephalopathy (HIE), hypothermia is a standard treatment today, however hypothermia treatment needs to be started within 6 hours to be effective (7). Even though hypothermia has long-lasting neuroprotective effect after HIE (8), there are still up to 25% of the infants born with severe HIE (grade 3) that will live with complex neurological handicaps despite hypothermia and 20% of the infants with severe HIE will not survive their first years of life (9). Moreover, it's important to note that preterm infants are not eligible for hypothermia treatment. Consequently, there arises a need

for additional treatments that can augment the efficacy of hypothermia treatment. These adjunct treatments should also have the potential to serve as standalone therapies for both preterm and term-born infants with brain injuries.

BRAIN INJURY IN THE TERM BORN INFANT

CLINICAL BACKGROUND AND PREVALENCE

Around 10/1000 term infants develop birth asphyxia and approximately 1-2/1000 suffer from neurological symptom in the newborn period due to HIE (9). HIE is diagnosed as either mild, moderate, or severe. Infants with moderate or severe HIE usually die or have serious complications while infants with mild HIE have a greater chance of a development the same as infants without HIE at the age of 18 months (10). Dyskinetic or tetraplegic CP is common neurological disabilities in term infants, where most often the basal ganglia and thalamus are affected, where spastic CP is predominant in preterm born infants where white matter is the dominant area of injury (5, 11). The only available treatment of HIE is hypothermia, where the infant is cooled down to 33-34°C for 3 days. The treatment needs to be started within 6 hours of birth. This has shown to significantly increase the survival and improving the neurological outcome of infants born with HIE (12).

PATHOGENESIS

The term birth asphyxia refers to the abruption of exchange in oxygen and reduction of blood supply to the infant intrapartum or shortly before birth (13). In clinics, with a clear abruptio placentae or cord prolapse, followed by low fetal pH, there is a well-defined asphyxiating injury, that usually is followed by an infant exhibiting signs of neonatal depression and low Apgar score, need for neonatal intensive care and later signs of neonatal encephalopathy (13). The clinical cause of damage is not always obvious, neonatal encephalopathy can

occur in infants with no clinical signs of asphyxia (14). Due to these findings it's important to remember that there could be several factors producing asphyxia, and maternal infection is one of them (15). Chorioamnionitis and fever during labor are risk factors for developing asphyxia and HIE, and maternal infection and chorioamnionitis has a clear association with CP at term and the ongoing infection can worsen the outcome (15).

MECHANISMS OF TERM BRAIN INJURY

Hypoxia-ischemia and two-phase response

The injury after HIE is not a single event but rather a complex and progressive process (16), where perinatal asphyxia occurs initially, followed by a latent phase of reperfusion with a recovery of energy metabolism. This is followed by a secondary phase with energy loss (6-18 h after birth), with accumulation of cytotoxins, seizures and edema (8). This suggests that the primary insult is only a small part of the total brain injury and that the secondary cascade of toxic substances starting during reperfusion will decide the extent of the final brain injury (17). When treating an asphyxiated infant with hypothermia, the overall hypothesis is that by lowering the cerebral temperature to 33-34°C after HI there is a possibility to modulate the cascade processes that occurs during the first phase of injury, and that will extend into the secondary phase (8).

Primary injury

When brain damage occurs after HI, there is a first phase/primary injury with a rapid energy depletion. This ATP loss leading to failure in the Na^+/K^+ pump and depolarization of the cell membrane, resulting in the mitochondria to produce energy without the presence of oxygen, increasing lactate and ROS production (16). This in turn results in increased intracellular calcium levels, and high levels of glutamate (an excitatory amino acid EAA) in the

extracellular space due to neuronal depolarization and a disrupted uptake (18). The increased intracellular calcium levels, caused by glutamate, lead to influx of Ca^{2+} via the glutamate receptor, into the cytosol and mitochondria, causing swelling of the mitochondria and the release of reactive oxygen spices (ROS), disruption of the blood brain barrier and cell death of neurons starts due to energy deprivation and membrane damage (19). After HI there is an increase of hydrogen peroxide (H_2O_2) in the immature brain, which is detrimental because of the increased levels of free iron that it generates due to degradation of heme proteins, that is a base for the release of ROS and exacerbation of oxidative stress (19).

Secondary injury

It is well established that the therapeutic window and initiation of the secondary phase takes place around 6 hours after HI (20). This latent phase, following the primary injury, cell metabolism is restored for those cells surviving the primary insult (16), followed by a subsequent decline in cell energy metabolism. From the first injury and then from the early reperfusion phase (6-12h), there is a cascade of neurotoxicity initiated, and the secondary injury is characterized by high levels of Ca^{2+} inside the cells, release of more ROS (that is most prominent during reperfusion) and nitric oxide (NO) (free radicals), proteases and an acute inflammatory response leading to mitochondrial damage and cell death via apoptosis. Apoptosis is an important part of the secondary injury (21-23). During the secondary phase, neutrophils are partly responsible for production of ROS. Mitochondrial dysfunction is suggested to be important in the development of secondary brain injury (24). Recently a tertiary phase might be present after HIE occurring weeks or months after injury, involving more chronic inflammation and tissue remodeling which could be an additional potential target for treatment (25).

BRAIN INJURY IN THE PRETERM INFANT

PERINATAL BRAIN INJURY-WHITE MATTER INJURY AND INTRAVENTRICULAR HEMORRHAGE

Brain injury appears differently in preterm versus term born infants. More specific to infants born preterm is the cerebral white matter damage (WMD), which is the predominant form of brain injury in these infants, where the major risk of neurological and cognitive variations is due to WMD, especially infants born with a weight under 1500g (26-28). In this chapter WMD includes periventricular leukomalacia (PVL), focal necrosis and the more diffuse cerebral white matter damage with loss of pre myelinating oligodendrocytes. The pathophysiology of brain injury in preterm born infants is complex. The undeveloped and delicate cerebrovascular anatomy of the preterm infant is particularly sensitive to ischemia and inflammation, especially in the white matter due to arterial end border and cerebral blood flow regulation (27). The long vessels mainly from the middle cerebral artery, ends in the deep periventricular white matter, and the terminations of these vessels are the ones most vulnerable to a decrease in the cerebral blood flow, which in combination with impaired cerebrovascular autoregulation, due to changes in blood pressure, could lead to ischemia and necrosis in these areas (27). Even though brain injury in the preterm infant is mainly focused on the white matter, there is also an indirect damage to the cortical gray matter, due to ventricular swelling, caused by white matter substance loss, and this particular damage could possibly explain some of the cognitive deficits seen in these infants (28, 29). White matter injury, characterized by the loss of pre-myelinating oligodendrocytes (pre-OL), which have been shown to be sensitive to free radicals due to lack of sufficient antioxidant defenses. This results in the inhibition of pre-OLs from maturing into mature OL, consequently leading to white matter injury (27).

There is not only a direct WMD occurring in preterm infants, but other pathological causes as well such as intraventricular hemorrhage (IVH), which is a common cause of injury in extremely preterm infants leading to neurological impairments and mortality (30). The most commonly detected hemorrhage is the germinal matrix hemorrhage (GMH). This occurs mainly in extremely- and very preterm infants (gestational week 23-32) due to the rich vascularized area but poor vascular support of the germinal matrix (GM) tissue, prone to rupture causing hemorrhage. GM is located close to ventricular ependyma and caudate nucleus and is a source of future neurons and glial cells; this area is important for neurodevelopment (31). After a GMH, usually within 72 h, there is a release of free iron with following production of free radicals (30, 32). After the hematoma is resolved, a secondary phase of tissue loss is prominent, due to the accumulation of CSF (hydrocephalus) causing tissue compression. If blood flow is re-established, further increase of free radicals and oxidative stress can exacerbate the injury, and then in particular affect the pre-oligodendrocytes. This in turn, activates a prolonged inflammatory response, which could further damage the white matter (33). IVH is usually defined as grade I-IV, where hemorrhage grade I is confined to the GM, grade II-III is extended into the ventricle but grade III attributes also with associated hydrocephalus and grade IV is bleeding extending into the white matter and has the worst prognosis, with a mortality rate around 90% (34).

MECHANISMS OF PRETERM BRAIN INJURY

There are some differences between the cellular mechanisms in the preterm and term brain injury, even though there are also similarities with those cascades described for term brain injury. The preterm brain injury is often characterized by damage to the immature oligodendroglia cells, while in the term brain injury often targets neurons due to HI (29, 35). The loss of oligodendroglial cells generate brain injury and atrophy, but there is also

questions raised about the impact on axonal maturation (36). A previous study has shown that oligodendroglia are essential for axonal maturation and loss of oligodendroglia could affect the function and neuronal plasticity (37). Immature oligodendroglia are known to be sensitive to HI injury and ROS toxicity, and immature oligodendroglia also have a low anti-oxidant defense and are vulnerable to free radicals, which make them extra sensitive to death after brain injury, figure 1 (38). Furthermore, immature oligodendroglia seem to be very prone to glutamate induced cell death (38). The neuronal death after HI occurs in several phases, and involves energy depletion (19).

Figure1

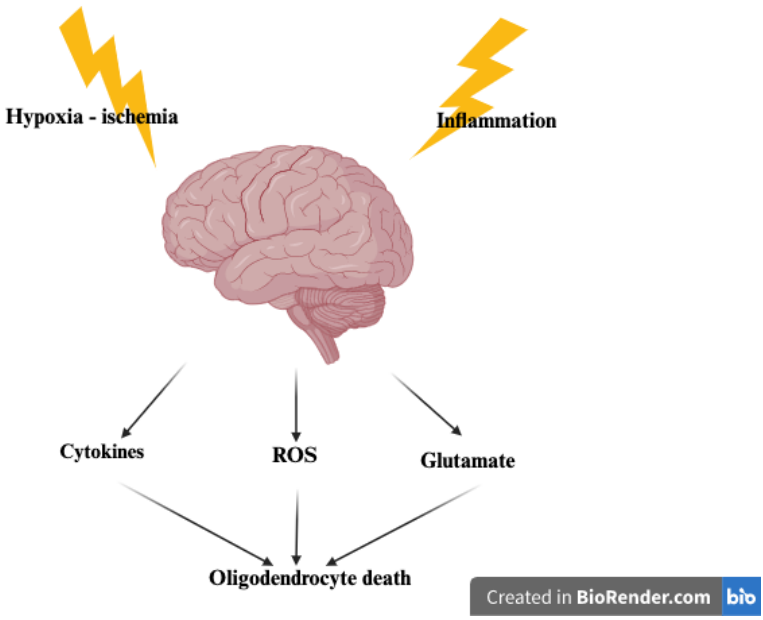

Figure 1. White matter damage and oligodendrocyte death. *Illustrated by Andrea Jonsdotter.*

PHAGOCYTOSIS AND MICROGLIA

Microglia are the macrophages of the brain, and after harmful stimuli they are activated within hours, but reach highest levels after 2-3 days and are present until 14 days after HI (21). There is a discussion about microglia polarization and activating either towards pro-inflammatory (M1) or anti-inflammatory (M2) properties. However, recent studies have indicated that it's not solely one or the other, rather it is more likely that microglia can inhibit both phenotypic polarizations, suggesting a more dynamic and heterogeneous form (39, 40). They inhibit the possibility to change their phenotype depending on activation and their activated form is seen within hours after HI (21). Microglia has an important role in clearing cellular debris and maintaining homeostasis in the

brain (41, 42). Even though microglia are important in tissue regeneration and clearing dead cells, activated microglia is suggested to also contribute to cell loss after brain injury (6, 42). It all seems to depend on the way they are activated, either towards the pro-inflammatory pathway or the anti-inflammatory pathway (42).

However, according to previous studies in the adult brain (43-45) neuronal death also can occur as a result of microglia phagocytosing stressed but viable neurons, a process named phagoptosis, resulting in cell death (43, 44). Whether a cell is being phagocytosed or not depends on the exposure of 'eat me signals' binding to respective opsonins and then activating the phagocytic receptor (such as MerTK) that are present on microglia (43). This is shown in studies where specific phagocytic receptors are deleted and thereby preventing neuronal loss and brain atrophy in models of ischemic brain injury (43, 46).

NEUROINFLAMMATION

Infection and the following inflammatory response include the involvement of chemokines and cytokines, which can worsen the outcome of HI in the brain even if the infection is distant from the brain (47, 48). It has also been demonstrated that a feto-maternal infection increases the risk of CP, and that raised levels of cytokines in the blood from a maternal infection, leads to elevated brain cytokines levels in the infant, and in turn leads to brain damage and CP (49). Cytokines are a large group of soluble proteins that act as messengers, and chemokines are a distinct subgroup of cytokines. Interleukin-6 (IL-6) is a pro-inflammatory cytokine that is known for its immunomodulatory and acute phase properties, such as fever and inflammation and is produced by macrophages (41, 49, 50). IL-6 in cerebrospinal fluid is also a marker of the extent of brain injury in newborns with HIE (51). Monocyte chemoattractant protein-1 (MCP-1) is a chemokine

that is released from microglia in the brain, and reacts with increased, high levels within a few hours after HI and up to 48 hours (52). Signal transducer and activator of transcription 3 (STAT3) is a signaling protein that in response to cytokines translocate to the nucleus where it acts as a transcription activator and can modulate a variety of genes. STAT3 is known to have an impact on macrophages and its polarization, and high levels of STAT3 has shown to be involved in the inflammatory response (53, 54).

EXENDIN-4

Exendin-4 is an analogue of the human gut hormone peptide glucagon-like peptide-1 (GLP-1), which works through regulating blood sugar by increasing insulin levels from the pancreas. Exendin-4 was approved by the European Medicines Agency in 2006 for treatment of type II diabetes in adults. Exendin-4 is a desirable alternative to GLP-1 due to its longer half time of 60-90 min (55) compared to mammalian GLP-1 that only reaches 1.5 min due to degradation by dipeptidyl peptidase IV (DDP-IV), which exendin-4 is resistant to (56). The potential neuroprotective effects of exendin-4 were first seen in patients treated for type II diabetes mellitus, where neuropathic and cognitive improvements after the treatment was detected (57). Exendin-4 also reduced deficits in motor and cognitive outcome in patients diagnosed with Parkinson's disease (58). Previous studies showed that the GLP-1 receptor exists throughout the brain and that exendin-4 readily crosses the blood brain barrier (59, 60). With its extra-pancreatic effects, exendin-4 can influence several pathways in the brain, such as cellular proliferation, neuroinflammation, enhancing cell survival and improving mitochondrial function (58). Previous reports demonstrate that the neuroprotective and anti-apoptotic effect of exendin-4 involve the protein kinase A (PKA) and PI3 kinase/Akt pathways (61). Kappe et al (62) found that microglia can increase GLP-1 and its receptor

in response to inflammation, proposing that activation of the GLP-1 receptor could be an endogenous protective response to dangerous stimuli (58).

MAGNESIUM SULFATE

Magnesium sulfate ($MgSO_4$) is a widely recognized therapy utilized worldwide for its neuroprotective properties (63). Magnesium is an important mineral in the body and is required for nerve transmission, protein synthesis and regulation of muscular contraction. Over half of the total amount of magnesium is mainly located in the bone, and the rest in skeletal muscle and soft tissue (64). The neuroprotective effect of magnesium is thought to derive from its ability to increase the structural integrity of membranes through decreasing the excitability and increase the threshold in membranes of neurons. The decreased excitotoxic effect seen by $MgSO_4$ is mediated by the suppression of calcium dependent transmission release and magnesium also has the effect of blocking the NMDA receptor, hence decreasing excitotoxicity. Other neuroprotective effects by magnesium are effects on NO and altering free radicals (65). Previous studies have shown a neuroprotective effect when $MgSO_4$ is administered antenatally to women with imminent risk of preterm birth or as prophylaxis for preeclampsia, with reduced risk of IVH/PVH as a result (63, 66) as well as a reduced risk of CP or severe motor disability by 30-40% (67). Previous study suggested that $MgSO_4$ inhibits a possible preconditioning effect (68), therefore, Koning et al conducted a study where $MgSO_4$ was given prior to HI between 6 days and 12 hours and hypothesized that $MgSO_4$ would exhibit a preconditioning effect. Animals who received $MgSO_4$ markedly had a reduced brain injury, with a maximal protection when $MgSO_4$ was administered 24 hours before HI (68).

Many countries internationally use $MgSO_4$ as a neuroprotective drug, but the dose varies between countries (63). There is still no consensus in the optimal

dose to use for a neuroprotective purpose, where considerations need to be taken for both short- and long-term outcome of the infant (63).

MERTK

The Myeloid-epithelial-reproductive tyrosine kinase (MerTK) receptor is a phagocytic receptor on microglia. The MerTK receptor can mediate phagocytosis of neurons, and is activated through binding its opsonin growth arrest specific 6 (Gas-6), complement factors or through osteopontin (fig. 2), which in turn binds to phosphatidylserine (PS) exposed on the damaged neurons surface as an 'eat me signal', and then phagoptosis occurs of the stressed neurons (43, 44). In previous study it is shown that MerTK and Gas-6 are both upregulated by inflammation in the brain, it is also shown in adult animals that MerTK is upregulated after focal brain ischemia on day 2-3, which could cohere with the resolution phase of inflammation. The same report could conclude that when deleting the MerTK gene there was a marked reduction in brain atrophy 7-28 days after brain ischemia compared to WT. The reduction of brain injury in MerTK gene deficient animals was accompanied by an improvement of the long term neurological functional outcome. The total amount of microglia containing neurons were reduced in the MerTK gene deficit animals compared to WT. These studies suggest that the protection of neurons in the MerTK KO animals found after brain ischemia, is due to that the neurons being engulfed in the WT animals after brain ischemia, were alive when phagocytized (44).

Figure 2

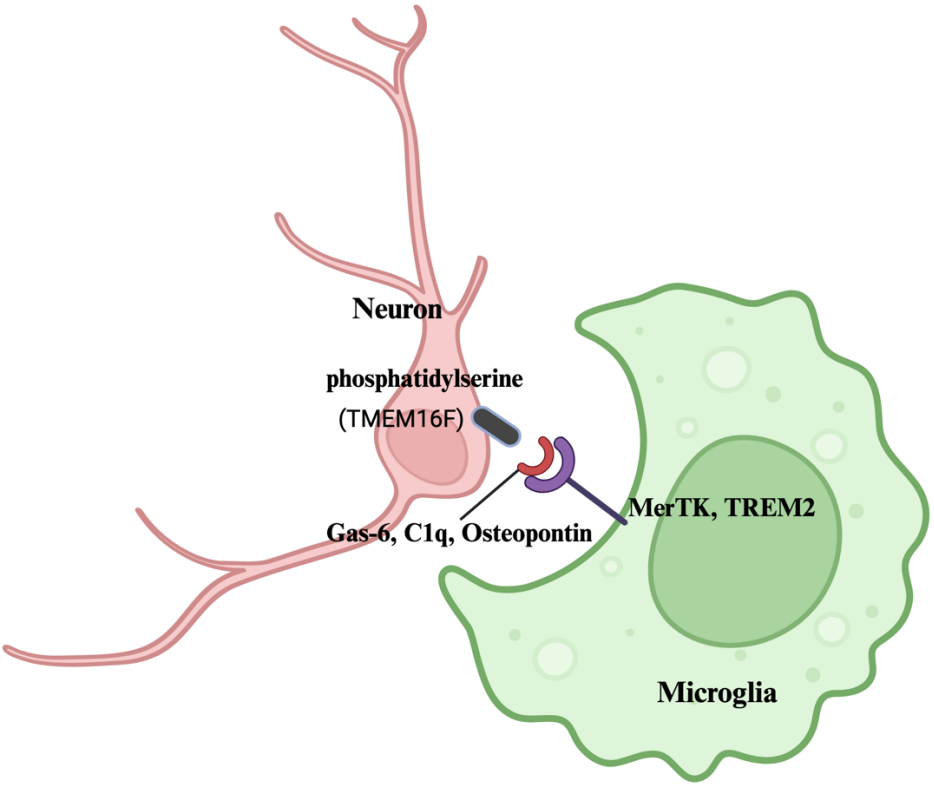

Figure 2. Possible signaling pathway of MerTK

Stressed neurons expose 'eat me' signals on their surface, such as phosphatidylserine. TMEM16F is a scramblase that flips phosphatidylserine from the inside of the neuron to the outside on the surface and is then recognized by activated microglia and its phagocytotic receptors (MerTK and TREM2) through opsonins, here shown; Gas-6, C1q, osteopontin. Illustrated by Andrea Jonsdotter.

AIM

The overall aim for this thesis is to explore basic mechanisms of perinatal brain injury and thereby develop new strategies for neuroprotection.

The specific aims are:

Paper I: Explore the therapeutic effect of exendin-4 administered alone or in combination with hypothermia in a neonatal mouse model of HI.

Paper II: Study the effect of an antenatal bolus dose of $MgSO_4$ on maternal and neonatal side effects as well as serum levels of magnesium in both the mother and the neonate.

Paper III: Investigate the role of phagoptosis in perinatal brain injury by deletion of the phagocytotic receptor MerTK and explore the effect on brain injury and phagocytosis of neurons in a term mouse model of HI.

Paper IV: Explore the therapeutic effect of exendin-4 in a preterm rat model of GMH.

MATERIAL AND METHODS

A more detailed description of material and methods can be found in every individual paper. A more general description will follow below.

All studies except for paper number two are preclinical animal studies on mice and rats (both female and male), performed at the Centre for Perinatal Medicine & Health, Institute of Clinical Sciences, Sahlgrenska Academy, and paper number one was partly performed at EGA Institute for Women's Health and UCL School of Pharmacy, both at University College London, UK.

Paper number two is a clinical study including pregnant women and their unborn offspring, performed at the maternity ward at Sahlgrenska University Hospital during 2017-2018.

ANIMAL MODELS (PAPER I, III, IV)

HYPOXIA-ISCHEMIA MODEL

In this model brain injury was generated through occlusion of the left common carotid artery followed by hypoxic exposure in a hypoxic chamber. The brain damage occur through a decrease in cerebral blood flow ipsilateral to the occluded vessel and depending on the duration of hypoxia, the severity of brain injury can be adjusted (19). The desirable injury is generated through the combination of hypoxia and carotid artery ligation. The cerebral blood flow is reduced by approximately 40-60% during hypoxia in the ipsilateral hemisphere, leading to brain injury due to the combination of tissue hypoxia and partial ischemia (19). Brain damage develops primarily in cerebral cortex, hippocampus, thalamus and striatum, areas that are supplied mostly by the middle cerebral artery (19). This model in mice shows that they have a more prominent damage in the hippocampus area, and less in cortex (69) (41) when

compared to a rat HI model, probably due to variations in vascular anatomy. One advantage of this model is that the contralateral side is not damaged and hence can be used as a control (70). Another advantage within this model is that it allows a long-term evaluation of the injury. The combination of hypoxia and ischemia followed by reperfusion and cell energy metabolism being affected, shows similarities to birth asphyxia in human neonates. However the model exhibits some disadvantages as well, such as in the model there is only a unilateral damage and no multi organ impact, which differs from birth asphyxia in human neonates and also that there is a variability between the animals and litters (70, 71).

The well-known Vannucci model of HI in rats (72), modified for mice (42), was used in **Paper I.** CD1 mice of PND7 mimic late preterm/early term human infants and PND 10 (term infants), were used to study the neuroprotective effect of exendin-4 after HI. Mouse pups were anaesthetized with isoflurane and the left common carotid artery was ligated. After 1 h recovery followed and then the mice were put in a hypoxic chamber with 8 % oxygen in 36 degrees C for 30 minutes (PND 7) or 20 minutes (PND10).

In **Paper III** mice of C57BL/6 strain were used and the effect of deleting MerTK gene in PND10 (to mimic term human infants) mice was explored. HI was induced as described above but the duration of hypoxia was 50 min and exposure to 10% oxygen.

The changes made in **paper I** and **paper III** in hypoxia and oxygen exposure are due to the strain and their differences in response to HI (73).

GERMINAL MATRIX HEMORRHAGE MODEL

IVH/PVH is a serious complication of prematurity and if the GMH is severe, then IVH/PVH is usually a progression of GMH. The vasculature of GM is fragile because the lack of angiogenic vessels that lack pericytes, immaturity of basal lamina and lack of GFAP in the end-feet of astrocytes. The pathogenesis of IVH/PVH is complex but often described as; fragile vessels of the GM, cerebral flow disturbance and coagulation disorders (31).

For **Paper IV** PND5 (to mimic preterm human infants) rats were used to evaluate the neuroprotective effect of exendin-4 after a brain injury caused by hemorrhage. The rat pups in our collagenase model were anesthetized with isoflurane and GMH was induced via injection of 0.3U/mL collagenase VII into the striatum in the sub ventricular zone where the germinal matrix is located, using a needle connected to an infusion pump (1µl/min during 2 min). The position of the needle is based on a previous study (Fig.1) (74). The needle then remained in position for 1 minute to avoid backflow. The animals then rested on a heating pad set on 35° C and were returned to their dams when recovered from anesthesia.

The model applied was a modification of the Lekic et al. (75) model where a bleeding in the GMH is induced by injection of collagenase (74). The hemorrhage caused by injecting collagenase resembled a spontaneous bleeding, similar to what happens in preterm born infants suffering from GMH, where high levels of collagenase released from injured cells cause hematoma expansion and edema. An important part in the animal model is the inflammatory response and the development of secondary brain injury in the brain parenchyma especially in the gray and white matter in striatum. The collagenase versus the blood injection model showed that at day one after induced GMH the neutrophils were present in both models but only to a

significant extent in the collagenase model (76). At day 3 the neutrophils were almost all gone in the blood injection model but still present in the collagenase model (76). Another GMH model is the one with a direct single injection of blood into the ventricle. This model has undergone modifications over the years, and Roppert et al. (77) utilized blood from another rat, potentially introducing a disadvantage due to an inflammatory response not typically observed in the clinical settings. Regarding functional outcome between these two models, it is reported that in the blood injection model complete recovery was seen at day 21, where testing was done once a week suggesting a time dependent resolution of injury and a spontaneous recovery. Regarding the collagenase model there was no recovery observed at 10 and 15 days after brain injury and when testing for neurological deficits there was no improvement as far as up to 2 months after GMH, suggesting that no spontaneous recovery occurs and that the model is useful for evaluating long term neurological development disabilities (76).

Another often used GMH model is the rabbit pup model. This model is slightly different from the above-mentioned models since this is a model of IVH in prematurely delivered animals. The animals are delivered preterm with cesarian section in gestational week 29 and then given glycerol i.p. at 2-3h postnatally in order to induce IVH (78). There are several advantages of this model; the brain of the rabbit resembles the human infant with its gyrencephalic brain, perinatal growth, and an extensive GM, unlike rodents. The model also induces an inflammatory response around the ventricle, similar to what is reported in preterm infants. Neurological long-term outcome is possible to observe in this model. Preterm infants have a risk of spontaneous GMH, just like this model shows and increases the risk after i.p. administration of glycerol. Glycerol causes hyperosmolarity and rupture of the vessels in the GM leading to IVH (78, 79). There are a few shortcomings of this model that

have been described (78) firstly, the pups are orphaned after delivery and need the supervision of humans including hand feeding which requires a lot of experience, time, and staff. The rabbit pups are fragile, and the mortality is high, unlike the models in rodents. Another disadvantage is the high frequency associated subarachnoid hemorrhage seen in this model compared to what is reported in clinical studies (80).

Figure 3

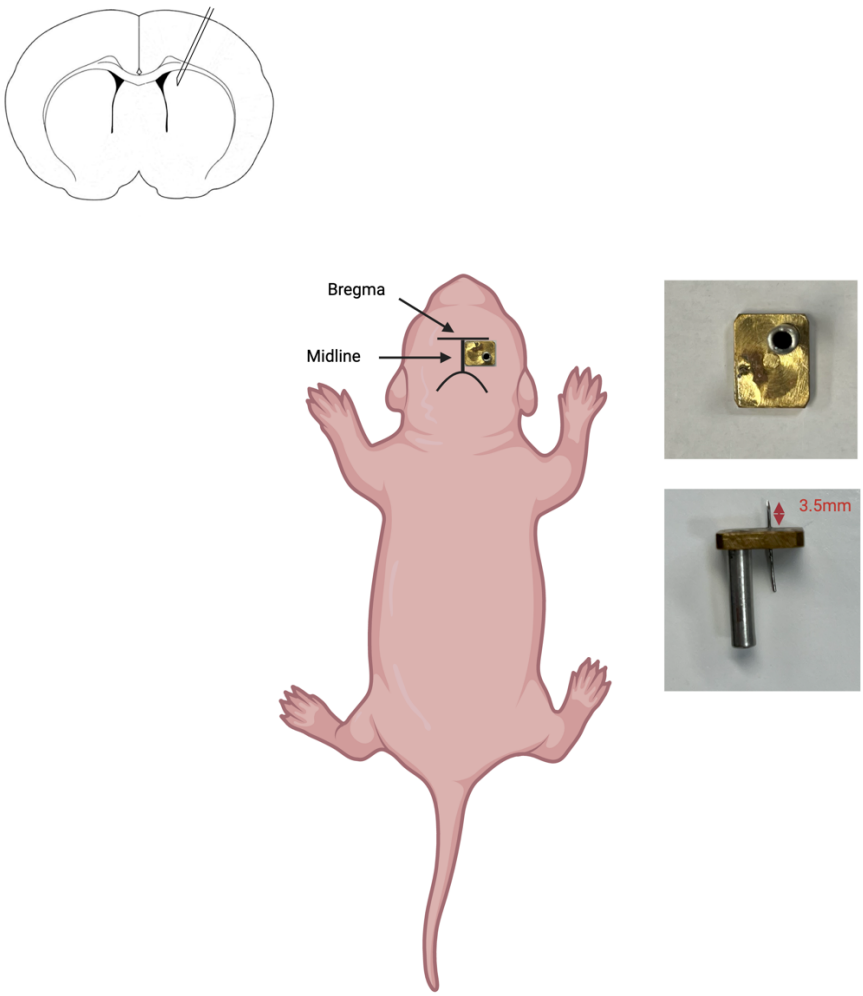

Figure 3 GMH model. *Position of the animal and tools for performing GMH surgery (medial striatum location). A 27G needle is placed in a plate with a holder to secure the right location, angle and depth for every injection made, as shown above. Illustrated by Andrea Jonsdotter.*

PATIENT RECRUITMENT (PAPER II)

In **Paper II** women who are at risk of preterm delivery were asked to participate in the study. The recruitment took place at the maternity ward at East hospital, Sahlgrenska University Hospital between 2017-2018. In total 49 women participated, both simplex and duplex pregnancies.

CRITERIA OF INCLUSION

Week of gestation 23+0 – 32+0

possibility of understanding the information and age over 18

cervix dilated 3 cm or more

rupture of membranes

preterm labor

expected to give birth within 24h

weight between 60-100 kg

CRITERIA OF EXCLUSION

Severe kidney dysfunction

Earlier reactions to $MgSO_4$

Severe liver disease

Fetus with known anomalies

$MgSO_4$ administered for other reasons e.g. preeclampsia

Estimated delivery within one h

Myasthenia gravis

IUGR with impaired blood flow

Patients who fulfilled the criteria of inclusion were given both written and oral information about the study and then signed the form of consent. The participation in the study was voluntary and the women could withdraw at any time.

CASE REPORT FORM

A case report form (CRF) was used to monitor every patient for adverse events who received $MgSO_4$. The midwife stated the weight and BMI before start and also checked the patient's creatinine to secure a normal kidney function. Then the parameters general condition, reflexes, hot/flush feeling, and respiratory rate were stated in the CRF before starting the infusion of $MgSO_4$. Directly after finishing the infusion and at time points 1h, 2h, 6h and 24h the midwife monitored the same parameters as mentioned above, but now also including diuresis.

DRUG ADMINISTRATION

INTRAPERITONEAL INJECTION OF EXENDIN-4 (PAPER I, IV)

For **Paper I** different dose regimens of exendin-4 were used to verify the most optimal treatment in the PND7 HI injury model, with initiation within the therapeutic window. Mouse pups were randomized to: (1) saline; (2) one high dose exendin-4 (0.5 μg/g) administered immediately after HI ; (3) four high doses of exendin-4 administered every 12 h, starting immediately after HI ; (4) four low doses of exendin-4 (0.05 μg/g) administered every 12 h, starting immediately after HI; and (5) four high doses of exendin-4 administered every

12 h, starting with a 2 h delay after HI. In the PND10 animals, exendin-4 (0.5 μg/g) and hypothermia treatment were combined, resulting in the following groups of animals: normothermia + saline; normothermia + single dose of exendin-4; hypothermia + saline; hypothermia + single dose exendin-4.

For **Paper IV** exendin-4 was given intraperitoneally (0.5μg/g) as a four-dose regimen 12h apart, starting within 10 minutes after GMH. Saline-treated animals were used as controls and saline ip injection was given at the same time points as exendin-4. Body weight was measured at each time-point of injection and daily afterwards for all different groups.

INTRACRANIAL STRIATAL INJECTION OF COLLAGENASE (PAPER IV)

GMH was induced by injecting collagenase VII into the striatum where the germinal matrix is located. The skull of a rat pup (PND5) is soft and can easily be penetrated by a sharp needle. To be able to find the exact place of injection a lamp was placed under the chin of the pup's head and important structures such as lambda, bregma and the midline were illuminated in the head and could easily be visualized. To be sure to inject at the exact right place during every procedure, a holder with a special needle (27G) and a metal plate has been developed. The depth of the needle is 3.5 mm, and the corner of the metal plate is placed at bregma and along the midline, to ensure the same location of injection every time, fig. 3.

IV INJECTION OF MAGNESIUM SULFATE (PAPER II)

After signing the informed consent form the patient was given 6 g of $MgSO_4$. The medication was diluted according to a specific manual written by a pharmacologist, with a duration of 20-30 minutes, administered either through an infusion pump or directly through a peripheral venous catheter. The patient was supervised for adverse advents when receiving the $MgSO_4$ and up until 24

hours afterwards. After the administration of $MgSO_4$ was finished, blood samples from the mother were analyzed at the following time points: directly after end of infusion and 1h, 2h, 6h and 24h after $MgSO_4$ administration. A blood sample from the umbilical cord was also taken directly after birth to analyze the concentration of $MgSO_4$.

HYPOTHERMIA TREATMENT

Hypothermia is now the only standardized treatment for term infants born with severe asphyxia and showing signs of HIE (grade II/III). The infants are cooled down to 33°C within 6h from delivery and during 72h (12). Within 10 minutes post HI, PND10 mice were given a single high dose of either exendin-4 (0.5 μg/g) or saline and were placed in individual sections within a hypothermia (33°C) or normothermia (36°C) chamber for 5 h, (no four-dose regime were applied after hypothermia, only single dose regime). One probe for each chamber monitored the temperature and one animal in each chamber was randomly selected to function as a temperature-monitoring sentinel to measure core temperature using a rectal probe. The sentinel was hereafter excluded and were only used as temperature controls, due to the stress the probe could cause and thereby interfere with the outcome of brain injury (81).

IMMUNOASSAY – ELISA (PAPER I, III, IV)

For **Paper I, III** and **IV** the immunoassay technique ELISA was used. In **Paper I** cAMP was used to analyze the transfer of exendin-4 from blood to the brain. cAMP immunoassay kit (Abcam) was used to analyze cAMP from brain tissue followed by a single dose of either exendin-4 or saline injection, at different time points (2h, 4h, 8h and 12h). In **Paper III,** ELISA was used to analyze the protein expression of MerTK and Gas-6 in naïve controls (PND 9, 10, 12 and 16) and in mice 6h, 24h, 72h and 7 days after HI to explore to what

extent these proteins were affected by HI. For **Paper IV** the technique was used to evaluate if exendin-4 had any effect on insulin in rat blood at different time points (0.5h, 2h, 6h and 12h) using a rat insulin ELISA kit (ERINS, Invitrogen).

BRAIN INJURY EVALUATION (PAPER I, III, IV)

IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

These two antibody-based techniques immunohistochemistry (IHC) and immunofluorescence (IF) were used in **Paper I, III** and **IV** in this thesis. When performing the staining procedures, a general protocol was followed, involving tissue preparation, the use of primary antibody to detect the antigen in the tissue and blocking steps. Depending on what technique to use, a secondary antibody conjugated to biotin (IHC) or fluorophore (IF) is used. To evaluate and visualize the staining a confocal microscope is often used for the IF.

In **Paper I** PND7 mice were killed 12h after they received the last high dose of exendin-4 (4 dose regime, 12h apart). Naïve, saline and exendin-4 treated animals were used and organs collected for staining were brain, heart, spleen, liver, lung, pancreas, and kidney. Staining chosen for histopathological assessments were hematoxylin and eosin. For the same paper the evaluation of neuropathological markers was done by using immunohistochemistry. PND7 mice were perfused with 4% paraformaldehyde (PFA) 48h post HI and snap frozen. PND10 animals were perfused the same way but 7 days after HI. 40 μm thick coronal brain sections, starting from corpus callosum, were collected for immunohistochemistry, histochemistry or immunofluorescence. Postmortem brains from preterm neonates were used and fixed in 4% formalin

before anatomical sections from frontal, occipital and parietal lobe were selected. The sections were then cut at 6 μm thickness using a microtome.

For **Paper III** (PND13 and PND17 mice) **and IV** (PND6, PND7, PND10, PND16 and PND40 rats) brains were dissected out and immersion fixed in paraformaldehyde 6%, (Histofix). The brains were then dehydrated (series of different alcohol concentrations and clearance of xylene) and embedded in paraffin and then cut (Meditome A550, for thinner, cryostat-thicker) into 7μm thick sections throughout the brain.

In **Paper I** (only MAP-2)**, III and IV** antibodies against MBP and MAP-2 were used to analyze white and gray matter respectively. MBP identifies the white matter and myelin present, and the loss of MBP after staining can serve as a measure of the reduced amount of myelin (82). MAP-2 labels neurons and the gray matter can be delineated and loss of MAP-2 staining is thereby a marker of neuronal death (83). For **Paper III** we analyzed microglia and neuron morphology through staining with NeuN (neurons) and lectin (microglia). While the counting and analysis were conducted in 2D, additional validation was performed through 3D sectional sampling to ensure accuracy and consistency.

MICROSCOPY AND IMAGE ANALYSIS

Brain sections stained (IHC and IF) were used for analyzing and imaging by different microscopical techniques. In **Paper III and IV** images stained with MAP-2 and MBP were captured using a light microscope Olympus BX60. The images were then analyzed using Fiji-build of ImageJ software. The injured areas were analyzed and manually outlined, and area loss was calculated using the form: (contralateral side – ipsilateral side)/ contralateral side x 100%).

MAP-2 staining in **Paper I** was calculated the same way as described for Paper III and IV.

In **Paper III** a 3D reconstruction of microglia was done (visualizing neurons engulfed by microglia) using immunofluorescence images captured using a confocal microscope Carl Zeiss LSM800, for **Paper I** a confocal microscope Zeiss LSM710 (Carl Zeiss) was used for animal sections, and for postmortem neonatal sections a Leica SP5 confocal microscope was used. Z-stacks were acquired for 3D visualization.

RNA SEQUENCING (PAPER I, III)

GENE ANALYSIS

Gene analysis was performed to examine the gene expression in the mice brain after HI. The sequencing data shown in **Paper III** was performed by Qiagen and generated using Illumina Inc. technology. The total RNA was extracted from tissue from naïve, MerTK WT and MerTK KO mice at different time points, 6h, 24h and 72h after HI and for naïve animals-PND10, 11 and 13 days by using miRNeasy mini kit from Qiagen, RNA quality check and mRNA Next Generation Sequencing was done by Qiagen.

QUANTITATIVE POLYMERASE CHAIN REACTION (PAPER I)

To analyze the distribution of the glucagon-like peptide-1 receptor (GLP-1R) gene expression, PCR was performed at different brain regions using naïve brains at PND7, PND10 and 10 weeks. RNA was isolated according to description from Qiagen RNeasy Mini kit. The extracted RNA was transcribed into cDNA using High-Capacity cDNA Reverse Transcription kit (Applied

Biosystems) and analyzed using RT-qPCR (retro PCR) using a StepOnePlus (Applied Biosystems). Primers were set to detect mouse GLP1R.

CYTOKINES AND CHEMOKINE ASSAY (PAPER III)

In **Paper III** we performed an immune assay technique to quantify chemokines and cytokines from protein samples from WT and KO animals at 6h and 72h. The assay used was 7-Bioplex according to Bio-Rad instructions. In the Bio-Plex method the antibodies are bound to different beads and after incubation with samples, biotinylated secondary antibodies, and Streptavidin-PE the Bio-Plex 200 machine (Bio-Rad) sort the beads and measure the fluorescence signals.

MOTOR FUNCTION AND BODY WEIGHT (PAPER I, IV)

BODY WEIGHT

Body weight of mouse pups in **Paper I** and rat pups in **Paper IV** measured daily, with start of PND4 (**Paper IV)** and PND7 and PND10 (**Paper I**) and at every time of injection for all groups.

MOTOR FUNCTION

In **Paper IV** different developmental behavioral tests were performed. *Negative geotaxis* was performed PND7 - 10 to evaluate the time required for the rat pup to rotate 180° upwards from being placed downwards on a 20° slope. This abnormal body-head position will stimulate the rat to turn around upwards. This test is an automatic movement against gravity and is used in newborn rodents to test geotaxis(84).

Eye opening latency was noted on both eyes daily until PND16, when all animals had both their eyes open.

To evaluate motor coordination and balance the *Rotarod test* was used at PND20 and PND40. This was done with animals sampled from a separate cohort. The rats were placed on the rod with an initial constant speed of 4 rpm that was over time accelerated up to 40rpm over a time period of 300 seconds, where fall latency was recorded, every animal underwent three trials, with a rest of 15-20 minutes between each trial. Another motor function test to assess potential motor asymmetry was the *Cylinder rearing test.* Rats were put in a transparent glass cylinder at PND21 and PND41 and were recorded by a video for 5 minutes to quantify forepaw preference during lateral exploration and full rearing. A mirror was placed below the cylinder for a better observation.

Regarding the animals in **paper III** and **IV,** all the behavioral tests, measurements and immunohistochemistry were analyzed by researchers blinded to group belonging.

STATISTICAL ANALYSIS

For **Paper I** Data was analyzed using the GraphPad Prism v6.0. All assessments were performed without knowledge of group belonging to avoid bias. Average ± standard error of the mean (SEM) was recorded for all data and was first analyzed with the Kolmogorov–Smirnov normality test. As the data did not follow Gaussian distribution, the Kruskal–Wallis non-parametric test was applied, followed by Dunn's test. In **Paper II** GraphPad PRISM 8 version 8.1.1 was used. *t*-test, Mann–Whitney test, simple linear regression analysis and Spearman rank were applied as appropriate. Pearson's test was used for normal distribution analysis. A two tailed p-value <0.05 was

considered statistically significant. For **Paper III** GraphPad Prism version 9.0 was used and for **Paper IV** GraphPad Prism version 10 for the statistical analyses and the average ± standard error of the mean (SEM) was obtained for all the data. The normality was first checked using the D'Agostino and Pearson. The data that passed the test was analyzed using unpaired t test. If the data did not follow the Gaussian distribution, then the Mann-Whitney test was used for comparisons involving two groups and the Kruskal-Wallis's test followed by Dunn's or Tukey´s multiple comparisons post hoc analysis for comparisons involving three or more groups. A p-value less or equal to 0.05 was considered statistically significant.

ETHICAL PERMISSION

The ethical approvals for these four studies were received prior to study initiation. All studies have ethical permission from the Regional Ethical Committee in Gothenburg (Dnr 61-2014/62-2016, 385-17/2020-07258, 1-2016/ 5.8.18-04092/2019, 5.8.18-06407/2020) and from Läkemedelsverket (Medical Product Agency, Dnr 5.1-2018-12254). Paper 1 also have ethical permission approved by the Ethics Committee of the University College London and UK Home Office Guidelines (PPL PCC 436823) and REC: 07/H0707/139

Written informed parental consent was acquired in accordance with the National Health Services (NHS) UK guidelines, and ethical approval was obtained from the National Research Ethics Services (West London), UK (ethic number: 07/H0707/139). For paper II all women and their partners signed written informed consent after given both oral and written information about the study, before entering and participation in the study. All participation was voluntary.

SUMMARY OF RESULTS

This is a summary of the results from each paper attached. More detailed information about the results can be found in the individual papers. Despite that the four papers focus on different subjects and are both preclinical and clinical studies they all have a common goal, i.e. to investigate and achieve a greater understanding of the mechanisms and responses in the immature brain after brain injury and thereby, develop novel treatment strategies.

PAPER I

The saline treated groups of HI animals, after staining and measurements, showed consistent 50% (± 6.9%) hemispheric tissue loss. A single dose (0.5 μg/g body weight) of exendin-4 reduced tissue loss to 17% (± 8.6%; p=0.027) (Fig. 4) and the four-dose regime group provided almost a complete protection (tissue loss: 2% ± 1.8%, p=0.021). A diluted version (0.05μg) of the high dose exendin-4 was also given directly after the HI insult but did not result in any protection. For many newborns that develop brain injury there is a desire to have the possibility to delay the treatment. Therefore, we aimed to evaluate if it was possible to delay the treatment and still obtain the same protection. Exendin-4 was given in a high dose regime with start 2h after the HI insult with a four-dose administration, and again we found a significant (p=0.033) neuroprotective effect (hemispheric tissue loss: 11.0% ± 6.9%) (Fig 3). Microglial activation was measured and showed significantly lower activity in the four-dose regime of exendin-4 (p=0.00006) and in the 2h delayed group (p<0.0001) (Fig 5). It's known that exendin-4 reduces appetite (58) and the weight in exendin-4 treated animals were significantly lower than in the saline treated group with a peak at 24h (p=0.007) and continued until 48h for the four-

dose high regime (fig. 6). For the single high dose exendin-4 this was not found.

The high dose of exendin-4 used in this paper to gain the neuroprotective effect is a higher dose than used in clinical setting (0.1μg/kg) to treat patients with diabetes type II. The high dose of exendin-4 (0.5μg) used in paper I and IV is based on the report from Teramoto et al (85). Hypoglycemia is a serious condition for infants with HIE. Therefore, we monitored blood glucose at different timepoints after injection with high dose of exendin-4, and blood glucose was not altered in PND7 naïve mice after high dose of exendin-4 (four dose regime every 12 h over 48 h) compared to saline treated (Fig 6). Weight loss was also observed in exendin-4 treated naïve animals due to loss of appetite, but weight partly recovered to baseline after 48h. High dose exendin-4 treatment was evaluated together with hypothermia in PND10 mice and exendin-4 enhanced the neuroprotective effect of hypothermia when given together resulting in a significantly decreased tissue loss evaluated 7 days after HI, compared to the saline + hypothermia treated group (Fig 8). Since exendin-4 is administered systemically, several organs were sampled and analyzed for adverse outcomes due to exendin-4 treatment. Staining of these organs did not show any abnormalities, or fibrosis compared to saline treated or naïve mice. Inflammatory response in the organs was not altered in exendin-4 treated animals compared to controls using a macrophage specific marker, CD68 (Fig. 7).

PAPER II

In **paper II** we aimed to evaluate the concentration of Mg, in both the mother and the infant after giving a bolus dose of 6 g $MgSO_4$. It's well-known that Mg can cause flushes and discomfort, therefore all women were supervised using the CRF as mentioned in material and method, and none of the women showed

signs of illness or affected general condition during the infusion or after. Therapeutic concentration in the women was set between 2.0-4.0 mmol/L according to a previous study (86) and none of the included women reached a concentration of Mg > 3.3 mmol/L. $MgSO_4$ was given either through an infusion pump or manually and those women receiving $MgSO_4$ manually reached a higher concentration of Mg (1.8 - 3.3 mmol/L) compared to (1.4 – 2.8 mmol/L) in those administered with an infusion pump. BMI was recorded of all women included and, as previously shown in reports (87), patients with a higher BMI might need higher doses compared with patients with lower BMI. We confirmed a descending concentration of Mg with increasing BMI of the woman, ($p=0.03$). Concentration of Mg in the blood of newborns did not exceed 2.5mmol/L in any of the samples (range: 0.87 – 1.4 mmol/L). According to previous reports, levels over 2.5mmol/L in Mg concentration is associated with adverse outcomes and an increased need of neonatal intensive care unit (88, 89). None of the neonates had any adverse outcomes when checked after delivery.

PAPER III

In **paper III** we conducted a study to explore if the MerTK receptor was present in the neonatal brain and what would happen after HI brain injury in MerTK animals with and without the MerTK receptor. MerTK was present in the neonatal brain. MerTK and its binding protein Gas-6 were both upregulated after HI at different timepoints and MerTK reached a significant peak at 24h, ($p<0.01$) compared to controls, no statistical differences in other time points. Gas-6 reached a significant peak at 24h ($p<0.002$), 72h ($p<0.004$) and 7 days ($p<0.002$) compared to controls (fig. 4). When deleting the gene for the MerTK receptor we saw a significant decrease in brain damage/tissue loss in gray matter (by 48%) and in white matter (by 32%) 7 days after HI in MerTK knock-

out (KO) animals vs wild-type (WT) animals, ($p=0.002$ and $p=0.04$ respectively) (fig. 5). Confocal microscopy using NeuN (neurons) and isolectin (microglia) staining (fig. 6) showed a significant reduction in neurons engulfed by microglia in striatum, ($p=0.03$) enhancing the results concerning less brain atrophy in MerTK KO vs WT animals, the mean percent of microglia with NeuN immunoreactivity in MerTK KO was ($11 \pm 3.0\%$) vs ($18.0 \pm 2.4\%$) in MerTK WT. Genes related to inflammation, phagocytosis, cell death and Wnt/β catenin pathway were significantly differentially expressed (FDR < 0.05) in MerTK KO vs WT animals, where chronic inflammation indicated as a top biofunction (Fig. 7,8). To further investigate the inflammatory pathway different cytokines were analyzed and there was a marked reduction in IL-6 and MCP-1 in MerTK KO vs WT animals (fig. 9). Also, caspase-3 was found to have reduced levels in MerTK KO vs WT animals.

PAPER IV

In the GMH model, exendin-4 showed neuroprotective effects including reduction of caspase-3 positive cells in striatum, microglia inflammation and reduced number of neutrophils in the hemorrhage area compared to saline-treated animals. Exendin-4 demonstrated neurodevelopmental improvements such as reduced eye-opening latency (fig. 5A) and improved negative geotaxis compared to saline-controls after completion of four doses of exendin-4 (0.5μg) (fig. 4A). Long-term functional improvements shown in the rotarod test where the falling latency was comparable with naïve controls, indicating improved/normalized motor coordination in exendin-4 treated animals (fig. 6A). This study showed that initially after a first high dose of exendin-4 there was an increase of glucose levels, that remained until 2h but then a glucose tolerance was noticed. Ketone bodies were altered in exendin-4 treated animals, as expected due to the known satiating effect caused by exendin-4

(90). This satiating effect, known in animal models (91) is also the reason to the initial weight loss in exendin-4 treated animals. However, by 24h they started to gain weight and by PND8 the weight was comparable to saline-controls. The exendin-4 neuroprotective properties seem to function in a multifactorial way. It's known that after a GMH insult, microglia and caspase-3 are activated (80) which in this study were shown to be markedly reduced by exendin-4 treatment, where caspase-3 counts were decreased by 50%, ($p=0.006$) in line with exendin-4's known anti-apoptotic properties (92). Exendin-4 treated animals after GMH showed a significant decrease in brain injury (MAP-2) compared to saline treated controls as soon as 48h after brain injury ($p=0.05$ striatum, $p=0.03$ in the hippocampus level) and persisted all the way until PND40 in gray matter ($p=0.0001$) and in white matter ($p=0.0001$). The vessels around the injured area after GMH is known to break down after injected collagenase (74). We analyzed matrix metalloproteinase-9 (MMP-9) a known protease that degrades the membrane of endothelial walls (93), and found that MMP-9 is reduced by 65% in exendin-4 treated animals compared to saline treated controls. Neutrophils infiltration was also shown to be reduced in exendin-4 treated animals compared to saline treated controls.

DISCUSSION AND CONCLUSION

The pathophysiology of neonatal brain injury is complex. Perhaps this is why it is difficult to determine when and how to treat and why it might be hard to find an optimal treatment. Even though neonatal care is improving, and many more preterm born infants survive, there is still no treatment for brain injury for preterm infants and for those born at term, the only option is hypothermia. Many major breakthroughs within this field have been done and hopefully many more are on its way. This thesis will hopefully contribute to valuable information about mechanism of injury and a potential new therapeutic strategy.

EXENDIN-4 NEUROPROTECTIVE EFFECTS IN THE IMMATURE BRAIN

Experiments conducted in this thesis consistently demonstrate that a high dose (0.5µg/g body weight) of exendin-4 significantly reduces brain damage, following both HI and GMH in the immature brain. This is in agreement with previous studies, that show brain protection in the adult brain (58, 85) after treatment with exendin-4. In **paper I** we showed that exendin-4 reduced brain injury in PND7 and PND10 old mice after HI, both if administered immediately or with a delay of 2h after HI. This result is highly promising because many infants born with severe asphyxia are not delivered at hospitals equipped with neonatal intensive care units and require transportation. It is also promising for those infants born in low resource countries where hypothermia is not available, since exendin-4 provides marked neuroprotection given alone without hypothermia. Another aspect where hypothermia has no effect, is infection, (94) which could cause and/or aggravate brain damage in both preterm and term infants, and here is also an area where exendin-4 could have

a place as a therapy, but so far no reports have been published on the effect of exendin-4 in models of combined infection and HI. The efficacy also with a 2h delay of exendin-4 administration is promising as it expands the therapeutic window.

Exendin-4 is known to easily cross the blood brain barrier (BBB) and quickly, within minutes, enters the brain vascular endothelial cells actively and exendin-4 is then transported across the BBB, via PKA that bind and activates the GLP-1R (95). There are other GLP-1 analogues, and lixisenatide is one of them. Lixisenatide as well as exendin-4 cross the BBB easily and interact with the GLP-1R, they both have about the same half-time (2-3 h) and similar molecular weight (3-4 kDa), but exendin-4 expresses more homology with human GLP-1 which might suggest that it could in our case, exhibit a better neuroprotective effect (96). This is in line with our results in **paper I** where cAMP was measured after administration of one dose of exendin-4, and a significantly higher cAMP level in the brain at 2h and 4h was observed when compared to saline treated controls, suggesting that exendin-4 does cross the BBB easily. Gilland et al (83) reported that glucose utilization is markedly decreased and a MAP-2 loss was noticed already at 24h, pointing towards that most of the brain injury had become irreversible by that time (83), which emphasizes the importance of early intervention. In **paper I** we could conclude a significantly reduced brain damage in animals treated only with exendin-4 as well as the combination of hypothermia and exendin-4 (Fig. 8**)**. This makes exendin-4 an even more valuable drug, due to its neuroprotective qualities alone, and also as an additive drug to hypothermia, and hence a future candidate in the treatment of neonates with HIE. Hypothermia as treatment is not enough for those infants born with severe HIE and severe abnormalities on EEG (97), therefore there is an urgent need for an add-on treatment for these infants. Due to exendin-4 neuroprotective effects in the HI model, we wanted

to further explore exendin-4 neuroprotective effects in a preterm model. In a review by Singhi et al they described that delayed cord clamping could be associated with decreased IVH but this was not statistically significant compared to controls (98). Our results showed a significant increase in ketone bodies (equivalent to mild ketonemia) after exendin-4 treatment that could be explained by the satiating effect of exendin-4. We also noticed the reduced weight gain during treatment in those animals compared to naïve. This is resolved after the last exendin-4 injection and may not be a problem in extremely born preterm infants who are treated in the neonatal intensive care unit, monitored intensively which also involves their caloric intake and measurements of blood glucose. In our study we could show that many of the pathological processes occurs around 24h. This is in line with previous studies, which described that the cascade of toxic substances usually occurs after the reperfusion state and secondary injury and set the degree of the final brain injury (17). GMH happens quickly and most of the cases are present by the first 24h of life, with hemorrhage causing tissue destruction (99). During this period there is an overload of free iron, free radicals, and inflammation due to the release of blood products after the hemorrhage (77), that will contribute to neuronal and myelination loss, and the final brain injury (99, 100). Due to these results in **paper IV** and the knowledge about phases in brain injury development, there is a need to find treatments that can be administered easily and quite directly after the assumed insult to be able to inhibit the toxic cascade leading to brain damage. It is known that exendin-4 has the possibility to cross the BBB (58, 85), and in our study we could show that exendin-4 inhibits several neuroprotective effects, such as anti-inflammatory with reduced microglia activation, reduced neutrophil infiltration and reduced MMP-9 counts in exendin-4 treated animals compared to saline treated. Previous reports show that GMH increases MMP-9 levels (101) and that it breaks down the membrane of the endothelial walls. This could, together with the

knowledge that exendin-4 is present in the endothelial cells (85) suggest that exendin-4 protects the vascular unit through inhibiting MMP-9, hence stabilizing the vascularity of the GM, followed by less microglia activation due to reduced infiltration of neutrophils. In severe GMH the bleeding could expand into the periventricular white matter, which could lead to adverse neurodevelopmental outcome in infants (102). In this study there is a delay in neurodevelopment in saline treated GMH animals, on the contrary, exendin-4 treated animals resulted in improved negative geotaxis as well as improved eye opening where 70% of exendin-4 treated animals had opened their eyes at PND14 compared to 55% in saline treated controls. These positive results were also in line with the histologically measured protection in gray and subcortical white matter. Motor function impairment is another sequelae of GMH (99) and in this study we could report improved outcome in motor coordination (rotarod test) in exendin-4 treated animals compared to saline treated. This improved recovery was also in agreement with the reduction in tissue loss seen in MAP-2 and MBP measurements after four high doses of exendin-4. Manaenko et al conducted a report about different GMH models, where the collagenase model mimics spontaneous IVH seen in the clinical setting and would be a good choice of model to evaluate both the inflammatory response and functional outcome (76).

As exendin-4 is easily administered, low risk of side-effects and without expensive equipment needed (as with hypothermia), exendin-4 is a potential upcoming candidate for treatment of term and maybe preterm infants at risk of developing brain injury, either alone or for term infants in conjunction with hypothermia.

THE OPTIMAL DOSE OF MAGNESIUM SULFATE

Uncertainties persist regarding the optimal dosage for achieving neuroprotection, as well as concerns regarding the gestational week up to which $MgSO_4$ administration can effectively yield neuroprotective effects (63). Due to these aspects, Sweden has actively awaited the implementation of $MgSO_4$ as a neuroprotective drug to women at risk of preterm birth. A relatively recent report by Koning et al presented that 1.1 mg/kg in rat pups (equivalent with 6 grams in infants) was acquired as an optimal dose for neuroprotection (103) due to its preconditioning effect. We could conclude in our paper that 6 g of $MgSO_4$ was well tolerated by all the women and infants receiving $MgSO_4$ and no adverse events were noticed in either the women or their newborn infants. All women and infants receiving $MgSO_4$ were within the desired s-Mg concentration of what is stated as safe levels (104). As shown in previous clinical trials, the dose probably needs to exceed 5 g to gain neuroprotection, and no differences in neuroprotective effects were noticed if given as a bolus dose without infusion or with infusion (63, 66, 105). Previous reports have raised a concern regarding severe adverse events in infants receiving high doses of $MgSO_4$, Rouse et al gave as much as 33 g as a mean (66) but this has not been reproduceable by any other research group (63), but on the other hand, a dosage that is too low appears to lack the desired neuroprotective properties (106). Rouse et al performed a study where they administered 6g as a bolus dose followed by an infusion of 2g/h (62). This study showed that mortality was higher in preterm born infants if serum magnesium exceeded 2.25 mmol/L (104). None of our infants reached a level higher than 1.4 mmol/L in magnesium in the umbilical cord, all 36 blood samples ranged between 0.87 – 1.4 mmol/L. These values represent infants born as early as 34 minutes after completed infusion up to 23h after infusion

and none of these infants exceeded 2.5 mmol/L in serum magnesium that may require neonatal intensive care (86, 89). Another aspect of choosing a single bolus dose as in **paper II** is the logistic and assets of staff at the delivery wards. Chollat et al described in their report that compliance was lower in the group of patients receiving a bolus dose followed by infusion due to the need for maternal monitoring (107-109). In our follow up study regarding the feasibility of receiving a single bolus dose of $MgSO_4$ we could show a nearly 90% administration rate of $MgSO_4$ (110) suggesting one of the factors contributing to this is the relatively easy way of administering the drug. Another important aspect of only administer once, as a bolus dose is the maternal side effects, including nausea, sweating and flushing due to the vasodilatory effects of $MgSO_4$ (67). Another concern regarding drugs and administration is the BMI of the patients. When consulting guidelines for medication administration, typically there is only one guideline for adults based on weight, without subdivisions, unlike those based on parameters such as renal clearance. This is likely a widespread issue within healthcare and likely, a lot of medications would need adjustments based on the patient's weight. In our study we could show that patients with lower BMI tolerated $MgSO_4$ well and achieved the targeted concentrations in the blood. We could show that patients with higher BMI, tended to have lower concentrations of s-Mg compared with those patients with a lower BMI. This is in line with what Vilchez et al showed in their report where obese women did not gain the neuroprotective effect of $MgSO_4$ suggesting that they might need a higher dose (87). This suggests that patients with a higher BMI need a higher dose of $MgSO_4$ than 6 g to reach the same neuroprotective effect. This conclusion is scarce, due to the few patients included in this subgroup analysis, and a larger group of patients are needed to draw any firm conclusions. Gentle et al reported that extremely preterm born infants benefit from the combined treatment of antenatal $MgSO_4$ and corticosteroids with reduced risk of neurodevelopmental disability or death

compared to those infants only receiving antenatal corticosteroids (111). Since this was a prospective cohort study, further clinical randomized studies are needed to confirm that this association indeed exists, as well as additional studies overall for $MgSO_4$ regarding dosage and type of administration.

THE ROLE OF MERTK IN BRAIN INJURY

The MerTK receptor is a known phagocytic receptor located on microglia and contributes to removing dead cells and maintaining homeostasis (43). Due to the need of better understanding of the underlying pathophysiology, we decided to explore the role of MerTK in the immature brain after brain injury. Brown et al (44, 45) has conducted studies in animal models of adult stroke where the gene for MerTK was deleted which resulted in a neuroprotective effect in these animals. We wanted to investigate whether phagocytosis also plays a role in the immature brain and whether gene deletion could confer a protective effect in a neonatal animal model of HI as well. MerTK is present in many different cells and inhibits many biofunctions which makes it promising as a therapeutic target but also difficult due to its many functions (112). MerTK is intensely investigated as a target among cancer therapies and clinical trials are ongoing (112). However, little is known about MerTK and the immature brain. In line with the Brown et al findings (45) we found a marked reduction in tissue loss of both gray (48%) and white matter (32%) in MerTK KO animals compared to WT using MAP-2 and MBP, suggesting that neuronal cell death by phagoptosis also occurs in the neonatal brain and deletion of the MerTK gene is protective, however the mechanism for this protective effect is not fully understood. The MerTK gene deficiency influenced the inflammatory response which proposedly could contribute to the neuroprotection.

Gentle et al report the extracellular domain of MerTK as a potential target, where it can be cleaved from the surface by a metalloprotease, producing a soluble product of MerTK acting as a ligand, and thereby inhibiting the MerTK phagocytotic function (112). The removal of debris and dead cells by phagocytes is a known process (113) including recognition and engulfment of dead cells. Ligands appear on the dead cell as 'eat me signals' and bridging molecules and phagocytotic receptors on the macrophage act towards an engulfment and thereby the inflammatory response is attenuated, (fig.2) (45, 114). An interesting finding is that in MerTK KO animals the macrophages recognize and bind to dead cells but were not able to engulf them, suggesting that MerTK is needed in the phagocytotic process (112). The term phagoptosis describes the process whereby phagocytosis can execute the death of stressed and damaged but still viable neurons (43, 44), hence if these neurons would be saved the brain damage would be reduced. Conditions that stress neurons (inflammation due to infection for example) will lead to the release of a cascade of toxic substances and microglia will be activated to induce phagocytosis (46). We cannot know for sure what the protection provided by MerTK gene deletion is entirely due to and how exactly the diminished phagocytosis of still viable neurons occurs. Is it the absence of the MerTK gene? Or the TMEM16F that regulates the PS activation and can flip the ligand back so that no 'eat me signal' is present and thereby no phagocytosis will occur? Or is it the decreased inflammatory response, that we saw in the IL-6/STAT3 expression, activating less microglia? When 'eat me signals' are not present, but there is still an injury to the neurons, but still viable, could this promote microglia towards a more reparative and tissue modeling state, instead of phagocytosis? Or could it point towards less activation of microglia and thereby less activation of IL-6/STAT3? To better understand what is happening during brain injury we conducted RNA sequencing and made a further analysis of different genes involved in the injury process (genes

involved in cell death, inflammation, and phagocytosis). What is interesting is that when conducting a heat map (fig. 7 in paper III) there is something happening in KO control P10 animals already before the injury, where some genes are upregulated. Could this suggest that by deleting the MerTK gene we modulate the response to HI which theoretically could exert a preconditioning effect and thereby contribute to brain protection. However, scrutinizing the genes up- or downregulated by MerTK gene deletion did not reveal any change in gene pattern previously associated with preconditioning protection (115, 116).

FUTURE PERSPECTIVES

There are still many questions to be answered concerning brain injury among newborns, but with the findings within this thesis together with its clinical importance, hopefully it might lead to improved novel treatment for those infants.

Exendin-4 is a promising new upcoming treatment in both preterm and term born after a hypoxic-ischemic event and has shown great neuroprotective properties in animal studies and in human adult studies so far. Even though we showed promising results in both **paper I, IV,** these are rodent studies and exendin-4 need to be further explored before it can be used in infants. There are promising preliminary reports also in newborn rabbits and fetal sheep models and ongoing experiments in piglets which hopefully will tell us more about the potential for clinical implementation of exendin-4 in HIE.

In **Paper III,** we try to highlight the role of phagocytosis in neuronal cell death after HI and specifically the role of the phagocytic receptor **MerTK**. What is currently understood is that many stressed but viable neurons are phagocytized by microglia and maybe also by invading macrophages which needs to be investigated in the future. Hypothetically, there could be a window of opportunity to rescue these neurons by preventing phagocytosis and neuronal death. What needs to be further explored is the possibility to do imaging of live cell phagocytosis. Our results showed a protection when deleting the MerTK gene, and a change in mainly the inflammatory response, but what exactly in this complex cascade of opsonins, cytokines and microglia phagocytosis is critical to provide neuronal rescue is unknown. This study only included mouse pups equivalent to term born infants, a future study could be of interest regarding the role of MerTK inhibition in a model relevant for brain injury in preterm infants.

There are still many questions regarding the optimal time point when **$MgSO_4$** should be given and more studies need to be done within this field to confirm the optimal dose, both through a neuroprotective as well as a safety perspective. The conclusion from our study contributes with the knowledge that 6 g of $MgSO_4$ is safe for both the mother and the infant, and that this dose achieves what we think is an optimal concentration for offering neuroprotection. Hopefully the follow up study will point us closer to an answer, by evaluating the potential effects of MgSO4 on motor function and cognitive variations at 2 and 5 years of age of those infants receiving $MgSO_4$ compared to those who did not receive any treatment (before $MgSO_4$ was introduced in Sweden as a national guideline). A subset of infants that needs further attention concerning receiving $MgSO_4$ are those infants born between gestational week 22-23. This attention is warranted due to advancements in neonatal care resulting in a higher survival rate among these infants. Consequently, they have been underrepresented in the studies conducted internationally so far. A growing issue is also obesity and as shown in this paper and by Vilchez et al (87) patients with higher BMI don't reach the same concentrations of s-Mg as those with lower BMI. We need to further explore if these patients need a higher dose of $MgSO_4$ to gain the same neuroprotection as the ones with lower BMI. Another question being unanswered when initiating this treatment is the infants being born at term, but who received $MgSO_4$ during pregnancy due to complications that could cause preterm birth. Is 6 g of $MgSO_4$ harmful for those infants?

In summary, many breakthroughs have been performed within this field the past years, but a lot remains. Hypothermia is now the standard care after HIE but only for a few infants. We need to find an add-on treatment to hypothermia to be able to help these infants that suffer from a severe HIE where hypothermia is not sufficiently effective, here exendin-4 may hopefully be a

potential candidate in the future. Corticosteroids are a well-documented drug used in preterm birth and is known to improve lung maturation and reduce neonatal death, perinatal death and IVH (98). Corticosteroids are also shown to stabilize the germinal matrix vasculature, which could possibly be a cause of the decrease in IVH (98, 117). Remaining questions when and if exendin-4 will be approved as a drug for infants in the future, is to assess the potential effect when combining corticosteroids and exendin-4. Finally, the mechanisms behind brain injury in term and preterm born infants are complex, involving various pathways. Phagocytosis has been shown to be an important part of neonatal brain injury development. There is likely no single treatment that universally benefits all individuals; instead, a multimodal approach is required to achieve the best outcome. $MgSO_4$ and hypothermia have demonstrated the ability to mitigate the effects in the brain after an insult, and hopefully in the future there will be even more therapies available.

ACKNOWLEDGEMENT

Ylva Carlsson my main supervisor, who guided me through this PhD with patience and engagement. Your energy for research is amazing and a true inspiration! Thank you for your time and great knowledge and for letting me be a part of these research projects!

Henrik Hagberg my co-supervisor, who found me at the coffee machine at the department of obstetrics and asked me if I was interested in research. Thank you for finding me and inviting me to an amazing group at PMC. Thank you for letting me experience the world of science. And thank you for all your expertise, fun talks, and genuine engagement, always making me a bit smarter for every discussion we have.

Eridan Rocha Ferreira my co-supervisor, thank you! I'm so glad to have got to know you better during this travel in life! Thank you for your encouragement, support, and great knowledge within a lot of fields, but a special thanks to your great knowledge and amazing patience with me and my total lack of computer knowledge when I needed it the most. Always there to help, always there to support, always up for a zoom meeting answering all questions, no question stupid enough. Hopefully, not too far away, there will be a house in the southern of France, with vineyards gathered around and a lot of great red wine where we can continue our fun talks and relieving laughs.

Anna-Lena Leverin, thank you for your expertise, for always answering the same dumb repeated questions, for always helping out. Thank you for all the fun and good talks. I will miss you and the good times together.

Thank you **Syam Nair** for always helping out and always answering to my questions**, Gisela Nilsson** for contributing to interesting and valuable questions

about my projects, and for all fun times around lunch table, **Pernilla Svedin** for all the technical expertise in the lab, always helping out and especially for all the laughs and fun times. **Joakim Ek** and **Kerstin Ebefors** for saving me the last minute before Christmas, you know what I mean. I'm truly grateful for that! I also want to say thanks to all my present and past colleagues of PMC for making my time at the lab so interesting and inspiring, and last but not least, thank you for all the fun, it's truly an amazing place to work at.

Thank you, **Maria Forslund** and **Helena Hognert** current and previous head of department, of gynecology at Sahlgrenska University Hospital, for giving me the time to finish my research.

Randa Akouri, Anders Elfvin and **Elisabet Jerlhag** for attending and giving me constructive feed-back on my half time seminar, thank you.

Thank you to all my wonderful colleagues at the department of Gynecology and Obstetrics and reproductive Medicine for your support and for working at the clinics so I could finish my research. A special thank you to my dear friend and colleague **Josefin** with whom I shared a lot of thoughts and great laughs with during the time we wrote our thesis. Our talks and meetings made these months so much easier and so much more fun! Thank you for being there.

A great thank you to all the wonderful staff at the maternity ward 314 and 315 who helped me out with the magnesium study. And to all the women and their unborn babies who participated.

To all my friends, especially **Birgitta** who encouraged me when I needed it the most, who helped me set the date and made me move forward when I wanted to give up. Thank you for always being there for me.

To my beloved **father Jon**, who always believed in me and that anything is possible. To my amazing **sister Mikaela with family** who is always there for me and my family, always helping out, for always giving me so much love and joy. Thank you for being in my life.

My love **Nisse**, you know, it doesn't need to be said but thank you for all the love and the never-ending support you give me every day. I love you.

To the most beautiful in my life, my children, the coolest persons I know, **Nike**, **Minna** and **Lo**, thank you for all the love, fun and beauty you bring to me every day. I love you.

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