# THE ROLE OF NEONATAL IMMUNITY IN PRETERM BRAIN INJURY

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Gothenburg 2016

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ISBN 978-91-628-9973-8

Printed in Gothenburg, Sweden 2016
Ineko AB

# The role of neonatal immunity in preterm brain injury

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## ABSTRACT

Perinatal brain injury is an important cause of mortality and morbidity and is associated with neurological disabilities such as those seen in cerebral palsy. Prematurity, especially in combination with very low birth weight, is associated with elevated risks for developing brain injuries, and the leading causes of perinatal brain injury are hypoxia-ischemia (HI) and infection/inflammation. The aims of this thesis were to establish a mouse model of HI-induced preterm brain injury, to determine the immune response after preterm brain injury, to explore the role of γδT-cells and the immune regulatory protein osteopontin (OPN) in preterm brain injury, and to evaluate the impact of *Staphylococcus epidermidis* bacteremia on the developing mouse brain.

We found that HI-induced preterm brain injury elicited a Th1/Th17-skewed immune response in the mouse brain, but in contrast to adult ischemic brain injury, the inflammatory cytokine IL-17 did not contribute to injury. Furthermore, we showed that γδT-cells are found in the mouse brain after HI, in the brains of asphyxiated fetal sheep, and in postmortem brains of human preterm infants with white matter injury. Genetic depletion of γδT-cells reduced the HI-induced preterm brain injury in mice, suggesting that γδT-cells contribute to preterm brain injury. We also showed that administration of OPN immediately before HI or the genetic depletion of OPN do not affect the outcome of brain injury in the mouse model of HI, while administration of the OPN-derived peptides N134-153 and C154-198 aggravate brain injury, which contrasts to what has been seen in adult ischemic brain injury. Finally, we showed that *S. epidermidis* bacteremia impair gray and white matter development in the mouse brain even without entry of bacteria into the central nervous system (CNS), providing evidence that systemic infections in the neonate can affect brain development.

In our studies, several findings indicate that the developmental state of the CNS and immune system is of great importance for the outcome of injury as well as for

possible therapeutic strategies. Thus identifying specific therapeutic targets for different age groups is of great importance.

**Keywords**: preterm brain injury, hypoxia-ischemia, immature brain, inflammation, neonatal immunity, γδT-cells, osteopontin, Staphylococcus epidermidis

**ISBN:** 978-91-628-9973-8

# SAMMANFATTNING PÅ SVENSKA

Hjärnskador som inträffar strax före, under eller strax efter födseln kan orsaka livslånga handikapp, t.ex. cerebral pares, inlärningssvårigheter och neuropsykiatriska problem. I Sverige föds 5-6 % av alla barn för tidigt, det vill säga före graviditetsvecka 37. Risken att drabbas av hjärnskada är högre hos för tidigt födda barn, vilket resulterar i en ökad frekvens av handikapp. T.ex. så sker ca 40 % av alla fall av cerebral pares hos för tidigt födda barn. Syftet med denna avhandling har varit att studera vilken roll immunförsvaret har i utvecklingen av hjärnskada hos för tidigt födda barn.

Nedsatt syre- och blodtillförsel (hypoxi-ischemi) till hjärnan är en vanlig orsak till hjärnskador hos för tidigt födda. Genom försök i en musmodell fann vi att hypoxi-ischemi leder till inflammation i hjärnan, och att en viss immunförsvarscell (γδT-cell) ökar i antal i hjärnan efter skada samt bidrar till förvärrad skada. Antalet γδT-celler ökade även i hjärnan i en fårmodell, samt hos för tidigt födda barn med hjärnskada. Till skillnad från vad som tidigare visats i musmodeller av syrebristhjärnskada hos vuxna, fann vi att det inflammatoriska proteinet interleukin 17, som kan produceras av γδT-celler, inte bidrar till hjärnskada hos för tidigt födda, vilket tyder på att immunförsvaret reagerar olika beroende på ålder. Vidare undersökte vi effekten av osteopontin (OPN) och syntetiskt framställda specifika fragment (peptider) av OPN. Vi fann att peptiderna förvärrade skadan medan OPN inte hade någon effekt, vilket antyder att effekten är åldersberoende då båda visat sig ge skydd hos vuxna möss.

Förutom hypoxi-ischemi så är även infektioner i livmodern under graviditeten, och i barnets blod efter födsel, vanliga bidragande orsaker till hjärnskada hos nyfödda. En av de vanligaste bakterierna som orsakar blodinfektion hos för tidigt födda är *Staphylococcus epidermidis*, som är en normal komponent av bakteriefloran på huden men som via t.ex. sår kan leda till blodinfektioner. Med en musmodell visade vi att blodinfektion av *S. epidermidis* kan orsaka inflammation i hjärnan och leda till hjärnskada trots att inga bakterier passerar över från blodet till hjärnan. Detta visar att blodinfektioner hos nyfödda kan ha allvarliga konsekvenser och att specifika behandlingsstrategier behövs för att minska infektionsrelaterade hjärnskador.

Sammanfattningsvis antyder resultaten att hypoxi-ischemi i den omogna hjärnan leder till inflammatoriska effekter som skiljer sig från dem i den mogna hjärnan, samt att blodinfektionsinducerad inflammation kan vara tillräckligt för att inducera hjärnskada i den omogna hjärnan. Detta indikerar att det är ytterst viktigt att hitta behandlingsstrategier som är specifika för hjärnskador hos för tidigt födda.

# LIST OF PAPERS

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

I. **Albertsson A-M**, Bi D, Duan L, Zhang X, Leavenworth J W, Qiao L, Zhu C, Cardell S, Cantor H, Hagberg H, Mallard C, and Wang X. The immune response after hypoxia-ischemia in a mouse model of preterm brain injury. *Journal of Neuroinflammation*. 2014; 11:153.

II. **Albertsson A-M**, Zhang X, Vontell R, Bi D, Xu Y, Bronson R, Baburamani A A, Supramaniam V, Xia L, Song J, Zhu D, Shang Q, Hua S, Nazmi A, Cardell S, Mallard C, Hagberg H, Xing Q, Zhu C, Cantor H, Leavenworth J, and Wang X. Preterm brain injury is gamma/delta T-cell dependent and IL-17/IL-22 independent. *Manuscript in preparation.*

III. **Albertsson A-M**, Zhang X, Leavenworth J W, Bi D, Nair S, Qiao L, Hagberg H, Mallard C, Cantor H, and Wang X. The effect of osteopontin and osteopontin-derived peptides on preterm brain injury. *Journal of Neuroinflammation*. 2014; 11:197.

IV. Bi D, Qiao L, Bergelson I, Ek C. J, Duan L, Zhang X, **Albertsson A-M**, Pettengill M, Kronforst K, Ninkovic J, Goldmann D, Janzon A, Hagberg H, Wang X, Mallard C, and Levy O. *Staphylococcus epidermidis* Bacteremia Induces Brain Injury in Neonatal Mice via Toll-like Receptor 2-Dependent and -Independent Pathways. *The Journal of Infectious Diseases.* 2015; 212:1480–90.

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| APC | Antigen presenting cell |
| BBB | Blood-brain barrier |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CNS | Central nervous system |
| CP | Cerebral palsy |
| CSF | Cerebrospinal fluid |
| DAMPs | Danger-associated molecular patterns |
| DAP12 | DNAX activation protein of 12kDa |
| DC | Dendritic cell |
| DNA | Deoxyribonucleic acid |
| EAE | Experimental autoimmune encephalomyelitis |
| EGFP | Enhanced green fluorescent protein |
| GW | Gestational week |
| HI | Hypoxia-ischemia |
| ICV | Intracerebroventricular |
| IFN | Interferon |
| IL | Interleukin |
| iOPN | Intracellular OPN |
| LPS | Lipopolysaccharide |
| MAP-2 | Microtubule-associated protein 2 |

| | |
|---|---|
| MBP | Myelin basic protein |
| mRNA | Messenger-RNA |
| MS | Multiple sclerosis |
| OPN | Osteopontin |
| PAMPs | Pathogen-associated molecular patterns |
| PCR | Polymerase chain reaction |
| PND | Postnatal day |
| PRR | Pattern recognition receptor |
| PVL | Periventricular leukomalacia |
| Rag1 | Recombination activating gene-1 |
| RNA | Ribonucleic acid |
| RT-qPCR | Real-time quantitative PCR |
| *S. epidermidis* | *Staphylococcus epidermidis* |
| SNP | Single nucleotide polymorphism |
| sOPN | Secreted OPN |
| TCR | T-cell receptor |
| Th | T-helper |
| TLR | Toll-like receptor |
| TNF | Tumor-necrosis factor |
| T-OPN | Thrombin-cleaved OPN |
| TREM | Triggering receptor expressed on myeloid cells |
| WT | Wild-type |

# INTRODUCTION

Preterm birth, and especially preterm birth in combination with very low birth weight, is a serious global health problem. In Sweden, the preterm birth rate (birth before gestational week (GW) 37; a full-term gestational period is 40 weeks) is 5.9/100 live births (Chang, Larson et al. 2013), and the annual number of preterm births worldwide is estimated at about 14.9 million (Blencowe, Cousens et al. 2012).

The development of modern neonatal intensive care has increased the survival rate among preterm and extremely preterm (born before GW 28) infants. Today, the majority of preterm infants in Sweden survive the neonatal period, and the 1-year survival of live-born extremely preterm infants in Sweden in 2004–2007 ranged from 9.8% when born in GW 22 up to 85% when born in GW 26, with a total of 70% survival when born in GW 22–26 (Fellman, Hellstrom-Westas et al. 2009). However, many of these survivors suffer from neurological deficits such as cerebral palsy (CP) and behavioral, social, attention, and cognitive defects associated with preterm brain injury (Khwaja and Volpe 2008, Volpe, Kinney et al. 2011).

A study examining the neurodevelopmental outcome of extremely preterm infants in Sweden at 2.5 years of age showed that 3 out of 4 (73%) of the extremely preterm infants had no (42%) or mild (31%) disabilities. However, 16% of the extremely premature infants displayed moderate disability and as many as 11% displayed severe disability, and the incidence of disability was higher the earlier in gestation the infant was born (Serenius, Kallen et al. 2013). These findings agree with the Swedish study describing the prevalence of CP in the birth-year period 2003-2006. According to that study, the overall prevalence of CP was approximately 2 per 1000 live births, but the prevalence differed with gestational age, where the highest prevalence (71.4 per 1000 live births) was found among extremely preterm infants and decreased through very preterm (39.6 per 1000 live births for GW 28–31), moderately preterm (6.4 per 1000 live births for GW 32–36) and near term (1.41 per 1000 live births for infants born after GW 36) (Himmelmann and Uvebrant 2014). Today there are no efficient therapeutic strategies for preterm brain injury, thus, seeking methods for preventing or treating injuries to the preterm brain are of great importance.

## NEONATAL BRAIN INJURY

Hypoxic-ischemic brain injury is an important cause of death in the perinatal period and is a major cause of neurodevelopmental disorders in newborn infants (Wyatt,

Edwards et al. 1989). Perinatal hypoxia can result from occlusion of the umbilical cord due, for example to prolapse, as well as from placental abruption, immaturity of the infant's lungs, and vasculature and cardiac arrest. Often, hypoxia-ischemia (HI)-induced neonatal brain injury is not dependent on a single event, but is rather a series of pathologic events resulting in brain injury. This series of events evolves over time and is initiated by a "primary energy failure", which, depending on its extent, can lead to a later "secondary energy failure".

The primary energy failure phase is initiated by the hypoxic-ischemic event which reduces the cerebral blood flow and thus also reduce the oxygen and nutrient (glucose) supply to the brain (Gunn and Bennet 2009, Ten and Starkov 2012) leading to numerous detrimental effects and finally to cell death. The secondary energy failure appears to be related to inflammatory processes, oxidative stress, and excitotoxicity, but its mechanisms are not as well-known as the primary energy failure (Cotten and Shankaran 2010, Allen and Brandon 2011). The time between the primary and the secondary energy failure, "the latent phase", can be of varying length, ranging from hours to days, and allows for a brief period of recovery (Wyatt, Edwards et al. 1989, Cotten and Shankaran 2010, Allen and Brandon 2011, Ten and Starkov 2012). Recently it was also suggested that there is a tertiary phase in the injury process where long-lasting effects such as glial scars, accumulation of immature oligodendrocytes, epigenetic changes, and persistent inflammation are possible factors that sensitize the brain and promote further damage after the initial insult (Fleiss and Gressens 2012). The series of events including the primary and secondary energy failure is well characterized for term infants with HI-induced brain injury, but it is not known whether secondary energy failure occurs in HI-induced injury in the preterm infant.

The manifestation of injury changes depending on the maturity/immaturity of the infants, with the more mature (full term) infants usually manifesting gray matter injuries while the more immature preterm infants most often display white matter injury (Ferriero 2016). Volpe has proposed the term "encephalopathy of prematurity" as a name for the combination of the specific form of cerebral white matter injury, periventricular leukomalacia (PVL), and neuronal/axonal abnormality most commonly seen in preterm brain injury (Volpe 2009).

Apart from ischemia, inflammation/infection is the other major initiating factor for neonatal brain injury (Dammann and Leviton 1997, Volpe 2009). Systemic infection can occur at any time during pregnancy or neonatal life and can cause central nervous system (CNS) inflammation leading to altered brain development and brain

injury (Hagberg, Mallard et al. 2015). The infection/inflammation can by itself cause injury to the brain, but it also enhances the vulnerability to injury from subsequent ischemic insults (Eklind, Mallard et al. 2005) (summarized in reviews (Wang, Rousset et al. 2006, Hagberg, Mallard et al. 2015)).

Maternal intrauterine infection is strongly associated with an increased risk of preterm birth and it is highly associated with increased risk of white matter injury, intraventricular hemorrhage, and subsequent development of CP (Dammann and Leviton 1997). In addition, postnatal infections can affect and contribute to progression of white matter injury in the preterm infant (Graham, Holcroft et al. 2004, Volpe 2009, Chau, Brant et al. 2012, Hagberg, Mallard et al. 2015, Ferriero 2016). *Escherichia coli*, the bacteria that is responsible for about 40% of the early-onset cases of bacteremia among preterm infants of very low birth weight, has been found to induce white matter injury in a rat model of neonatal sepsis (Loron, Olivier et al. 2011), as well as in models of intrauterine infection (Bo Hyun, Chong Jai et al. 1997, Debillon, Gras-Leguen et al. 2003, Pang, Rodts-Palenik et al. 2005, Yuan, Yu et al. 2005, Wang, Hagberg et al. 2007, Mallard and Wang 2012). Today the most common cause of late-onset sepsis in preterm infants is coagulase-negative staphylococci (Ohlin, Bjorkman et al. 2015), such as the gram-positive, ubiquitous skin commensal *Staphylococcus epidermidis* (Stoll, Hansen et al. 2002, Power Coombs, Kronforst et al. 2013, Strunk, Inder et al. 2014). In human newborn infants with gram-positive bacterial infection, Toll-like receptor (TLR)2 is activated in peripheral blood mononuclear cells (Zhang, Yang et al. 2010), suggesting that sepsis caused by *S. epidermidis* contribute to brain injury. This is supported by the observation that systemic administration of the TLR2 agonist Pam(3)CSK(4) impairs neonatal mouse brain development (Du, Fleiss et al. 2011),

## NEONATAL IMMUNITY

Neonatal mice and humans have immature immune systems making them highly sensitive to infections compared to adults (Adkins, Leclerc et al. 2004, Levy 2007). The immature immune responses partly depend on the lack of adaptive immune memory in the neonates (Adkins, Leclerc et al. 2004), as well as to the smaller number of peripheral immune cells in neonates compared to adults (Adkins, Leclerc et al. 2004). Also the limited exposure to antigens in utero leaves the adaptive immune cells naïve and in need of antigen presentation and maturation in order to mount a response to infection (Adkins, Leclerc et al. 2004, Levy 2007, Kumar and Bhat 2016).

T-cells play a central role in the adaptive immunity and can be categorized into subgroups depending on the T-cell receptor (TCR) they express. Most T-cells are αβT-cells that express TCRs that consist of an α- and a β-chain. The αβT-cells can be divided into CD4+ T-helper (Th) cells, which by cytokine production mainly aid the functions of other cells, and CD8+ cytotoxic T-cells which mediate killing of infected cells. The Th-cells can be further divided into subgroups, including the effector cells Th1, Th2, Th17, Th22, and T regulatory (Treg) cells, which have distinct cytokine profiles. A small population of T-cells (<5% of peripheral T-cells in mouse) consists of γδT-cells that express TCRs consisting of a γ- and a δ-chain (Pardoll, Fowlkes et al. 1987, Davis and Bjorkman 1988). The γδT-cells and conventional αβT-cells differ significantly in their mode of activation, and αβT-cells activation requires antigen processing and presentation by professional antigen-presenting cells (APCs), while γδT-cell activation is not restricted to antigen presentation by APCs (Shibata, Yamada et al. 2008, Kalyan and Kabelitz 2013, Vantourout and Hayday 2013).

During development, the γδTCR is the first TCR to be expressed on murine (Pardoll, Fowlkes et al. 1987, Carding, Kyes et al. 1990) and human thymocytes (McVay and Carding 1996). The γδT-cells are considered to be a heterogeneous population of T-cells and they can be divided into subsets depending on the specific variable segment (V) in the γ-chain of the TCR they express. The γδT-cell subsets are established at different time points during fetal development where the Vγ5+ cells are the first to develop in the thymus at around embryonic day 12 (E12), which is followed by the Vγ6+ cells from E14 to birth, Vγ4+ cells from E16 and onwards, and Vγ1+ cells from E18 and onwards (Figure 1) (Carding and Egan 2002). The different waves of γδT-cell development produce γδT-cells that home to specific tissues. The first wave of γδT-cells, the Vγ5+ cells, mainly homes to the epidermis, the second wave mainly homes to the genital tract and the following waves of γδT-cells homes to tissues such as the gut and lungs (Carding and Egan 2002, Vantourout and Hayday 2013). The γδT-cells are capable of producing numerous inflammatory mediators such as interleukin (IL)-17, IL-21, IL-22 (Sutton, Lalor et al. 2009), IL-13 (Han, Lee et al. 2014, Dalessandri, Crawford et al. 2016), interferon (IFN)-γ (Gao, Yang et al. 2003), and perforin (Lafont, Sanchez et al. 2014). Certain γδT-cells subsets, such as the Vγ4+ γδT-cells (Martin, Hirota et al. 2009, Sutton, Lalor et al. 2009), are preprogrammed to produce IL-17 and can rapidly start to produce IL-17 upon activation (Shibata, Yamada et al. 2008, Eberl 2012).

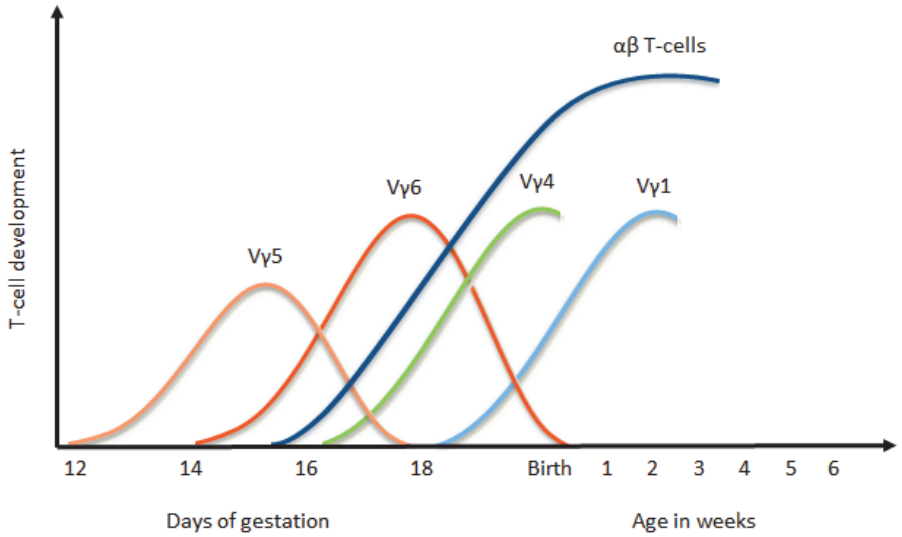


*Figure 1. Schematic figure of γδT-cell and αβT-cell development.*

The immunity of the neonatal mouse and human is skewed to Th2 type responses (Adkins and Du 1998, Adkins, Bu et al. 2003, Adkins, Leclerc et al. 2004, Rose, Lichtenheld et al. 2007), with the potential for mounting a robust Th2 associated IL-4, IL-5, and IL-13 cytokine response (Adkins 2013). Neonatal mouse Th1 cells express high levels of the IL-13R alpha1 receptor, which forms a heterodimer with the IL-4R alpha receptor and induces apoptosis of Th1 cells upon activation by IL-4 (Lee, Hoeman et al. 2008). This is believed to be one of the reasons for the Th2-skewed neonatal immune response. However, IL-12 can trigger the downregulation of IL-13R alpha1 and protect the Th1 cells from IL-4-induced apoptosis (Lee, Hoeman et al. 2008). In humans, this skewing of the cytokine response towards a Th2-biased response instead of a pro-inflammatory Th1 response is important for the maintenance of pregnancy because the Th1-associated cytokines tumor-necrosis factor (TNF) and IL-1β are associated with increased risks of preterm labor and preterm birth (Levy 2007, Morein, Blomqvist et al. 2007, Ygberg and Nilsson 2012).

Dendritic cells (DCs) are potent professional APCs and are important in T-cell activation, and have important functions as IFN producers in the innate immune responses against viral infections (Willems, Vollstedt et al. 2009). In the neonatal mouse, the numbers of DCs are lower in the lymphoid organs compared to adult mice, and the capacity of these cells to produce IFN-γ and IL-12p70 and to induce antigen-specific activation of T-cells is reduced (Dakic, Shao et al. 2004). However, by approximately postnatal day (PND)7, the neonatal mouse has a similar DCs to T-

cells ratio as seen in adults (Adkins, Leclerc et al. 2004). From approximately day 6 or 7 after birth there is gradual maturation of conventional DCs including the start of IL-12p70 production, which leads to the ability of neonatal mice to generate Th1 responses and overcome the Th2 skewing (Lee, Hoeman et al. 2008, Willems, Vollstedt et al. 2009). From this point of view, PND6-7 might represent an important switch point for the neonatal immunity in mice, which in turn could have an impact on the immune responses early in life.

## INFLAMMATION IN THE BRAIN

The CNS is considered to be an immune privileged site, which is a beneficial feature for an organ of low regenerative capacity in order to limit damage during inflammation (Galea, Bechmann et al. 2007). The swelling of tissues and accumulation of cells which is common in peripheral inflammation, is not as well tolerated by the brain because it is enclosed by the skull, and thus the immune privilege is important to maintaining homeostatic CNS functions (Carson, Doose et al. 2006). This immune privilege is constrained to the cerebral parenchyma and is not present in the meninges, choroid plexus, circumventricular organs, or ventricles (Carson, Doose et al. 2006, Galea, Bechmann et al. 2007).

Under pathological conditions, such as infection and traumatic or hypoxic injury, the immune privilege is breached and the CNS is exposed to the peripheral immune system (Carson, Doose et al. 2006). When the immune privilege is degraded, both the central and peripheral immune systems will contribute to CNS inflammation (Hagberg, Mallard et al. 2015), and peripheral immune cells can be recruited to the CNS (Stridh, Ek et al. 2013, Hagberg, Mallard et al. 2015). Inflammation is an important contributor to both injury outcome as well as to normal development in the immature brain (Hagberg, Gressens et al. 2012, Hagberg, Mallard et al. 2015), and it is recognized that both the innate and adaptive arms of the immune system are involved in neonatal HI-induced brain injury (Bona, Andersson et al. 1999, Hedtjarn, Mallard et al. 2004, Winerdal, Winerdal et al. 2012).

The barriers of the CNS include the blood-brain barrier (BBB), which is composed of endothelial cells of the parenchymal capillaries together with pericytes, basal lamina, and astrocytes; the arachnoid epithelium; and the epithelium of the choroid plexus that makes up the blood-cerebrospinal fluid barrier (Engelhardt and Sorokin 2009, Abbott 2013). The barriers of the CNS are crucial to maintaining the ionic balance in the CNS by preventing ionic fluctuations as well as preventing toxins and proteins in the blood from affecting the CNS (Abbott 2013). Furthermore, the BBB also prevents circulating immune cells and immune components, including T-cells,

B-cells, cytokines, and antibodies, from entering the parenchyma (Kivisäkk, Mahad et al. 2003, Engelhardt and Sorokin 2009, Bitzer-Quintero and Gonzalez-Burgos 2012), although there is evidence that T-cells might enter the cerebrospinal fluid (CSF) through the choroid plexus (Kivisäkk, Mahad et al. 2003, Reboldi, Coisne et al. 2009).

## TREMs AND DAP12

Triggering receptor expressed on myeloid cells (TREMs) are innate immune receptors, belonging to the immunoglobulin (Ig) superfamily of receptors (Painter, Atagi et al. 2015), and they play an important role in fine-tuning the inflammatory response (Ford and McVicar 2009). TREM-1 is considered to amplify innate immune responses, such as neutrophil- and monocyte-induced inflammatory responses (Bouchon, Dietrich et al. 2000), and TREM-1 signaling result in the production pro-inflammatory cytokines and chemokines including MCP-1, TNF-α, IL-1β and IL-6 (Bouchon, Dietrich et al. 2000, Fan, He et al. 2016, Varanat, Haase et al. 2016). However, TREM-2 is generally considered to be an anti-inflammatory receptor, and TREM-2 signaling has been shown to protect against excessive pro-inflammatory responses induced by lipopolysaccharide (LPS) (Zhong, Chen et al. 2015) and might dampen TLR-induced inflammation (Painter, Atagi et al. 2015).

TREM-2 is expressed on the cell membrane of macrophages, monocyte-derived DCs, osteoclasts and microglia (Painter, Atagi et al. 2015). TREM-2 is also expressed on microglia in the mouse brain during development (Klesney-Tait, Turnbull et al. 2006, Chertoff, Shrivastava et al. 2013, Genua, Rutella et al. 2014). TREM-2 can sense both pathogen-associated molecular patterns (PAMPs), such as gram-positive and gram-negative bacteria, and danger-associated molecular patterns (DAMPs), such as myelin-associated lipids (Paradowska-Gorycka and Jurkowska 2013, Painter, Atagi et al. 2015). Thus TREM-2 may sense both pathogen-derived antigens and self-antigens associated with tissue injury. Data from both in vitro and in vivo experiments show that TREM-2 signaling facilitates phagocytosis (Takahashi, Prinz et al. 2007, Hsieh, Koike et al. 2009, Kleinberger, Yamanishi et al. 2014) and apoptotic neurons can be engulfed by microglia after TREM-2 stimulation in vitro (Hsieh, Koike et al. 2009).

TREM-2 signaling is mediated via the transmembrane signaling adaptor protein DNAX activation protein of 12kDa (DAP12), which is expressed in various cell types, including γδT-cells, natural killer cells and in myeloid cells such as DCs, macrophages, and microglia (Xing, Titus et al. 2015). DAP12 is the signaling adaptor protein for both TREM-1 and TREM-2 (Klesney-Tait, Turnbull et al. 2006, Tessarz and Cerwenka 2008), and contain an intracellular cytoplasmic immunoreceptor tyrosine-

based activation motif, through which the recruitment and activation of downstream signaling molecules is facilitated (Xing, Titus et al. 2015).

In experimental autoimmune encephalomyelitis (EAE), which is the mouse model of multiple sclerosis (MS), TREM-2 is up-regulated in the CNS both during the early inflammatory and the chronic phases of the disease, and administration of a TREM-2 antagonist enhances the severity and progression of the disease (Piccio, Buonsanti et al. 2007). Microglia that expresses TREM-2 clear degenerated myelin during EAE, contributes to an anti-inflammatory environment and mediates recovery from injury (Takahashi, Prinz et al. 2007). In addition, DAP12 gene expression is upregulated in the brain after HI-induced neonatal brain injury in mice (Hedtjarn, Mallard et al. 2004), and deficiency of DAP12 signaling causes hypomyelinosis in the mouse thalamus (Kaifu, Nakahara et al. 2003), and white matter lesions in human Nasu–Hakola disease (Paloneva, Kestila et al. 2000, Tanaka 2000) suggesting that DAP12 is associated with oligodendrocyte pathology.

### OSTEOPONTIN

Osteopontin (OPN) is a glycoprotein that exists both as a secreted (sOPN) and an intracellular (iOPN) protein (Patarca, Saavedra et al. 1993, Inoue and Shinohara 2011, Uede 2011). sOPN is expressed by various immune cells including macrophages, DCs, neutrophils, NK-cells, T-cells, and B-cells, and promotes cytokine production, cell migration, cell activation, and cell adhesion (Wang and Denhardt 2008, Buback, Renkl et al. 2009). sOPN binds to a series of different integrins, including the α9β1, α4β1, α4β7, α5β1 and the αv-containing integrins αvβ1, αvβ3, αvβ5, and αvβ6 through two main integrin binding sequences, the RGD sequence and the SLAYGLR (SVVYGLR in humans) sequence (Rittling and Singh 2015). iOPN serves as an adaptor molecule and modulate signaling pathways downstream of innate immune receptors, including some TLRs (Inoue and Shinohara 2011, Uede 2011, Fan, He et al. 2015), and is an important regulator of NK-cell function and homeostasis (Leavenworth, Verbinnen et al. 2015).

OPN has long been regarded as a survival factor in part by inhibiting apoptosis induced by pathological events such as growth factor deprivation (Khan, Lopez-Chua et al. 2002). Under inflammatory conditions, OPN serves as a Tbet-dependent pro-inflammatory cytokine that is produced by activated Th1 cells. However, OPN also stimulates anti-inflammatory processes under certain circumstances, showing that the effect of OPN is context dependent and can be either protective or detrimental depending on the situation (Wang and Denhardt 2008, Cantor and Shinohara 2009).

In the human *Opn* locus, genetic polymorphisms have been linked to increased susceptibility to infections, autoimmune disease, cancer (Inoue and Shinohara 2011), and CP (Shang, Zhou et al. 2016). In MS and EAE animal models, OPN plays an important role in the pathogenesis of adult white matter injury (Girgrah, Letarte et al. 1991, Chabas, Baranzini et al. 2001, Jansson, Panoutsakopoulou et al. 2002, Kim, Cho et al. 2004, Selvaraju, Bernasconi et al. 2004, Back, Tuohy et al. 2005). Furthermore, OPN is up-regulated in the rat brain after stroke (Ellison, Velier et al. 1998, Lee, Shin et al. 1999), and has neuroprotective effects in an adult mouse model of stroke (Meller, Stevens et al. 2005, Doyle, Yang et al. 2008). In the human neonatal brain, strong OPN immunoreactivity was found in the axons at the periphery of the ischemic zone in subacute and chronic PVL lesions, indicating a role in PVL (Tanaka, Ozawa et al. 2000).

### T-CELLS IN THE CNS

There is accumulating evidence that lymphocytes, especially T-cells, are recruited to the CNS after both adult and neonatal brain injury (Benjelloun, Renolleau et al. 1999, Bona, Andersson et al. 1999, Hedtjarn, Mallard et al. 2004, Winerdal, Winerdal et al. 2012, Yang, Sun et al. 2014).

In animal models of stroke, the number of T-cells increased in the infarction area after the injury (Schroeter, Jander et al. 1994, Jander, Kraemer et al. 1995), and immune-deficient mice lacking T-cells as well as those in which T-cells were depleted exhibit significant reductions in infarct volume (Yilmaz, Arumugam et al. 2006, Hurn, Subramanian et al. 2007). Furthermore, both the Th1-type inflammatory response cytokine IFN-γ, and the Th17-type inflammatory response cytokine TNF-α, are highly toxic to premyelinating oligodendrocytes but not at all toxic to mature oligodendrocytes (Baerwald and Popko 1998, Horiuchi, Itoh et al. 2006), suggesting that Th1- and Th17-associated inflammatory responses might play a role in the pathogenesis of preterm brain injury.

Brain infiltrating γδT-cells play an important role in ischemic brain injury in adult mice through the secretion of the cytokine IL-17A (Shichita, Sugiyama et al. 2009), and these cells were identified in demyelinating lesions in the CNS in both animal models and human patients with white matter injury (Selmaj, Brosnan et al. 1991, Wucherpfennig, Newcombe et al. 1992, Salerno and Dieli 1998). These cells have also been shown to kill human oligodendrocytes in vitro (Freedman, Ruijs et al. 1991), and the absence of γδT-cells results in milder disease symptoms in the EAE model (Rajan, Gao et al. 1996, Spahn, Issazadah et al. 1999, Odyniec, Szczepanik et al. 2004).

Conventional αβT-cells acquire their effector function by being exported as naïve T-cells to the lymph nodes where they come into contact with antigens and subsequently acquire the effector Th cell phenotype. The fact that γδT-cells, in contrast to αβT-cells, are already mature and differentiated in the fetal thymus indicates that they might be important contributors to immune responses early in life (Shibata, Yamada et al. 2008), therefore we hypothesize that γδT-cells play a role in the development of preterm brain injuries.

# AIMS

The overall aim of this thesis was to investigate the mechanisms of preterm brain injury by exploring the role of neonatal immunity in preterm brain injury, to identify possible therapeutic targets. More specifically, we aimed to:

- Develop a mouse model of preterm brain injury.
- Explore the immune response after preterm brain injury in the mouse.
- Explore the role of γδT-cells in preterm brain injury.
- Explore the role of OPN, an inflammatory regulator, in preterm brain injury.
- Explore the contribution of *S. epidermidis* bacteremia to preterm brain injury.

# METHODS

## SUBJECTS USED IN THE STUDIES

### HUMAN SUBJECTS (PAPER II)

In paper II, blood samples from 715 patients with CP and 658 healthy control participants as well as post mortem brain tissue from human preterm infants were used. For the use of patient blood samples, ethical approval was obtained from the ethics committee of Zhengzhou University and the Medical Academy of Henan Province, China. Written informed consent was obtained from the parents. A total of 715 CP patients (average age 18.3 ± 15.1 months) and 658 healthy control participants (average age 19,5 ± 17,1 moths) were enrolled from the Third Affiliated Hospital of Zhengzhou University, the Zhengzhou Children's Hospital and the First Affiliated Hospital of Henan Traditional Chinese Medical College from 2011 to 2014. Children in either the CP or control group with myopathy or metabolic anomalies were excluded. Controls that presented with any neurological condition (CNS infection, developmental delay, seizure disorder, attention-deficit/hyperactivity disorder, or migraine headache) or predefined medical conditions (juvenile diabetes mellitus or growth retardation) were excluded from the study.

For the use of postmortem brain tissue from human preterm infants, a written informed parental consent form was acquired according to National Health Service UK guidelines. Ethical approval was obtained from the National Research Ethics Service (West London) UK, and five preterm postmortem brains (<35 weeks' gestational age) of vaginally delivered neonates were used in this study. The primary cause of death of each case was assessed by a pathologist.

### ANIMALS (PAPERS I-IV)

For all animal studies included in the thesis, ethical approval was obtained from the Animal Ethical committee of the University of Gothenburg. All animal experiments were performed in, and all animals were housed in, the Experimental Biomedicine animal facility at the University of Gothenburg.

This thesis is mainly based on data from experimental studies using mouse models of preterm brain injury. The mouse is a good model organism because mice have a short generation time, they share many genetic, anatomical, and physiological similarities with humans, and they are of reasonably low cost to maintain compared to larger animals. Mouse models also enable the use of a wide range of genetically modified animals, which is an advantage when studying the molecular mechanisms

involved in the development of preterm brain injury. The specific mouse strains used in this thesis are listed in Table 1.

However, some differences between humans and mice are obvious when using the mouse as a model organism for preterm brain injury. The rodent brain is lissencephalic as compared to the human brain that is gyrencephalic and the small size of mice at an age equivalent to the human preterm infant is an obstacle that makes repeated sampling (Hagberg, Bona et al. 1997) and monitoring of physiological parameters, such as blood pressure, difficult.

As a complement to mice, sheep were used in paper II for examining the presence of γδT-cells in the brain after preterm brain injury.

*Table 1. Summary of mouse strains used in this thesis.*

| Strain | Strain hereafter referred to as | Paper I | Paper II | Paper III | Paper IV |
|---|---|---|---|---|---|
| C57BL/6J | WT | + | + | + | + |
| B6.129S7-Rag1tm1Mom/J | Rag1 −/− | | + | | |
| B6.129P2-Tcrdtm1Mom/J | γδT −/− | | + | | |
| Tcrd-H2BEGFP | γδT-EGFP | | + | | |
| B6;129S5-Il22tm1.1Lex/Mmucd | IL-22−/− | | + | | |
| B6.129S6(Cg)-Spp1tm1Blh/J | OPN −/− | | | + | |
| B6.129-Tlr2tm1Kir/J | TLR2−/− | | | | + |

+ indicates in which paper each mouse strain was used; -/- = knock-out; EGFP = Enhanced green fluorescent protein; Rag1−/− = Recombination activating gene-1 knock-out (mice deficient in all T- and B-cells); γδT −/− = mice deficient in γδT-cells; γδ-T-EGFP = mice with EGFP expressing γδT-cells; IL-22 −/− = mice deficient in IL-22; OPN −/− = mice deficient in OPN; TLR2 −/− = mice deficient in TLR2.

# ANIMAL MODELS

## MOUSE HYPOXIA-ISCHEMIA BRAIN INJURY MODEL (PAPERS I-III)

The most commonly used method for studying HI-induced damage in the neonatal brain is a rodent HI model often referred to as the Rice–Vannucci model. This model was first developed and used in rat pups (Rice, Vannucci et al. 1981), but it has subsequently been adapted and is now also used in neonatal mice (Hedtjarn, Leverin et al. 2002, Vannucci and Vannucci 2005).

In the HI model brain damage is induced by unilateral ligation of the common carotid artery and subsequent systemic hypoxia. During hypoxia the blood pressure decreases systemically leading to reduced cerebral blood flow in the hemisphere ipsilateral to the ligation. This results in cell death and brain damage. The brain damage is restricted to the hemisphere ipsilateral to the ligation, and the duration of hypoxia and the age of the animal can affect the severity of the brain injury (Vannucci and Hagberg 2004, Vannucci and Vannucci 2005). Importantly, injury only occurs when combining artery ligation and hypoxia, and neither ligation nor hypoxia alone results in injury (Rice, Vannucci et al. 1981, Towfighi, Zec et al. 1995).

The Rice–Vannucci HI model of neonatal brain injury allows for long term survival, and as long as the conditions are strictly controlled the model has high reproducibility, although there is still some variation in the degree of injury between animals. The variability in injury can be compensated for by using large sample sizes. However, some limitations to the HI model are inevitable. In severe clinical asphyxia, multi-organ involvement is present, which is not seen in the rodent HI model (Hagberg, Bona et al. 1997). Also, the unilateral distribution of injury in the rodent HI model does not correspond to the injury distribution in human infants with severe HI brain injury (Hagberg, Bona et al. 1997).

HI-induced brain injury in near-term infants is commonly modeled using PND9 mice, and the developmental stage of the CNS in PND9 mice correlates well with the developmental stage of the CNS in human near-term infants (Semple, Blomgren et al. 2013). However, the incidence of preterm brain injury consisting of PVL is the highest during GWs 23-32 (Back, Luo et al. 2001). Thus, to study HI-induced brain injury at an age equivalent to the preterm human infant, the neonatal mouse HI model was adapted to more immature mice.

We adapted the HI mouse model for the use of PND5 mice, an age when mouse CNS development is equivalent to the human preterm infant within the time frame when PVL is the most likely to occur (Craig, Ling Luo et al. 2003). PND5 is also an age

when the size and developmental stage of the mice make them reasonably easy to handle during surgery as well as during drug administrations. Thus PND5 mice were used in this thesis, to establish a reproducible model of HI in neonatal mice that produces consistent local white/gray matter brain damage that is relevant to preterm brain injury in humans.

At PND5 (papers I–III) or PND9 (paper I), mice were anesthetized and an incision was made in the midline of the pups´ neck, through which the left common carotid artery was ligated, the wound was sutured, and the pups were allowed to recover from anesthesia before they were brought back to the mother to rest. Subsequently the pups were exposed to hypoxia (10% O2) for 50 minutes (paper I), 70 minutes (paper I–III) or 80 minutes (paper I).

### FETAL SHEEP ASPHYXIA MODEL (PAPER II)

The fetal sheep model of asphyxia-induced injury is a well-established model for preterm brain injury. In mid-gestation sheep, umbilical cord occlusion results in lesions in the periventricular white matter as well as in the subcortical gray matter (Mallard, Welin et al. 2003, Welin, Svedin et al. 2007, Back, Riddle et al. 2012). The fetal sheep model allows for monitoring of physiological parameters and repeated sampling, in contrast to preterm brain injury models using small animals, such as mice (Back, Riddle et al. 2012).

In paper II, the possible infiltration of γδT-cells into the brain in a large-animal preterm brain injury model was examined using brain tissue sections from asphyxiated fetal sheep.

At 95 days of gestation (full term gestation = 147 days), time-mated pregnant sheep were anesthetized and the fetus underwent aseptic surgery in order to implant catheters to the brachial artery and vein as well as to place a cuff around the umbilical cord. At day 99–100 of gestation the umbilical cord was transiently occluded for 25 min by inflating the umbilical cord cuff, and the fetus remained in utero for another 14 days after the occlusion until post mortem examination was performed and brain tissue was prepared for paraffin histology.

### BACTEREMIA MOUSE MODEL (PAPER IV)

Under physiological conditions, the blood stream is considered a sterile environment, and bacteremia occurs when bacteria enter the blood stream. During neonatal intensive care, the use of a central venous catheter and parenteral nutrition increase the risk for blood stream infections in the neonate, and the low gestational age of the preterm infant is a significant risk factor for bacteremia

(Olsen, Reinholdt et al. 2009). One of the most common bacteria causing bacteremia or sepsis in the newborn preterm infant is the coagulase-negative bacteria *S. epidermidis*, which is a commensal skin bacteria and part of the normal human bacterial flora (Power Coombs, Kronforst et al. 2013).

To model bacteremia, normal C57bl/6J wild-type (WT) mice and TLR2 -/- mice were intravenously injected via the intrajugular vein with 5 x $10^6$ live *S. epidermidis* bacteria at PND1 (within 24 hours after birth) according to an established neonatal bacteremia model (Kronforst, Mancuso et al. 2012). Many staphylococcal infection models in newborn mice utilize intraperitoneal or subcutaneous injection as routs of infection (McKay and Arbuthnott 1979, Maderazo, Breaux et al. 1990, Gallimore, Gagnon et al. 1991). However, because venous catheters are possible sites of bacteria to enter the blood stream in neonatal intensive care, we use intravenously administered bacteria so as to be comparable to the clinical rout of infection.

The small size of the PND1 mice pups makes intravenous injection via the intrajugular vein a delicate task, and thus thorough training is required. For example, the success of injections needs to be evaluated by examining the injection site. Tissue swelling around the injection site is an indicator of unsuccessful injections, as it indicates extravasation of the injected bacteria into the surrounding soft tissue.

## DRUG ADMINISTRATION

### INTRA-CEREBRAL VENTRICULAR INJECTION (PAPERS II AND III)

The BBB plays a protective role by shielding the brain from potentially harmful substances such as inflammatory cytokines and pathogens present in the blood stream. However, the BBB can by the same mechanisms also be an obstacle preventing possible therapeutic agents from entering the CNS (Pardridge 2002, Cardoso, Brites et al. 2010). In an experimental setting, the BBB can be bypassed by administering a drug via intracerebroventricular (ICV) injection. By ICV injection the drug directly enters the CSF and thus is delivered to the CNS. In order to examine their possible neuroprotective effects in preterm brain injury in mice, recombinant OPN protein or either of the OPN-derived peptides N134–153 or C154–198 (corresponding to amino acids 134–153 and 154–198 of the OPN protein, respectively) (paper III) or anti-IL-17 antibody (paper II) were injected into the left lateral ventricle immediately before HI at PND5. Animals serving as controls were administered the appropriate vehicle.

During ICV administration, the pups were anesthetized with isoflurane, and the injection was performed using a syringe connected to a microinjection pump at a speed of 1.25 µL/min and at a depth of 1.9 mm from the mouse skull skin surface.

### INTRANASAL ADMINISTRATION (PAPER III)

Intranasal drug administration is a noninvasive method for administering drugs to the CNS. By intranasal administration, a drug, peptide, or compound is believed to access the brain via the olfactory or trigeminal nerve systems (Dhuria, Hanson et al. 2010, Mittal, Ali et al. 2014). Intranasal administration rapidly distributes the administered compound in the brain, and both an anti-N-κB peptide (Yang, Sun et al. 2013) and radioactively labeled albumin (Falcone, Salameh et al. 2014) were shown to access the brain within 5–30 minutes after intranasal administration. Because intranasal administration is a clinically more convenient method for drug delivery compared to ICV injection (Dhuria, Hanson et al. 2010), we sought to evaluate the possibility of using this route of drug delivery in the preterm HI mouse model. Thus, in paper III recombinant OPN protein, thrombin-cleaved OPN (T-OPN) protein, or the OPN-derived peptide N134–153 were intranasally administered to mice immediately before and after HI. The outcome of HI-induced brain injury after intranasal administration was compared to the outcome of HI-induced injury after ICV injections of the same proteins and peptide to confirm the efficiency of the intranasal administration.

During intranasal administration, pups were awake, laid on their back, and held gently to prevent them from moving during administration. One drop of 1.5 µL protein or peptide was administered to each nostril of the pup immediately before and after HI. After each administration the pups were allowed to inhale the drop before administering the next drop into the other nostril. Due to the small size of the mouse pup nostrils, intranasal administration was performed under an operation microscope to ensure that the protein or peptide was properly administered.

Pretreatment with the enzyme hyaluronidase before intranasal administration has been shown to increase the delivery of intranasally administered cells to the brain (Danielyan, Schafer et al. 2009). Thus, we used pretreatment with hyaluronidase before intranasal administration in order to increase the delivery of the protein and peptide to the brain. The hyaluronidase treatment also helped to rinse the nostril of the pups making the subsequent administration of the OPN and OPN-derived peptides more reliable by providing easier entry into the nostril.

## HISTOLOGY (PAPERS I-IV)

Histology is a useful tool for examining the distribution, location, and morphology of a cell or a protein in a tissue and for examining the morphology of the tissue as a whole. Proper preparation of the tissue prior to histological examination is important for maintaining the integrity and morphology of the tissue. Fixation preserves the morphology of the cells in the tissue and prevents the tissue from undergoing autolysis and degradation. To avoid the possible interference of blood components during histological examination of the tissue, the tissues can be perfused with isotonic saline to remove remaining blood from the vasculature before fixation.

All tissues used for histology in this thesis were paraffin embedded and sectioned. Tissues destined for paraffin sectioning need to be dehydrated after fixation. Dehydration of tissues was done by a series of incubations in increasing concentrations of alcohol and finally xylene followed by infiltration of paraffin.

When collecting the mouse brain tissue for histology (papers I–IV), the mice were deeply anesthetized and intracardially perfused with isotonic saline to remove the remaining blood from the brain vasculature. The tissues were fixed by incubation in Histofix, a commercially available fixative containing 6% formaldehyde, before they were prepared for paraffin sectioning. Sheep brains (paper II) were perfused in situ during post mortem examination of the fetal sheep, and the brains were stored in fixative for at least 14 days before being processed for paraffin sectioning. Human postmortem preterm brains (paper II) were fixed in 4% formalin for 5 to 7 weeks, depending on the size, before processed for paraffin sectioning.

For evaluating the tissue loss after brain injury in mice, mouse brain tissue slides were stained with antibodies specific for microtubule-associated protein-2 (MAP-2) or myelin basic protein (MBP). MAP-2 is expressed in neurons and is a commonly used and sensitive marker for evaluating gray matter tissue loss because the loss of MAP-2 staining indicates neuronal death (Gilland, Bona et al. 1998). MBP is a protein present in mature myelin (Back, Luo et al. 2002) and it is a commonly used marker for white matter damage. Total brain tissue-loss (volume) in the gray or white matter was calculated as MAP-2 or MBP-positive volume in the uninjured hemisphere minus the MAP-2 or MBP-positive volume in the injured hemisphere. The MAP-2 or MBP-positive volumes were calculated as: $V = \sum A \times (1/S) \times T$, where V = the total volume, $\sum A$ = the sum of the areas measured, (1/S) = the section sampling fraction and T = the thickness of the brain sections. The area of brain tissue loss in gray or white matter in one representative section was calculated as

MAP-2 or MBP-positive area in the uninjured hemisphere minus the MAP-2 or MBP-positive area in the injured hemisphere. In paper I, a linear regression analysis comparing the total brain tissue loss (volume) and the tissue loss in one representative section (area) from the hippocampus level showed a significant positive correlation ($p$ <0.001, $R^2$=0.9689) (Figure 3C, paper I), and thus one representative section from the hippocampus level was used to evaluate tissue loss in several of the following experiments.

## RT-qPCR (PAPERS I-IV)

Real time quantitative polymerase chain reaction (RT-qPCR) was used in papers I-III to examine gene expression in the mouse brain after HI and in paper IV to examine gene expression in the mouse brain after bacteremia caused by *S. epidermidis* infection. For these purposes, mRNA was extracted from brain tissue and reverse-transcribed in to its complementary DNA (cDNA) and gene expression levels were assessed by RT-qPCR, using the cDNA as the template.

RT-qPCR is a widely used technique to examine gene expression, and it detects changes in gene expression between samples. In a RT- qPCR reaction, cDNA is exponentially amplified and, in our case, quantified by the binding of a fluorescent dye (SYBR Green) within the newly generated double-stranded cDNA copies. When SYBR Green binds to double-stranded DNA, it emits a fluorescent signal, and during each amplification cycle the fluorescence intensity increases as the number of cDNA copies double. In order to only amplify the cDNA sequences corresponding to the mRNA of interest, specific primers are used to find the correct cDNA sequence.

The efficiency of the reverse transcription reaction, where mRNA is reversely transcribed into cDNA, needs to be controlled by the use of a reference gene. We have seen that differences in the animals' age and treatment can affect the expression of genes that are normally believed to be constitutively expressed. Therefore, several reference genes are usually tested and evaluated in the specific setup to determine which reference genes are the most stable. In papers I–III, the reference genes selected were GAPDH and 18S, while in paper IV the reference genes selected were GUSB, HPRT, HSP90AB1, GAPDH, and ACTB.

## WESTERN BLOT (PAPER III)

Western blot is a widely used technique for detecting and quantifying proteins in a sample. Tissue samples need to be mechanically broken down and homogenized in order to prepare protein samples, and subsequently the proteins are separated by

molecular weight by gel electrophoresis. After gel electrophoresis, proteins are transferred (blotted) onto a nitrocellulose membrane, and the blotted proteins can be detected by antibody-based staining.

Western blot is also a useful tool for examining protein modifications such as phosphorylation, splicing, or protein-protein interactions. Thus, western blot was used in paper III to quantify OPN protein in the mouse brain during development as well as for determining the efficiency of thrombin cleavage of OPN.

## SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS (PAPER II)

Single nucleotide polymorphisms (SNPs) are the most common genetic variations among people and are defined as the replacement of a single nucleotide at a single position in the DNA sequence, where the replacement should be present in more than 1% of the population. The nucleotide replacement, the SNP, can be present both in non-coding regions of the DNA as well as in a gene. When the SNP is present in a gene, that specific gene is described as having more than one allele. SNPs usually have no harmful effect on health and development, but if they are located in a gene or in a genes regulatory area, they might affect the gene´s function. Some SNPs are associated with certain diseases and can thus be used to predict an individual's genetic predisposition to develop these diseases.

In paper II, SNP analysis was used to determine whether SNPs in the genes encoding the IL-17A, IL-17F, and IL-22 cytokines were associated with increased risk for developing CP. For this purpose, blood samples collected from patients with CP as well as healthy controls were analyzed.

# RESULTS AND DISCUSSION

## ESTABLISHMENT OF AN HI-INDUCED PRETERM BRAIN INJURY MODEL IN MICE (PAPER I)

In paper I we sought to establish an HI-induced preterm brain injury model in mice to resemble brain injury in premature infants. The incidence of PVL in preterm infants is the highest between GWs 23 and 32 and has been found to correlate with the presence of pre-oligodendrocytes that are abundant in the brain during these GWs (Back, Luo et al. 2001). At PND2 up to PND7, the rodent brain white matter and oligodendrocyte development is similar to that in the human preterm infant. The cerebral oligodendrocyte and white matter development in the PND2 rodent is suggested to correspond to the human infants between GWs 18 to 27 while the PND7 rodent correspond to a late preterm infant at between GWs 30 and 36. However, from PND6–PND7 the maturation of oligodendrocytes rapidly increases in the rodent brain and the immature oligodendrocytes will become predominant over the pre-oligodendrocytes (Craig, Ling Luo et al. 2003). Thus, we chose to adopt the Rice-Vannucci HI-model to PND5 mice since pre-oligodendrocytes are still abundant in the mouse CNS at PND5 and because the size of the mice pups at this age make them reasonably easy to handle during surgery as well as during drug administrations. At PND5, mice were subjected to HI with different hypoxia durations to find the optimal hypoxia duration to cause focal white/gray matter injury.

At PND5, mice were subjected to HI by unilateral carotid artery ligation and subsequently exposed to hypoxia (10% $O_2$) for 50 minutes, 70 minutes or 80 minutes. At 7 days after HI, the brain injury induced by the different hypoxia durations was evaluated by immunohistochemistry. We found that 50 minutes of hypoxia resulted in only a small percentage of animals being injured, while 70 minutes of hypoxia resulted in injury in 93% of the animals. The injury at 70 min hypoxia was characterized by hippocampal atrophy and local white matter injury and focal cortical injury, features that are similar to those seen in preterm brain injury in human infants, while 80 minutes of hypoxia resulted in extensive brain injury with infarction in the white matter as well as in the gray matter in the cortex, hippocampus, and thalamus.

When subjecting PND5 mice to HI we found that the subcortical white matter was significantly more vulnerable to HI than the surrounding striatum and cortex,

features that were not found in the PND9 mouse after HI (Figure 3F and 3G, paper I) and that resemble the features of preterm brain injury. These findings support the choice of using PND5 mice to model brain injury at a developmental time when the white matter is particularly vulnerable. Furthermore, 70 minutes of hypoxia resulted in significant white matter and gray matter tissue loss in the injured hemisphere compared to the uninjured hemisphere, and the subcortical white matter in the injured hemisphere displayed abnormal myelin structure (Figure 4, paper I). Taken together, we concluded that 70 minutes of hypoxia was the optimal duration to induce reproducible HI-induced brain injury in PND5 mice that produces consistent white and gray matter damage that is relevant to the type of injury seen in human preterm infants.

## IMMUNE RESPONSE AFTER PRETERM BRAIN INJURY IN MOUSE (PAPERS I AND II)

It is known that both the adaptive and innate arms of the immune system are activated after neonatal HI (Hedtjarn, Leverin et al. 2002, Hedtjarn, Mallard et al. 2004, Hedtjarn, Mallard et al. 2004, Hedtjarn, Mallard et al. 2005, Winerdal, Winerdal et al. 2012), but the inflammatory response after HI in mouse models of preterm brain injury has not previously been characterized. We examined the immune responses in the brain after HI at PND5 by using RT-qPCR and immunohistochemical staining.

### INCREASED INNATE IMMUNE RECEPTOR EXPRESSION IN THE MOUSE BRAIN AFTER HI

The innate immune receptor TREM-2 is known to be involved in fine-tuning innate immune responses (Ford and McVicar 2009), and to form a complex with the transmembrane signaling adaptor protein DAP12 (Klesney-Tait, Turnbull et al. 2006, Tessarz and Cerwenka 2008). Because deficiency in TREM-2 and in DAP12 is known to be associated with white matter injury (Paloneva, Kestila et al. 2000, Tanaka 2000, Piccio, Buonsanti et al. 2007, Takahashi, Prinz et al. 2007), and that blocking TREM-2 exacerbates EAE in the mouse model of MS (Piccio, Buonsanti et al. 2007), we hypothesize that TREM-2 and DAP12 are involved in the white matter injury in preterm brain injury.

We first examined the expression of TREM-2 and DAP12 in the mouse brain after HI at PND5. We found that both TREM-2 and DAP12 expression were increased in the injured brain hemisphere with a maximum expression at 24 hours after HI. In addition to the parenchyma close to the site of injury, TREM-2 and DAP12 were

found in the meninges, the choroid plexus, and along the blood vessels (Figure 5 and Figure 6, paper I). These locations suggest that TREM-2 and DAP12 pathway might act as a sensor mediating communication between the periphery and the CNS. The possible communication between the periphery and the CNS through TREM-2 is supported by a study where TREM-2 knock-out mice were shown to have reduced invasion of CD3+ T-cells into the brain after stroke (Sieber, Jaenisch et al. 2013). However, in contrast to earlier studies which show that TREM-2 contributes to reduced inflammation in the EAE model (Takahashi, Rochford et al. 2005, Takahashi, Prinz et al. 2007), Sieber et al. show that TREM-2 deficiency was associated with reduced levels of pro-inflammatory cytokines in the sub-acute phase after stroke (Sieber, Jaenisch et al. 2013). Whether TREM-2 and DAP12 act as pro- or anti-inflammatory mediators in preterm brain injury needs to be further explored to evaluate their possible protective or detrimental contribution to preterm brain injury.

### TH1/TH17-TYPE RESPONSE IN THE NEONATAL MOUSE BRAIN AFTER INJURY

HI-induced neonatal brain injury can activate adaptive immune responses with an increase in cytokine production and infiltration of adaptive immune cells into the brain (Bona, Andersson et al. 1999, Hedtjarn, Leverin et al. 2002, Hedtjarn, Mallard et al. 2004, Hedtjarn, Mallard et al. 2004, Hedtjarn, Mallard et al. 2005, Winerdal, Winerdal et al. 2012), and HI combined with the TLR4 agonist LPS leads to a Th17 type response that can be attenuated by the administration of the lymphocyte migration blocking compound FTY720 (Yang, Sun et al. 2014).

By analyzing the mRNA expression of the Th1, Th2, Th17, and Treg-associated signaling pathways in the brain after HI in PND5 mice (Figure 8, paper I), we found that Th1- and Th17-associated cytokines (IL-12a, IL-6, IL-23a, and IL-22) and transcription factors (T-bet and RORγt) were increased in the injured hemisphere after HI. However, the regulatory T (Treg)-associated transcription factor (Foxp3) and cytokines (TGFβ and IL-10) were not altered after HI, nor were the Th2-associated transcription factor GATA3, or cytokine IL-5, while the Th2-associated cytokine IL-4 was downregulated and IL-13 was upregulated after HI. This indicates that the immune response triggered after HI at PND5 is skewed towards a pro-inflammatory Th1 and Th17-type response. Accordingly, we also found that the total number of CD4+ cells was increased in the brain at 24 hours and 7 days after HI, suggesting the possible participation of CD4+ T-cells in the injury process after HI. Most of the CD4+ cells were located in the blood vessels, and only a few CD4+ cells were present in the brain parenchyma (Figure 7, paper I).

Notably, since many of the changes in cytokine/transcription factor expression in the brain were seen as early as 6 hours after HI, it suggests the participation of "innate" types of lymphocytes to the brain injury process. The γδT-cells are considered to be a link between the innate and adaptive immunity and can respond quickly to an insult (Born, Reardon et al. 2006). The γδT-cells can be activated through the TCR, but they also express other activating receptors such as the natural killer group 2D (NKG2D) (Vantourout and Hayday 2013), IL-1R, and IL-23R (Sutton, Lalor et al. 2009) through which γδT-cells can be rapidly activated. γδT-cells can respond to an infection or insult within hours, as compared to conventional αβT-cells that need days to become activated by a new antigen (Chien, Zeng et al. 2013, Vantourout and Hayday 2013).

## γδT-CELLS IN PRETERM BRAIN INJURY (PAPER II)

Already in early development, the γδT-cells are functionally competent and are of great importance in immunity in early life (Gibbons, Haque et al. 2009). The γδT-cells respond to infection and sterile-induced inflammation as well as lipid ligands (Born, Kemal Aydintug et al. 2013), that are often enriched upon CNS injury. Although γδT-cells are found in the CNS and participate in demyelinating CNS injury in both human patients and in animal models of white matter injury such as MS (Selmaj, Brosnan et al. 1991, Wucherpfennig, Newcombe et al. 1992, Odyniec, Szczepanik et al. 2004) as well as in ischemic brain injury in adult mice (Shichita, Sugiyama et al. 2009), their role in neonatal brain injury is not known.

### INCREASE OF γδT-CELLS IN THE MOUSE, SHEEP, AND HUMAN BRAIN AFTER PRETERM BRAIN INJURY

We first used RT-qPCR to examine if TCRγ-chain mRNA was present in the mouse brain after preterm brain injury, and we found that the gene expression of the TCRγ-chain was increased in the brain already at 6 hours after HI, indicating that γδT-cells are present in the brain after HI (Figure 2, paper II). Interestingly, when analyzing the subtypes of TCRγ-chain expressed in the mouse brain at 6 hours after HI, we found high mRNA expression of the TCRγ-subtypes Vγ4, Vγ5, and Vγ7, which are the same subtypes expressed by γδT-cells in the blood, spleen, lymph node, lung, skin and gut in the mouse (Carding and Egan 2002).

The presence of γδT-cells in the neonatal mouse brain after HI-injury at PND5 was further confirmed using immunohistochemistry. γδT-cells were enriched in the injured brain hemisphere early (6 hours) after HI-injury in the border zone of the injury area, in the hippocampus, periventricular area, subcortical white matter and

in the meninges (Figure 3, paper II). γδT-cells is a link between the innate and adaptive immunity and can be divided into innate-like cells, which are often found in barrier tissues and can be activated and respond to an insult within hours, and adaptive-like cells which are present in secondary lymphoid organs and need APCs for activation. It takes days before the adaptive-like γδT-cells become activated (Chien, Zeng et al. 2013), thus the rapid increase in γδT-cell number as well as in TCRγ mRNA expression after injury suggests that the γδT-cells found in the brain are of the innate-like subtype.

To further confirm the presence and increase in γδT-cells in the brain after preterm brain injury, brain tissue from fetal sheep subjected to asphyxia and postmortem brain tissue from human preterm infants with brain injury were examined by immunohistochemistry staining. γδT-cells were frequently found in both the postmortem preterm infant brains and in the sheep brains after injury. Similar as in the mouse brain, the γδT-cells in the sheep brains (Figure 4, paper II) and in the human postmortem brains (Figure 5, paper II) were most frequently found in the meninges area as well as in the border zone of the injury. Together, this is the first study to show the presence of γδT-cells in the fetal and neonatal brain after preterm brain injury in multiple species, and suggest that γδT-cells are involved in preterm brain injury.

Our finding of γδT-cells being frequently present in the meninges might support the finding in a recent study where γδT-cells were found in the meninges of adult naïve C57BL/6 WT mice, suggesting that γδT-cells are normal components of the meninges (Hatfield and Brown 2015). The γδT-cells found in the meninges by Hatfield and Brown were RORγt positive, and because RORγt is a transcription factor important in IL-17 producing cells (Ivanov, McKenzie et al. 2006), this suggests that the γδT-cells in the normal meninges are capable of IL-17 production.

### γδT-CELLS BUT NOT IL-17/22 CONTRIBUTE TO PRETERM BRAIN INJURY

Using the PND5 HI brain injury model with WT mice and genetically modified mice lacking γδT-cells (γδT−/− mice), we found that the absence of γδT-cells provides neuroprotection (Figure 6, paper II). This further confirmed that γδT-cells play an important role in preterm brain injury. In an adult mouse model of stroke (Gelderblom, Weymar et al. 2012) it was suggested that the pro-inflammatory cytokine IL-17 is an important mediator of brain injury. Furthermore, IL-17 can act synergistically with the cytokine IL-22 (Eyerich, Eyerich et al. 2010), and disrupt the integrity of the BBB and mediate leucocyte migration in vitro (Kebir, Kreymborg et al. 2007), and the expression of IL-17A, IL-17F and IL-22 correlate with increased

lesion volume and demyelination in patients with MS (Tao, Zhang et al. 2015). Since both IL-17 and IL-22 can be produced by γδT-cells (Martin, Hirota et al. 2009, Sutton, Lalor et al. 2009, Wolk, Witte et al. 2010, Gelderblom, Weymar et al. 2012, Sabat, Ouyang et al. 2014) we hypothesized that γδT-cells contribute to preterm brain injury through IL-17/IL-22 signaling pathways.

First, the gene expression of IL-22 and IL-17, as well as the expression of genes associated with their respective signaling pathways, were assessed by RT-qPCR. We found that gene expression of both IL-22 and IL-17 as well as some genes in their signaling pathway were upregulated in the mouse brain as early as 6 hours after HI at PND5 (Figure 1, paper II), the same time point as we see the highest increase in the number of γδT-cells in the brain. However, the most commonly expressed IL-17 family cytokine, IL-17A, was not detected in the mouse brain after HI at PND5, while IL-17F, the IL-17-family cytokine with the most homology to IL-17A, was increased at 6 hours after HI in the injured brain hemisphere.

To further, evaluate the contribution of the IL-17F and IL-22 cytokines in preterm brain injury, antibody depletion was used to block IL-17A and IL-17F in normal WT mice exposed to HI, while the effect of IL-22 was examined by subjecting IL-22 deficient (IL-22-/-) mice to HI. In contrast to adult stroke (Gelderblom, Weymar et al. 2012) and MS (Tao, Zhang et al. 2015), none of the IL-17 and IL-22 cytokines were found to contribute to the pathology of preterm brain injury, because neither the depletion of IL-17 nor the lack of IL-22 resulted in any improvement in injury outcome compared to control mice. Furthermore, γδT-cells do not seem to be the major producer of IL-17F and IL-22 in preterm brain injury because the expression of the cytokines after HI did not differ between WT mice, γδT−/− mice and mice lacking all B- and T-cells (Rag1−/− mice). These results therefore also differ from a study on EAE where Vγ4+ γδT-cells were identified in the brain and found to produce high levels of IL-17 (Blink, Caldis et al. 2014). Overall, our results show that γδT-cells contribute to HI-induced preterm brain injury, but further studies are needed to evaluate the mechanism behind the γδT-cell-mediated pathology as well as to evaluate the specific properties of the different subtypes Vγ4, Vγ5, and Vγ7, found in the brain after preterm brain injury.

### One gene SNP for IL-17A but none for IL-17F and IL-22 link to CP in patients

Preterm birth and neonatal brain injury has long been known risk factors for the neurodevelopmental motor function disorder CP, but more recently genetics have also been recognized as a possible contributor and risk factor for CP (McMichael,

Girirajan et al. 2014, McMichael, Bainbridge et al. 2015, Oskoui, Gazzellone et al. 2015). Thus, blood samples from CP patients and healthy controls were analyzed to evaluate if SNPs in the genes encoding IL-17A, IL-17F, and IL-22 were associated with CP. One of the SNPs selected for *IL17a* showed a significant association with CP, while none of the SNPs for *IL17f* or *IL22* did. The SNP analysis was made in a mixed population of patients with CP, including both patients born preterm and at term. Our original aim was to analyze SNPs in patients with CP born at term and preterm separately to compare the two groups. However, due to difficulties in enrolling enough patients born prematurely, this was not possible. When we did try to separate these two patient groups in the SNP analysis, no SNP was shown to be associated with CP in the patient group born preterm while the association with CP in the patients born at term remained. However, the number of patients born preterm was very small (n = 38), and thus no conclusion can be drawn from the results in the preterm group. A larger sample size of preterm patients with CP, in particular CP cases born extremely preterm, would be needed to evaluate the association of SNPs in the genes encoding IL-17A, IL-17F, and IL-22 in the development of CP in preterm versus term infants.

## OPN-DERIVED PEPTIDES AGGRAVATE INJURY TO THE IMMATURE MOUSE BRAIN (PAPER III)

OPN is an inflammatory regulator that can have either pro-inflammatory or anti-inflammatory functions depending on the situation (Wang and Denhardt 2008, Cantor and Shinohara 2009), and OPN is one of the most highly regulated genes in the brain in a mouse model of neonatal HI-induced brain injury (Hedtjarn, Mallard et al. 2004). The upregulation of OPN expression after HI was confirmed in our PND5 HI mouse model, where OPN mRNA expression (Figure 2, paper III) as well as OPN positive staining (Figure 3, paper III) was increased in the injured brain hemisphere already at 6 hours after HI.

Because previous studies show that administration of recombinant OPN protein has protective effects after HI-induced brain injury in neonatal rats (Chen, Ma et al. 2011) and in adult mouse models of stroke (Meller, Stevens et al. 2005, Doyle, Yang et al. 2008), and that OPN deficiency results in more severe HI-induced brain injury in neonatal mice (van Velthoven, Heijnen et al. 2011), we sought to evaluate the effect of OPN on brain injury outcome in the HI-induced preterm brain injury model in mice. We did not detect any significant differences in brain injury outcome between animals administered recombinant mouse OPN by ICV injection immediately before HI or by intranasal administration immediately before and after

HI compared to mice receiving control substance (Figure 5, paper III). Furthermore, no significant difference was detected in brain injury outcome between WT mice and mice lacking OPN (OPN−/− mice) after HI at PND5 (Figure 6, paper III).

In addition to full-length recombinant OPN, Doyle et al. also showed that thrombin-cleavage of OPN, which results in an N-terminal and a C-terminal fragment of the protein, with the N-terminal fragment containing both of the integrin-binding sequences RGD and SLAYGLR (Denhardt, Noda et al. 2001), enhances the protective effect of OPN (Doyle, Yang et al. 2008). They also showed that administration of a short peptide derived from the N-terminal side of the thrombin-cleaved site as well as a peptide from the C-terminal side of the thrombin cleavage site significantly reduces infarct volume (Doyle, Yang et al. 2008).

To evaluate the potential neuroprotective effect of the thrombin-cleaved OPN (T-OPN) and OPN derived peptides (Figure 2) in the mouse model of preterm brain injury T-OPN and the OPN-derived peptide N134–153, corresponding to amino acids 134–153 of the OPN protein, were administered by intranasal administration while the N134-153 peptide and a peptide corresponding to amino acids 154–198 (C154–198) of the OPN protein were administered by ICV injection (Figure 5 and Figure 7, paper III). The peptides chosen for administration had the same amino acid sequences as the peptides that had been proven to be protective in the mouse model of adult stroke (Doyle, Yang et al. 2008).

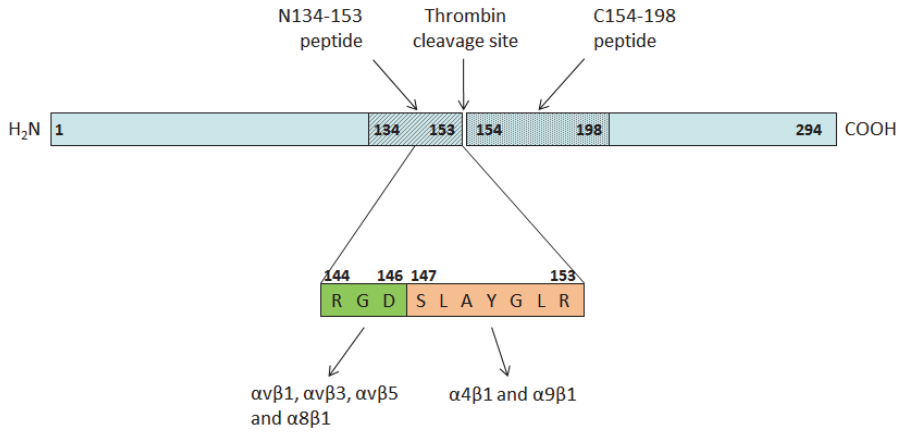


*Figure 2. Schematic figure of OPN protein. Arrows indicate the location of the thrombin cleavage site, the N134-153 peptide, the C154-198 peptide in the OPN protein, and the integrins that are bound by the integrin binding sequences RGD (green box) and SLAYGLR (orange box) of OPN.*

The extent of HI-induced brain injury was evaluated and compared between the animals receiving rmOPN, T-OPN, N134–153 and N154–198 and animals receiving control substance. We found no difference in injury outcome between animals receiving rmOPN and T-OPN and animals receiving control substance. However, in contrast to the adult mouse stroke model (Doyle, Yang et al. 2008), we found that administration of the N134–153 peptide aggravated gray matter injury while the C154–198 peptide aggravated both gray matter and white matter injury after HI compared to animals receiving control substance.

Our findings that the N134–153 peptide did not provide protection against neonatal HI are further supported by a recent study where the same short OPN peptide, but coupled to a HIV-TAT-sequence to facilitate cellular uptake in the brain, failed to elicit neuroprotection when administered intranasally, by intraperitoneal injection, or by ICV injection in the PND9 HI mouse model (Bonestroo, Nijboer et al. 2015). However, they did not see aggravated injury as we found in our study. Taken together, our results differ from previous studies (Meller, Stevens et al. 2005, Doyle, Yang et al. 2008, Chen, Ma et al. 2011) where OPN and OPN-derived peptides provide protection to brain injury.

A recent study on the effect of OPN on stroke has shown that intranasal administration of the same short OPN-derived peptide as the N134–153, provide significant protection by binding to the αvβ3 integrin (Jin, Lee et al. 2016). In the developing brain, integrin expression can vary during different developmental stages, and play important roles during for example the development of cortical neurons (Schmid and Anton 2003), and oligodendrocytes (Blaschuk, Frost et al. 2000) and during myelination (Relvas, Setzu et al. 2001). Thus, differing expression of integrins in the CNS during development might partly explain the contradictory results of OPN on brain injury in adult compared to neonatal ischemic brain injury. However, the C154–198 peptide, which in our setup resulted in both gray and white matter injury, do not contain the integrin binding sites RGD or SLAYGLR, but is suggested to bind the receptor CD44 (Doyle, Yang et al. 2008). However, the mechanisms behind the effect of the N134–153 and C154–198 peptides in our setup have not been evaluated.

## THE IMPACT OF *STAPHYLOCOCCUS EPIDERMIDIS* BACTEREMIA ON THE DEVELOPING MOUSE BRAIN (PAPER IV)

Epidemiological data suggest that sepsis in the neonatal period, caused by Gram-positive bacterial infections, is associated with neurodevelopmental impairments

(Schlapbach, Aebischer et al. 2011, Chau, Brant et al. 2012). Today the most common cause of gram-positive bacteremia in preterm infants is the coagulase-negative staphylococci (Ohlin, Bjorkman et al. 2015), such as the ubiquitous skin commensal *S. epidermidis* (Power Coombs, Kronforst et al. 2013). We used an established neonatal *S. epidermidis* bacteremia model (Kronforst, Mancuso et al. 2012) to evaluate the effect of bacteremia on the developing brain.

### BACTERIA DO NOT ENTER THE CNS DURING *S. EPIDERMIDIS* BACTEREMIA

When the neonatal *S. epidermidis* bacteremia model was established the bacteremia-induced systemic inflammatory response included increased expression of the innate immune receptor TLR2 and its adaptor molecule MyD88 mRNA in the liver (Kronforst, Mancuso et al. 2012) suggesting the TLR2 signaling pathway to be important in the neonatal immune response to *S. epidermidis*. Kronforst et al. also demonstrated that live bacteria were present in the liver, spleen, and blood after inoculation, and the bacterial infection was cleared by the pups within 24–48 hours after infection (Kronforst, Mancuso et al. 2012).

Because neonatal *S. epidermidis* bacteremia increase the expression of TLR2 (Kronforst, Mancuso et al. 2012) and systemic administration of the TLR2 ligand Pam(3)CSK(4) impairs neonatal mouse brain development (Du, Fleiss et al. 2011), we sought to examine the impact of *S. epidermidis*-induced bacteremia on the preterm brain and the possible role of TLR2 in this process. Normal WT mice or TLR2−/− mice were intravenously administered with live *S. epidermidis* bacteria at PND1 in order to induce neonatal bacteremia. Bacterial cultures showed positive *S. epidermidis* cultures of blood, spleen, and liver, but no viable *S. epidermidis* bacteria were found in the CNS (Figure 1, paper IV). Furthermore, the bacterial load peaked at 4 hours after inoculation and the bacteria were cleared by the WT mouse pups within 48 hours, in agreement with what was shown by Kronforst et al. However, the TLR2−/− mice had impaired clearance of bacteria, and did not clear the infection until 72 hours after inoculation, indicating that *S. epidermidis* clearance is TLR2 dependent.

### INFLAMMATORY RESPONSE IN THE CNS INDUCED BY *S. EPIDERMIDIS* BACTEREMIA

*S. epidermidis* induces the production of several inflammatory mediators such as IFN-γ (Stuyt, Kim et al. 2003), TNF-α, IL-1β (Wakabayashi, Gelfand et al. 1991, Kronforst, Mancuso et al. 2012), IL-6 and CCL2 (Kronforst, Mancuso et al. 2012) in the blood. We analyzed the cytokine and chemokine responses to *S. epidermidis* bacteremia in the blood and in the brain and found that the pro-inflammatory cytokines and chemokines IL-6, CCL2, CXCL1, and IL-12p40 were significantly

increased in the blood of both WT and TLR2−/− mice, while only CCL2 was increased in the brain (Figure 2, paper IV). Furthermore, *S. epidermidis* bacteremia was capable of inducing apoptosis in the brain of WT mice, but not in the TLR2−/− mice, suggesting that *S. epidermidis*-induced caspase-3 activation is TLR2 dependent (Figure 4, paper IV).

Even though no live bacteria entered the brain, *S. epidermidis* bacteremia was capable of inducing innate immune signaling pathways in the brain, as well as increasing the white blood cell numbers in the CSF already at 6 hours after infection (Figure 3, paper IV). The *S. epidermidis* bacteremia selectively triggered upregulation of TLR2 mRNA expression in the brain as none of the other TLRs examined (TLR1 and TLR3–9) were altered on mRNA level (Figure 5, paper IV).

### *S. EPIDERMIDIS* BACTEREMIA IMPAIRS WHITE AND GRAY MATTER BRAIN DEVELOPMENT

Previous animal studies have shown that inflammation induced by repeated systemic injections of the TLR2 agonist Pam3CSK4 negatively influence brain development in newborn mice (Du, Fleiss et al. 2011), and inflammation caused by systemic administration of the TLR4 agonist LPS has been shown to induce cerebral white matter injury, similar to that seen in preterm human infants (Mallard, Welin et al. 2003, Wang, Hellgren et al. 2009, Mallard and Wang 2012). However, few studies have been conducted where the effect of live bacteria on brain development has been explored. To evaluate if *S. epidermidis* bacteremia induced by intravenous administration of live *S. epidermidis* have an effect on brain development, the white and gray matter brain volumes of *S. epidermidis*-injected animals and control animals were analyzed. In both WT and TLR2−/− mice, *S. epidermidis* bacteremia resulted in significantly reduced gray and white matter brain volume, as well as disrupted myelin processes (Figure 6, paper IV). These results support the findings from human preterm infants where postnatal infections have been associated with abnormalities in brain development, even when CSF cultures are negative (Chau, Brant et al. 2012).

Our results show that even though *S. epidermidis* did not enter the CNS, the bacteremia led to impaired white and gray matter development in the neonatal brain as well as increased white blood cell numbers in the CSF. The effects of *S. epidermidis* bacteremia were both TLR2 dependent and independent, with TLR2 dependent systemic inflammatory responses, while the impairment of white and gray matter development was TLR2 independent.

# SUMMARY AND CONCLUSIONS

Since brain injury associated with preterm birth is a significant contributor to childhood disabilities, it is crucial to explore the mechanisms involved in preterm brain injury to identify possible therapeutic targets. In this thesis we established a mouse model of HI-induced brain injury relevant to the injury seen in human preterm infants. By using this model we characterized and explored the contribution of neonatal immunity to the development of preterm brain injury, and we examined the possible neuroprotective effects of modulating specific parts of the immune responses. Furthermore, we examined the effect of neonatal acquired bacteremia on the development of the brain and its possible contribution to brain injury in mouse. The main findings are summarized in figure 3.

We show for the first time, that preterm brain injury is reduced by the deletion of γδT-cells and that γδT-cells are present in the injury area in preterm brain injury models in mice and sheep as well as in the postmortem brains of human preterm infants with white matter injury. In addition, HI-induced preterm brain injury elicits a Th1/Th17-skewed immune response, but in contrast to ischemic injury in mature animals, the pro-inflammatory cytokine IL-17 does not seem to be an important mediator of HI-induced preterm brain injury.

At a developmental age corresponding to early preterm infants, bacteremia in the neonatal mouse caused by the commensal skin bacteria *S. epidermidis* induced impaired white and gray matter brain development even without entry of live bacteria into the CNS. These findings provide important evidence that systemic infections even with rather harmless opportunistic pathogen/bacteria in the neonatal period can have potentially deleterious effects on brain development.

We have also found evidence of the importance of the developmental state of the neonatal immunity and the CNS when predicting possible therapeutic agents for preterm brain injury. OPN, which has been shown to be protective in adult models of ischemic brain injury, does not provide protection in the mouse model of preterm brain injury. Furthermore, the injury is aggravated when administering OPN-derived peptides, which in adult ischemic brain injury showed high neuro-protective potential.

Taken together our findings highlight the unique features of injury in the developing brain, and emphasize that preterm infants are developmentally immature and have different responses to the same stimuli as compared to more mature individuals.

Thus, seeking specific therapeutic targets for different age groups is of great importance.

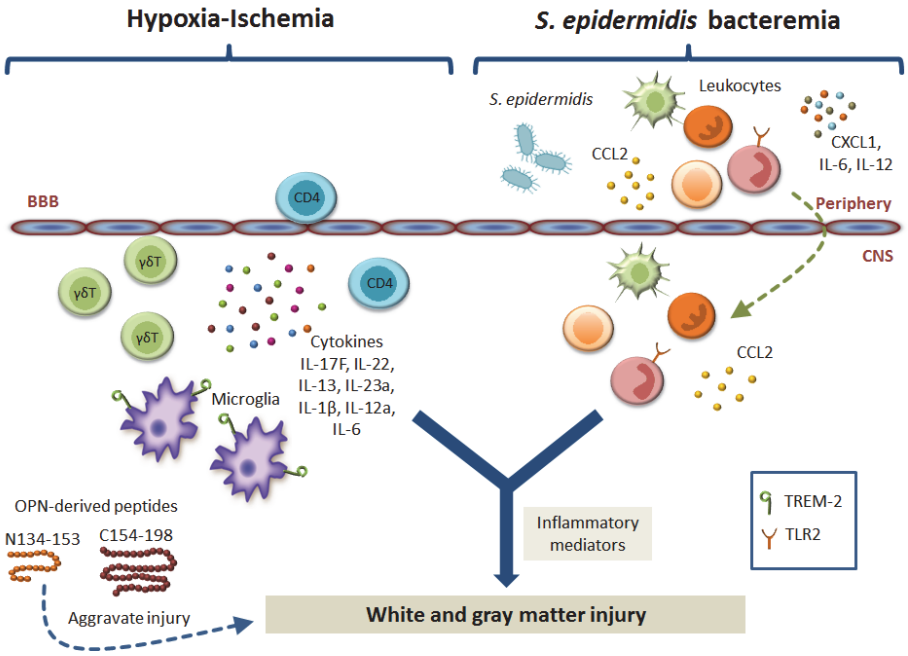


*Figure 3. Schematic figure summarizing the brain injury processes following experimental hypoxia-ischemia (HI) and S. epidermidis bacteremia in neonatal mice. HI at PND5 results in an early γδT-cell response and a late CD4+ T-cell infiltration in the brain, that is accompanied by an increased inflammatory response including Th1/Th17- associated signaling, and TREM-2 and OPN protein expression. γδT-cell deficiency protects the mouse from HI-induced brain injury; while administration of the OPN-derived peptides N134-153 and C154-198 aggravate brain injury. S. epidermidis-induced bacteremia results in a systemic inflammatory response including increased levels of cytokines and chemokines in the blood, increased TLR2 mRNA expression in the brain, and increased number of leukocytes in the blood and CSF, without bacteria entering the CNS. S. epidermidis bacteremia results in impaired gray and white matter development.*

# ACKNOWLEDGEMENTS

Many people have been involved in the making of this thesis. I deeply appreciate all your help! In particular I would like to express my thanks to:

My main supervisor **Xiaoyang Wang**, for introducing me to the science of preterm brain injury, for always being encouraging, and for providing support and ideas whenever needed.

My co-supervisors **Carina Mallard** and **Henrik Hagberg**, for great scientific input and inspiration.

All **co-authors** for excellent work and scientific expertise.

My wonderful past and present colleagues at the physiology department and Sahlgrenska Academy, including **Xiaoli**, **Dan**, **Arshed**, **Gisela**, **Anna-Lena, Pernilla, Gabriella**, **Linnea**, **Ana**, **Pete**, **Syam**, **Amin**, **Kristina**, **Joakim**, **Jacqueline**, **Eridan**, **Veronika**, **Sha**, **Barbara**, **Nina**, **Anna**, **Josefine**, and **Karolina**. Thank you all for making my workplace inspiring and fun, and for always being so helpful.

**Mamma**, **Pappa**, **Daniel**, **Linda**, **Maria**, **Josefin** och alla **respektive** och **barn**, för kärlek, stöd och uppmuntran.

**Gustav** och **Rufus**, min underbara lilla familj ♡

This work was supported by grants from: The Swedish Research Council (VR), Swedish governmental grants to researchers in the public health service (ALFGBG), VINNMER–Marie Curie international qualification (VINNOVA), Gothenburg Medical Society, the Frimurare Barnhus Foundation, the Chinese Scholarship Council, the National Natural of Science Foundation of China, the Department of Science and Technology of Henan Province, the Science and Technology Bureau of Zhengzhou, the Bill & Melinda Gates Foundation Grand Challenge Explorations and Global Health, Wilhelm and Martina Lundgren, the Åhlén Foundation, Wellcome Trust, the Byggmästare Olle Engqvist Foundation, the Leducq Foundation, the Swedish Brain Foundation, the Swedish Medical Society, Signhild Engkvists Stiftelse, the National Institutes of Health and National Institute of Allergy and Infectious Diseases, MedImmune, Crucell, the Åke Wiberg Foundation, and the Magnus Bergvall Foundation.

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