Developing brain and systemic inflammation: a “Toll-like” link with consequences

Amin Mottahedin

Department of Physiology,
Institute of Neuroscience and Physiology
at Sahlgrenska Academy
University of Gothenburg

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UNIVERSITY OF
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Cover illustration by Amin Mottahedin. Two-photon image of mouse choroid plexus immunolabeled for occludin (red) and CD31 (green).

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Amin.Mottahedin@gu.se

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To my dear family

Developing brain and systemic inflammation: a "Toll-like" link with consequences

Amin Mottahedin

Department of Physiology, Institute of Neuroscience and Physiology Sahlgrenska Academy at University of Gothenburg

ABSTRACT

The developing brain is vulnerable to external insults, and perinatal brain injury (PBI) is a major cause of life-long neurological syndromes such as cerebral palsy. Currently, no pharmaceutical intervention is available. Hypoxia/ischemia (HI), infections and inflammation are implicated in the pathogenesis of PBI. However, the crosstalk between these etiologies is not fully understood. Toll-like receptors (TLR) 3 and TLR2 are responsible for sensing viral and bacterial infections and initiating the inflammatory response. The aim of this thesis was to investigate the effect of systemic inflammation induced by activation of these TLRs on neonatal HI brain injury. We demonstrate that intraperitoneal administration of TLR3 and TLR2 ligands (PolyI:C and P3C, respectively) prior to HI increases the brain injury in neonatal mice. PolyI:C and P3C induced neuroinflammation and altered microglial phenotype as assessed by RT-qPCR, multiplex cytokine assay or flow cytometry. PolyI:C also upregulated the pro-apoptotic gene, *Fasl,* expression and reduced activation of pro-survival signaling molecule Akt. On the other hand, P3C suppressed mitochondrial respiration, a major mechanism of cellular energy production. P3C, unlike other TLR agonists, induced marked infiltration of leukocytes to the cerebral spinal fluid and brain of neonatal mice and rats. Confocal microscopy, Cre recombinase-mediated gene targeting and *in vitro* cell transmigration assay revealed the choroid plexus as a site of leukocyte entry. RNA sequencing of the choroid plexus followed by transcriptome cluster analysis and Ingenuity Pathway Analysis revealed potential mechanisms of leukocyte infiltration, including a specific chemotaxis signature and cytoskeleton-related pathways. Finally, we show that N-acetylcysteine treatment inhibits TLR2-mediated leukocyte trafficking *in vivo* and *in vitro*.

To conclude, this thesis describe a TLR-mediated link between systemic inflammation and developing brain with detrimental consequences on HI brain injury, suggesting potential novel therapeutic strategies.

Keywords: neonatal brain injury, hypoxia-ischemia, inflammation, infection, Toll-like receptor, choroid plexus

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Sammanfattning på svenska

Hjärnskador hos nyfödda barn är den vanligaste orsaken till neurologiska problem (såsom cerebral pares) bland barn. I Sverige diagnostiseras 2 av 1000 spädbarn med cerebral pares. Dessa hjärnskador uppstår i både fullgångna och för tidigt födda barn, men förekomsten är betydligt högre bland barn som fötts förtidigt. En vanlig orsak till hjärnskadorna hos spädbarn är brist på syre (hypoxi) och/eller lågt blodflöde (ischemi) till hjärnan. Ännu en riskfaktor är infektioner hos spädbarnet eller mamman någon gång runt födseln. Hur infektioner kan öka risken för hjärnskada i nyfödda var den huvudsakliga frågan vi ville försöka svara på i denna avhandling. Infektioner känns igen av vårt immunförsvar genom specifika receptorer som kallas Toll-liknande receptor (TLR) som finns på ytan av alla immunceller. Virus aktiverar framförallt TLR3 och en viss grupp bakterier aktiverar TLR2, och genom aktivering av dessa receptorer startar en immunologisk reaktion i kroppen.

Vi använder oss av en modell av hypoxi/ischemi (HI) hos nyfödda möss som producerar hjärnskador som liknar dem som kan ses hos spädbarn. Genom att administrera specifika ämnen aktiverades TLR3 och TLR2 innan HI. Vi upptäckte att aktivering av dessa receptorer ökar hjärnskadorna i både grå substans (nervceller) och vit substans (nervfibrer). Det visar på att virus- och bakterieinfektioner gör hjärnan känsligare för HI. Vi undrade sedan vad det är som orsakar denna ökade känslighet. Vi upptäckte att aktivering av TLR3 leder till en kraftig inflammatorisk respons i hjärnan, vilket ledde till specifika cellförändringar som pekade på minskad överlevnadsfunktion samt att hjärnans egna immunceller (s.k. mikroglia) blev mer reaktiva. Aktiveringen av TLR2 ledde till invasion av vita blodkroppar till hjärnan från blodet och minskad mitokondriefunktion (mitokondrierna ger celler energi) i hjärnans celler. Vi såg att de vita blodkropparna tar sig in i hjärnan via ett litet organ i hjärnan som kallas plexus choroidea. Därför analyserade vi genförändringar i plexus choroidea efter TLR2-stimulering och kunde fastställa de molekylära mekanismer som kan göra det möjligt för vita blodkroppar att ta sig in i hjärnan. Slutligen visade vi att behandling av neonatala råttor med N-acetylcystein, ett antioxidantläkemedel, blockerar invasionen av vita blodkroppar till hjärnan i denna modell. Sammanfattningsvis fann vi att virus- och bakterieinfektioner orsakar inflammation i hjärnan och gör hjärnan känsligare för HI-relaterade hjärnskador. Minskning av inflammationen i hjärnan genom riktade behandlingar mot plexus choroidea eller genom behandling med N-acetylcystein kan minska risken för hjärnskador hos nyfödda.

List of papers

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

I. Stridh L, **Mottahedin A**, Johansson ME, Valdez RC, Northington F, Wang X, Mallard C. *Toll-like receptor-3 activation increases the vulnerability of the neonatal brain to hypoxia-ischemia.* Journal of Neuroscience, 2013. 33(29): p. 12041-51.

II. **Mottahedin A**, Svedin P, Nair S, Mohn CJ, Wang X, Hagberg H, Ek J, Mallard C. *Systemic activation of Toll-like receptor 2 suppresses mitochondrial respiration and exacerbates hypoxic-ischemic injury in the developing brain.* Journal of Cerebral Blood Flow and Metabolism. 2017 Jan 1:271678X17691292.

III. **Mottahedin A**, Smith PL, Hagberg H., Ek CJ, Mallard C. *TLR2-mediated leukocyte trafficking to the developing brain.* Journal of Leukocyte Biology. 2017 Jan;101(1):297-305.

IV. **Mottahedin A**, Ek J, Truvé K, Hagberg H, Mallard C. *Differential analysis of TLR2- versus TLR4-induced alterations in transcriptome of choroid plexus reveals leukocyte trafficking mechanisms.* Manuscript.

V. **Mottahedin A**, Blondel S, Ek J, Babikian A, Hagberg H, Mallard C, Ghersi Egea JF, Strazielle N. *N-acetylcysteine inhibits TLR2-mediated neutrophil transmigration through the choroid plexus.* Manuscript.

Content

Abbreviations

ANOVA	analysis of variance
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCSFB	blood-cerebrospinal fluid barrier
CMV	cytomegalovirus
CNS	central nervous system
CP	cerebral palsy
CPEC	choroid plexus epithelial cell
CSF	cerebrospinal fluid
CVO	circumventricular organ
DAB	3,3′-Diaminobenzidine
DAMP	damage-associated molecular pattern
dsRNA	double-stranded RNA
EAE	experimental autoimmune encephalomyelitis
EONS	early-onset neonatal sepsis
ETC	electron transfer chain
FADH	flavin adenine dinucleotide
FCCP	carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FIRS	fetal inflammatory response syndrome
GBS	group B streptococcus
G-CSF	granulocyte-colony stimulating factor
GRK2	G protein-coupled receptor kinase 2
GW	gestational week
HI	hypoxia/ischemia
HIE	hypoxic/ischemic encephalopathy
ICV	intracerebroventricular
IgG	immunoglobulin gamma
IHC	immunohistochemistry
IL	interleukin
iNOS	induced nitric oxide synthase
IVH	intraventricular hemorrhage
JNK	c-Jun N-terminal kinase
LONS	late-onset neonatal sepsis
LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
MAP2	microtubule-associated protein 2
MBP	myelin basic protein

MCP-1	monocyte chemoattractant protein 1
MD2	myeloid differentiation protein-2
MEGF10	multiple epidermal growth factor-like domains protein 10
MERTK	mer tyrosine kinase
MHCII	major histocompatibility complex
MIP1-a	macrophage inflammatory protein 1 alpha
MIP1-b	macrophage inflammatory protein 1 beta
miRNA	microRNA
MyD88	myeloid differentiation primary response gene 88
NAC	N-acetylcysteine
NADH	nicotinamide adenine dinucleotide
NE	neonatal encephalopathy
NEC	necrotizing entrocolitis
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	pathogen-associated molecular pattern
PFA	paraformaldehyde
PND	postnatal day
Poly I:C	polyinosinic:polycytidylic acid
PRR	pattern-recognition receptor
PRX3	peroxiredoxin 3
PVL	periventricular leukomalacia
RANTES	regulated on activation, normal T cell expressed and secreted
RNAi	interfering RNA
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SOD2	superoxide dismutase 2
SVZ	sub-ventricular zone
TGF-β	transforming growth factor beta
TIR	Toll-like/IL1R
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
WB	Western blot
WBC	white blood cell

Introduction

Brain development

The brain is the most complex organ in the body; hence, its development consist of intricate processes including formation, differentiation, migration and connection of neurons. The cerebral cortex grey matter that constitutes more than 80% of the adult human brain mass contains 19% of the total brain neurons (16 billion), while cerebellum that has 10% of brain mass contains 80% of all neurons (69 billion). Almost half of the brain cells are non-neuronal, cells which are mainly located in white matter (15 billion) and a small proportion in grey matter (1.5 billion) (Azevedo et al., 2009). In humans, brain development starts as early as week 3 of gestation (GW3) when the neural plate emerge from neuroepithelial cells of the ectoderm (Stiles and Jernigan, 2010). This is followed by formation and closure of the neural tube that is the scaffold structure for development of the central nervous system (CNS). The neural tube is also eventually transformed into the ventricular system of the brain.

Progenitor cells, such as radial glial cells, line the internal side of the neural tube and start dividing and giving rise to pyramidal neural cells by GW5 (Budday et al., 2015). The human brain developmental time course from GW5 onwards is depicted in figure 1. The newborn neurons migrate radially from the subventricular zone (SVZ) outward to form the cortical plate (Budday et al., 2015). Differentiation of the migrated neurons give rise to two main inhibitory and excitatory neuronal types and several subtypes, of which sixteen has been identified by single-cell nucleus RNA sequencing (Lake et al., 2016). All six layers of cortex are shaped by GW18, and thereafter, dendritic and axonal growth and synaptogenesis begins (Budday et al., 2015). These processes are guided by intrinsic and extrinsic cues, and local mRNA translation plays a key role (Holt and Schuman, 2013). The migration of neurons continues postnatally in humans until 1.5-2 years of age (Sanai et al., 2011; Paredes et al., 2016). A large population of neurons migrate from SVZ to the frontal lobe during the first 5 months of human life and differentiate into inhibitory neurons (Paredes et al., 2016). Synaptogenesis is followed postnatally by elimination of unnecessary synapses by microglia and astrocytes in a process mediated by components from the complement system, MEGF10 (multiple epidermal growth factor-like domains protein 10) and MERTK (mer tyrosine kinase) phagocytic pathways (Stevens et al., 2007; Chung et al., 2013). Synaptic

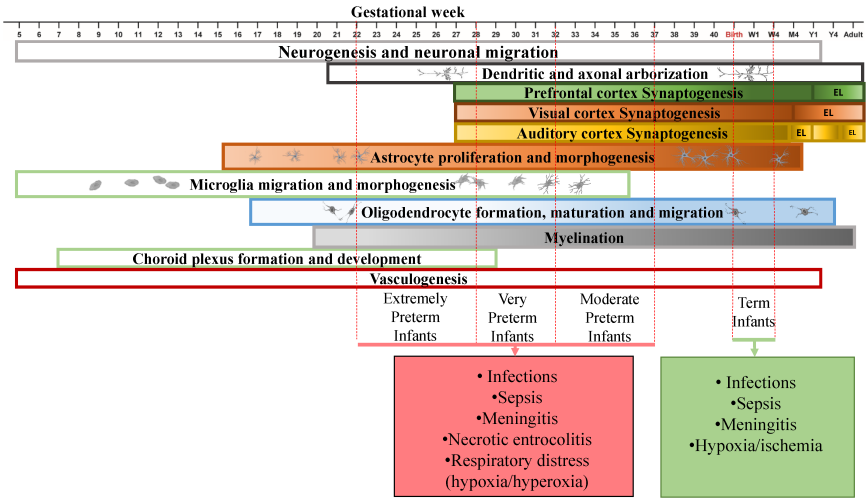

Figure 1. The time course of events in developing brain of human and potential perinatal challenges. EL=elimination

pruning is a critical mechanism of sculpting neural circuit and brain plasticity (Riccomagno and Kolodkin, 2015). Astrocytes emerge from either radial glial cells prenatally or progenitor cells in SVZ postnatally (Ge et al., 2012). They are detected in the human brain by GW15 (Roessmann and Gambetti, 1986). Microglia, the professional immune cells of the brain, originate from yolk sac primitive macrophages and migrate to the developing brain as early as GW5 (Monier et al., 2007; Ginhoux et al., 2010). Therefore, both microglia and astrocytes are instrumental in shaping the developing brain from early stages (Reemst et al., 2016). The progenitors of oligodendrocytes (OL), the myelinating cells, are generated in the fetal brain around GW17 and continue to proliferate and differentiate into mature OL until early childhood (Rakic and Zecevic, 2003; Yeung et al., 2014). Myelination, therefore, begins after OL appear in the brain around mid-gestation (Tosic et al., 2002; Yeung et al., 2014). Myelination in adult brain occurs as one of the mechanisms of brain plasticity induced by neural activity (Liu et al., 2012). Brain vasculature starts to form from GW5 (Budday et al., 2015) and choroid plexus from GW7 (Dziegielewska et al., 2001) and both contain the barrier structures that separates the CNS from the periphery.

Brain interfaces

Brain vasculature, choroid plexus and circumventricular organs (CVOs) constitute

the major brain interfaces. The CNS is protected from unwanted compounds, including immune system components, by the blood-brain barrier (BBB) in the brain vasculature and blood-cerebrospinal fluid barrier (BCSFB) in the choroid plexus as well as barrier structures surrounding CVOs. Essential compounds such as nutrients and ions pass these barriers into the CNS by, for example, passive diffusion, energy-dependent active transport and energy-independent facilitated diffusion (Banks, 2016). In addition, some large molecules such as albumin has been shown to access the CNS in small amounts through extracellular pathways (functional leaks) such as via vessels of the pial surface of the meninges and some CVOs (e.g. median eminence) (Banks, 2016).

Brain microvasculature comprises of pial arteries in the subarachnoid space, arterioles in the Virchow-Robin space, penetrating arterioles and capillaries. All these structures are equipped with BBB; however, capillaries lack the smooth muscle layer (Kulik et al., 2008). BBB consists of various junction proteins between the endothelial cells of brain vasculature, while similar structures are present between epithelial cells of the choroid plexus that constitutes the BCSFB (Redzic, 2011a). The structure includes adherence junctions, tight junctions, and gap junctions. These can be transmembrane proteins or cytoplasmic plaque proteins that interact with cell scaffolds to form the functional barrier (Redzic, 2011b). Claudins are a family of transmembrane proteins that are believed to have a central role in the function of tight junctions. It is known that claudin 3, 5 and 12 dominate in the

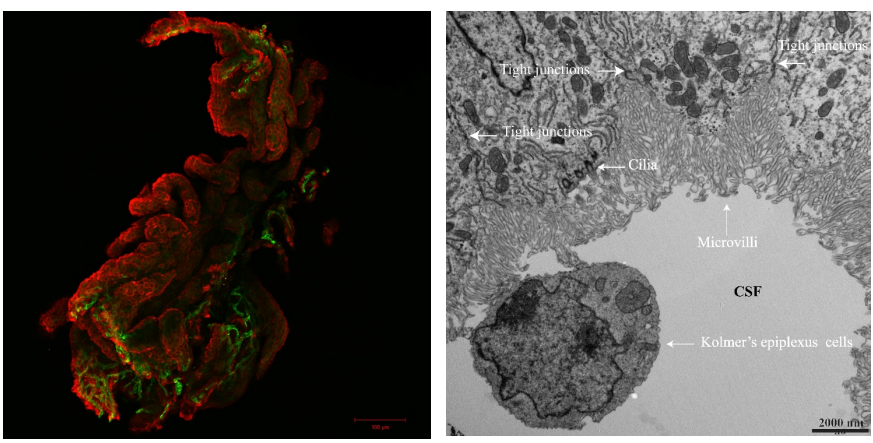

Figure 2. Left panel: two-photon image of a P9 mouse choroid plexus immunolabeled for tight junction protein occludin (red) of epithelial cells and CD31 (green) of vessels. Right panel: transmission electron microscopy image of the lateral ventricle choroid plexus of a P9 mouse.

BBB, while claudin 1, 2, 3 and 11 dominate in the choroid plexus epithelium barrier (Redzic, 2011b). BBB is supported and regulated by astrocyte end-feet and pericytes, which together with neurons, constitute the neurovascular unit (Hawkins and Davis, 2005; Armulik et al., 2010). Both BBB and BCSFB are formed and become functional early during development in humans, non-human primates and rodents (Saunders et al., 2012; Ek et al., 2015). In humans, BBB tight junction proteins occludin and claudin-5 were detected in the fetal brain at GW 16 (Ballabh et al., 2005). Moreover, peripherally injected dye (trypan blue) in aborted human fetuses (from ~GW12) did not diffuse into the CNS, supporting a functional BBB early in development (Grontoft, 1954; Saunders et al., 2012). The choroid plexuses are located in the brain ventricles and their development in humans starts as early as GW7 (Fig. 1). It consists of fenestrated vessels surrounded by a monolayer of epithelial cells (CPECs)(Mortazavi et al., 2013). The tight junction structure between the CPECs constitutes the BCSFB (Fig. 2). The stroma between CPEC and blood vessels contain some resident macrophages and dendritic cells (Quintana et al., 2015). The apical side (CSF side) of the choroid plexus is villous, which increases the choroid plexus surface area, and consequently, increases CSF production.(Lun et al., 2015). Special macrophages called Kolmer's epiplexus cells crawl on the microvilli (Fig. 2). Tufts of primary or motile cilia are also present on the apical side (Fig. 2) with roles in CSF circulation and osmo-/chemosensation (Wolburg and Paulus, 2010; Lun et al., 2015). The principal function of the choroid plexus is to produce of CSF. In addition to water and ions, CSF contains many proteins (e.g. neurotropic factors), metabolites, hormones and microRNAs, of which many are transported from peripheral blood and some are produced by the choroid plexus itself (Lehtinen et al., 2011; Lun et al., 2015). Therefore, the choroid plexus has a pivotal role in the CNS development and physiological functions. As an essential gatekeeper to the brain, the malfunction of the choroid plexus can-contribute to several CNS pathologies, including some neurodegenerative diseases (Balusu et al., 2016a).

Brain immune cells

Under normal physiological conditions, microglia cells are the sole professional immune cells in the brain parenchyma, playing important roles in brain development and maintenance. For some decades, it was assumed that microglia are dormant cells that become active only when there is an infection or injury. However, a substantial interest in microglia biology in the last decade has shed light on many functions of microglia in the normal brain. Synaptic pruning and remodeling during development and phagocytosis of apoptotic neurons without inflammation are among emerging functions attributed to microglia under physiological

conditions (Takahashi et al., 2005; Paolicelli et al., 2011; Schafer et al., 2012). Microglia originate from a subset of precursor monocytes in the yolk sac during early stages of embryo development (Ginhoux et al., 2010; Kierdorf et al., 2013). Recently, it was discovered that microglia cells express regional diversity in the healthy brain (Grabert et al., 2016). Other resident macrophages are also present in the meninges, choroid plexus and perivascular spaces. Meningeal and perivascular macrophages originate from embryonic hematopoietic precursors, and similar to microglia, have a long life span. However, choroid plexus macrophages, although having the same origin, are short-lived and replenished by blood monocytes after birth (Goldmann et al., 2016). Furthermore, a small number of T cells, mostly effector-memory T cells from adaptive immunity, are present in the CSF surveying the CNS for potential antigens (Engelhardt and Ransohoff, 2012). Recently, it was discovered that the brain is connected to the lymphatic system, challenging the long-held dogma that the brain is an "immune-privileged" organ (Louveau et al., 2015). However, the fact that immune homeostasis of the brain is strictly controlled remains unswerving.

Perinatal brain injury

As depicted in figure 1, the perinatal period, is a critical time for brain development and is vulnerable to external challenges. Injury to the perinatal brain can occur in 4 clinical settings: neonatal encephalopathy (NE) of term infants, perinatal stroke, preterm brain injury and systemic infections (Hagberg et al., 2015; Hagberg et al., 2016). NE occurs in 3/1000 live births and is attributed mainly to hypoxia and/or ischemia (HI), although this has been recently questioned (Badawi et al., 1998; McIntyre et al., 2015). Therefore, it has been suggested that the term "hypoxic/ischemic encephalopathy" (HIE) should be used only when criteria for intrapartum asphyxia, such as marked acidosis, is present (Volpe, 2012).

The clinical symptoms such as seizures, feeding difficulties, irritability and altered tonus, together with an altered electroencephalogram pattern, is associated with HIE in term infants (Pierrat et al., 2005; Kurinczuk et al., 2010; Stridh et al., 2011). Neonates that develop HIE more often present with low APGAR score, and umbilical cord acidosis at birth (Allen and Brandon, 2011). Insufficient supply of oxygen (hypoxia) and/or blood (ischemia) to the fetus/newborn can be due to antepartum, intrapartum and postpartum complications such as maternal hypotension, uterine rupture, cord prolapse, placental abruption or systemic inflammation. Moderate hypothermia (33.5ºC) started within 6 hour of birth and continued for 3 days is currently the only available treatment of HIE. Hypothermia significantly

increases the survival rate and improves the disability outcome, although not all infants benefit from this treatment (Edwards et al., 2010; Shankaran et al., 2012). The pattern of brain injury differs between term and preterm infants. In preterm infants, the injury appears most commonly in the form of white matter damage (e.g. if severe, periventricular leukomalacia; PVL) and intraventricular hemorrhage (IVH). In the term infants, focal grey matter injury in basal ganglia, thalamus, and cortex is most common (Mallard and Vexler, 2015b).

Perinatal stroke has two subtypes, perinatal arterial ischemic stroke and cerebral sinovenous thrombosis, with occurrence rate of 1/1600-1/5000 and 0.6-12/100000 live births respectively (deVeber et al., 2001; Lee et al., 2005; Laugesaar et al., 2007; Berfelo et al., 2010; van der Aa et al., 2014). Systemic infection, such as neonatal sepsis, is another clinical compromise that is associated with brain injury in the perinatal period (Mallard and Wang, 2012).

Perinatal brain injury can lead to permanent neurological impairment that affects motor and cognitive functions. Cerebral palsy (CP) is the leading cause of motor disability in children affecting 17 million people worldwide (Graham et al., 2016). The prevalence of CP in a large European population was 1.9 per 1000 live births in 1980 and 1.77/1000 in 2003, showing a slight decrease over time (Sellier et al., 2016). In western Sweden, 206 children who were born between 2003 and 2006 were later diagnosed with CP. This number indicats a prevalence of 2.18 infants per 1000 births. Strikingly, the CP prevalence was up to 71 per 1000 births for children born premature in GW<28 (Himmelmann and Uvebrant, 2014). Extremely preterm infants also present a higher prevalence of different cognitive disabilities when the outcome was measured at 2.5 or 6.5 years of age in a Swedish study (Serenius et al., 2013; Serenius et al., 2016). Preterm birth occurs in 5-18% of live births, and up to 10% of extremely premature infants suffer brain injury (Hagberg et al., 2015; Lancet, 2016; Serenius et al., 2016)

Hypoxic-ischemic brain injury

HI brain injury has a complex pathophysiology that consists of three phases. The primary phase of injury is due to the immediate energy failure after deficit in blood or oxygen supply. Lack of energy causes sodium/potassium pump failure leading to an influx of Na^+ ions, depolarization of neurons, and excessive release of the excitatory neurotransmitter glutamate (Hagberg et al., 2016). The glutamate-mediated activation of neurons results in a massive increase in intracellular calcium ions that activates apoptotic cell death pathways and induces the production of

reactive oxygen species (ROSs). The activation of these pathways causes mitochondrial damage (Hagberg et al., 2014), BBB disruption and brain edema, which altogether contributes to neural cell death by necrosis (if HI is severe) and apoptosis (Allen and Brandon, 2011; Thornton et al., 2012b). Following reperfusion, there is a latent or transient recovery period during which the oxygenation and metabolism of the brain returns to normal (blood reperfusion) (Gilland et al., 1998b; Thornton et al., 2012a). Following acute ischemia, this period normally lasts for 6-12 hour. Next, if the initial insult is severe, a secondary energy failure occurs that lasts from hours to several days. It is suggested that neuronal loss during this phase of brain injury is due to a combination of inflammation, oxidative stress and excitotoxicity, however the exact mechanisms are yet to be elucidated (Allen and Brandon, 2011; Hagberg et al., 2016). A tertiary phase of injury has recently been suggested to be important in the clinical outcome after HIE that involves chronic inflammation, gliosis, epigenetic changes and tissue remodeling (Bennet et al., 2012; Fleiss and Gressens, 2012). The role of inflammation is pivotal in secondary and tertiary phases of brain injury, suggesting it as a potential target for therapeutic intervention (Hagberg et al., 2015).

From systemic inflammation to neuroinflammation

Toll-like receptors mediate inflammation

Inflammation is a response to a stimulus, which can be a microbial component, an injured cell component, or a toxic compound. These stimuli all express unique and conserved molecular patterns such as: pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), which are recognized by host, pattern recognition receptors (PRRs). The main family of PRRs are Toll-like receptors (TLRs) (Takeuchi and Akira, 2010). The name was coined due to the similarity between the first discovered TLR gene sequence and *toll* gene in *drosophila melanogaster* (O'Neill et al., 2013).

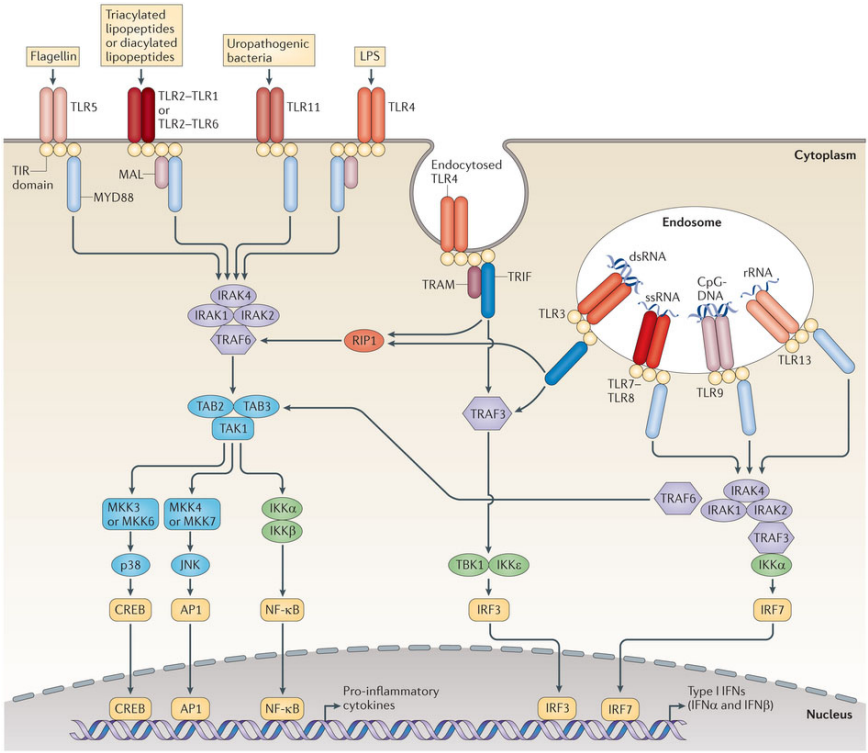

Figure 3. Toll-like receptor signalling. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] (O'Neill et al., 2013), copyright (2013)

TLR proteins are transmembrane glycoproteins that share an intracellular Toll-like/IL1R (TIR) domain and have various leucine-rich repeat motifs in the extracellular domain (Akira et al., 2006). They are located on the cell surface or associated with organelles in the cytoplasm. There are 10 TLRs in humans and 13 in mice, each recognizing certain PAMPs and DAMPs (Fig. 3). For example, TLR4 recognize the lipopolysaccharide (LPS) of the outer membrane of gram-negative bacteria, TLR2 recognizes the lipopeptides present in the cell wall of both gram-positive and gram-negative bacteria, and TLR3 recognizes the double-stranded RNA of viruses (Akira et al., 2006). Some TLRs need to form homo- or heterodimers in order to recognize specific ligands. For instance, the TLR1/TLR2 heterodimer recognize triacylated lipoproteins of *Mycoplasma pneumoniae* (Shimizu et al., 2007) as well as diacylated lipopeptides, while TLR2/TLR6 heterodimers can only recognize diacylated lipopeptides (Jin and Lee, 2008). TLR2 has also been shown to form heterodimer with TLR4 to respond to hemoglobin during intracerebral hemorrhage (Wang et al., 2014). TLR3 is located on endosomal membranes in the cytoplasm. Upon binding of the ligand, TLR3 form homodimers and a signal is initiated (Jin and Lee, 2008). TLR4 extracellular domain is in a complex with adapter molecule myeloid differentiation protein-2 (MD2) and form homodimers upon LPS binding (Kim et al., 2007).The various dimerization diversifies the immune responses in different immune cells (Depaolo et al., 2008; Netea et al., 2008; DePaolo et al., 2012).TLRs are expressed in the CNS. In the mouse brain the mRNA of TLR1-9 has been detected and shown to be developmentally regulated (Kaul et al., 2012; Stridh et al., 2013). The protein expression of some of these TLRs have also been reported in different brain cell types of neonatal mouse (Stridh et al., 2011). In the human brain TLR1-9 mRNA can be detected: TLR1-9 were detected in microglia and TLR2-3 in astrocytes and oligodendrocytes (Bsibsi et al., 2002). Some TLRs are also expressed on neurons of different species including human and mouse (Rietdijk et al., 2016). In addition to their principal function in the immune response, different TLRs play critical roles in neurogenesis and can have negative effects on regulation of axonal growth and neural progenitor proliferation (Cameron et al., 2007; Rolls et al., 2007; Lathia et al., 2008). Downstream of TLRs are various adapter molecules, which transfer the signal to corresponding transcriptions in the cytoplasm. The adapter molecules for TLR2 and TLR3 signaling are MyD88 (Myeloid differentiation primary response gene 88) and TRIF (TIR-domain-containing adapter-inducing interferon-β) respectively. Activation of transcription factors (such as NF-κB) by protein modification lead to their nuclear translocation and transcription of genes encoding, for example, various cytokines, including type I and II of interferons (Fig. 3) (Akira et al., 2006). Cytokines are signaling proteins produced by immune and non-immune cells. Chemokines are chemotactic cytokines involved in cell migration.

They are categorized into several subfamilies (e.g. CC and CXC) based on the arrangement of their N-terminal cysteine residues (Zlotnik and Yoshie, 2000). Some chemokines have other conventional names than the CC/CXC-based names. For example, CCL3 is also called MIP1a. Interleukins are a large family of cytokines with pro-inflammatory, anti-inflammatory or immunoregulatory functions (Turner et al., 2014). Tumor necrosis factor (TNF) constitutes another family of cytokines with various functions in immunity and cell death/survival (Brenner et al., 2015).

Perinatal systemic inflammation

Maternal and neonatal infections are the main cause of systemic inflammation in the perinatal period. Maternal infections that affect the umbilical cord and fetal membranes, such as funisitis and chorioamnionitis often originate from the lower genital tracts and are caused by multiple microorganisms, most commonly from the mycoplasma family, which are part of the vaginal flora in most women (Tita and Andrews, 2010). Other causative pathogens are gram-variable *Gardnerella vaginalis,* gram-negative *bacteroides* and gram-postive *Group B streptococcus* (Tita and Andrews, 2010). Human and animal studies show that maternal infections induce an elevation in certain inflammatory cytokines in maternal and fetal blood, as well as in the neonate's brain (Dammann and Leviton, 1997; Laborada and Nesin, 2005). For example, chorioamnionitis in humans cause an elevation of interleukin 6 (IL6) and granulocyte-colony stimulating factor (G-CSF) in both maternal and fetal blood, and an increase in IL6 and IL8 in the newborn's CSF (Dammann and Leviton, 1997; Laborada and Nesin, 2005). Maternal infection may also lead to fetal inflammatory response syndrome (FIRS) in preterm infants, an acute systemic inflammation characterized by elevated IL6 in cord blood plasma and neutrophilia (Bonadio, 2016).

Among the causes of inflammation are also viral infections. Many neurotropic viruses including herpes-simplex virus, herpes virus, cytomegalovirus (CMV) and Epstein-bar viruses are able to pass the placenta, and increase the risk of preterm birth and cerebral palsy (Gibson et al., 2006). CMV is the most common congenital viral infection in developed countries. It is transmitted from the mother with primary and recurrent CMV infection to the fetus during pregnancy with a transmission rate of 32% and 1.4% respectively (Kenneson and Cannon, 2007). CMV induces a pro-inflammatory cytokine release in the placenta and amniotic fluid of pregnant women (Scott et al., 2012). It also induces an interferon response in the developing brain of mice (van den Pol et al., 2007). Congenital CMV infection is associated with permanent disabilities such as hearing loss and mental retardation

(Naing et al., 2016).

Neonatal infections are major causes of mortality and morbidity in the first month of life. Approximately 1% of all newborns in developed countries are affected by infections in the neonatal period (Vergnano et al., 2011). These infections often lead to sepsis, meningitis and/or pneumonia (Heath and Jardine, 2014). Sepsis has recently been re-defined as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer et al., 2016). In other words, sepsis is a severe and serious systemic inflammation in response to infections. Neonatal sepsis and meningitis were the causative factors for ~7% (0.4 million) of all global deaths of children under the age of 5 in 2015, while preterm birth remained the leading cause of mortality in this group (~16%; 0.9 million) (Liu et al., 2016). Preterm infants are, in particular, susceptible to invasive infections (Strunk et al., 2014). Neonatal sepsis is divided into two categories: early-onset (EONS, 0-7 days of age) and late-onset (LONS, 8-28 days of age). EONS is believed to originate from the placenta or maternal genital tracts, while LONS can be nosocomial or from the community environment (Dong and Speer, 2014). In developed countries, the major pathogens for EONS are *Group B streptococcus* (GBS) and *Escherichia coli (E-coli)* (Simonsen et al., 2014; Schrag et al., 2016) and for LONS coagulase-negative staphylococci such as *Staphylococcus epidermidis.* In developing countries *Klebsiella* species, E-coli, *Staphylococcus aureus*, GBS are the main causes of EONS and gram-positives such as *Streptococcus* and *Staphylococcus* species are the pathogens causing LONS (Obiero et al., 2015). The very low birth weight infants are more vulnerable to EONS particularly to gram-negative infections (Wynn and Levy, 2010).

Necrotizing entrocolitis (NEC) is a severe intestinal disease, mostly in preterm infants, and associated with a systemic inflammatory response (Neu and Walker, 2011). Another cause of systemic inflammation, predominantly affecting the very preterm infant, is severe lung disease requiring mechanical ventilation. In addition to inflammation, respiratory support may cause fluctuations in cerebral blood flow that add to the increased risk of brain injury (Polglase et al., 2014). Severe respiratory conditions in the term infants, such as meconium aspiration, are also followed by a systemic inflammatory response (Reuter et al., 2014).

Inflammatory cells in circulation

In addition to a surge of various cytokines into the circulation, systemic inflammation is associated with a marked change in the diversity and quantity of blood leukocytes. The reference range for white blood cell counts in newborns in their first month of life are: 9100-34000 white blood cells (WBC)/μm^3, 32-67% segmented neutrophils, 0-8% band neutrophils, 25-37% lymphocyte, 0-9% monocytes, 0-2% eosinophils and 0-1% basophils (Andropoulos, 2012). Many factors might influence the values including: infant age in hours and gender, as well as maternal factors (Thomas et al., 2010). The reference is of limited value for preterm infants due to large variabilities (Maheshwari, 2014). There is also diversity in the subtype of cells present within different populations of leukocytes. For example, circulating monocytes of human and mouse are categorized into inflammatory ($CD14^+CD16^+$ and $CD14^+CD16^-$, human) and patrolling ($CD14^{dim}CD16^+$, human) based on their surface marker expression. The former has a role in inflammatory response to bacterial pathogens, while the latter has roles in tissue repair and inflammatory response to viruses (Cros et al., 2010; van de Veerdonk and Netea, 2010).

TLRs are expressed on human neonatal blood monocytes, neutrophils and lymphocytes at comparable levels to adults (Viemann et al., 2005; Dasari et al., 2011), however, some differences, for example in TLR3 expression, has been reported between neonatal and adult leukocytes (Slavica et al., 2013). During neonatal sepsis TLR2 (but not TLR4) expression is transiently increased on monocytes (Viemann et al., 2005). However, the phagocytic activity of neutrophils and monocytes is defective in newborns in their first 3 days of life (Filias et al., 2011). Moreover, exposure to pathogens also reduces the phagocytic activity of these cells, predisposing the neonates to sepsis (Silveira-Lessa et al., 2016). Newborns with infections have lower WBC and neutrophils count and larger proportion of immature neutrophils when assessed <24h of life compared to the age-matched non-infected newborns (Thomas et al., 2010). The number of $CD14^{dim}CD16^+$ and $CD14^{high}CD16^+$ monocytes is increased in newborns with sepsis (Skrzeczyñska et al., 2002). Lymphocyte count >95th percentile or <5th percentile is also associated with EONS (Christensen et al., 2012).

Neuroinflammation

What conditions lead to the breach of the tightly homeostatic system of the brain and neuroinflammation? CNS infections leading to meningitis or encephalitis are

naturally the first plausible cause of neuroinflammation. Meningitis is the inflammation of CNS meningeal membranes in response to infections (Kim, 2010). Neonatal meningitis is usually associated with sepsis, particularly EONS (Simonsen et al., 2014). GBS, *E-coli*, *Listeria monocytogenes*, *Streptococcus pneumoniae* are main pathogens causing meningitis. With development of vaccines, meningitis incidence due to *Neisseria meningitidis* and *Haemophilus influenza,* the classical meningitis pathogens*,* has decreased (Kim, 2010). Any local injury (e.g. HI) in the CNS also triggers an inflammatory response. Moreover, some autoimmune diseases such as multiple sclerosis also involves the CNS, resulting in severe inflammation in the brain (Becher et al., 2017).

Peripheral inflammation, particularly systemic inflammatory conditions like sepsis, can affect the immune homeostasis of the CNS and its function. Adult sepsis frequently leads to septic encephalopathy (Dal-Pizzol et al., 2014) and long-term cognitive decline (Annane and Sharshar, 2015). However, direct evidence for an association of systemic inflammation with neuroinflammation is scarce in humans, as accessing relevant tissue is difficult.. In postmortem brains of adult patients, that died of septic shock, an increased expression of the inflammatory cytokine TNF-a was observed in glial cells and induced nitric oxide synthase (iNOS) was detected in the vasculature, which was associated with apoptosis in neurons (Sharshar et al., 2003). In another study, an elevation of chemokines CXCL8, CXCL10, CXCL12, CCL13 and CCL22 was detected in brains of patients that died of sepsis complications (Warford et al., 2017). In preterm infants with EONS, the level of TNF-a and IL1-b was higher in plasma and CSF compared to age-matched controls. (Basu et al., 2015). There are more studies on human preterm infants with documented neuroinflammation; however, it is not possible to attribute the effect only to systemic inflammation or sepsis due to the often-multifactorial nature of the pathology and death. There are a few experimental studies in adult humans connecting systemic inflammation to brain function, however the presence of neuroinflammation was not investigated (Krabbe et al., 2005; van den Boogaard et al., 2010; Kullmann et al., 2014). For example, systemic administration of a low dose *E-coli* endotoxin (0.2 ng/kg) in humans did not change the plasma level of the stress hormone cortisol, but increased the cytokines TNF-a and IL6, which was associated with a decline in declarative memory performance (Krabbe et al., 2005). It should also be noted that the dosages used in these endotoxemia models are understandably far lower than those measured in plasma of sepsis patients (Marshall et al., 2002).

Altogether, the human data suggest that peripheral systemic inflammation can in-

duce neuroinflammation and alter the brain function. However, there is more supporting evidence from data in experimental animal models. In adult mouse and rat models of sepsis, induced by cecal ligation and perforation, increased leukocyte trafficking was observed in brain vasculature in addition to release of several cytokines and chemokines in the brain (Comim et al., 2011). This was followed by an increase in BBB permeability in several regions of the brain and brain autonomous dysfunction. Likewise, systemic infection of neonatal mouse with *Staphylococcus epidermidis,* without bacteria presence in the CSF, significantly upregulated expression level of several inflammatory genes in the brain (Bi et al., 2015). In an LPS (5mg/kg)-induced sepsis model in the adult mouse, a sustained activation of microglia cells and release of pro-inflammatory cytokines was identified in the brain (Weberpals et al., 2009). Similar inflammatory responses have been reported with LPS administration in neonatal rat, mouse and sheep (Mallard et al., 2003; Smith et al., 2014; Patil et al., 2016). Moreover, intra-amniotic inflammation is associated with subsequent neuroinflammation in neonates in experimental animal models (Bell and Hallenbeck, 2002; Schmidt et al., 2016). Thus, these experimental studies show that systemic inflammation can induce neuroinflammation.

Several mechanisms have been suggested to communicate the systemic inflammation to the CNS (Fig. 4). Systemic infections (e.g. sepsis) result in the release of several PAMPs and DAMPs, as well as several inflammatory cytokines in the circulation. These compounds can stimulate the PRRs at the brain interfaces (e.g. choroid plexus), leading to the release of inflammatory mediators into the CNS and/or leukocyte recruitment. In support, it has been shown that activation of TLR4 in the adult mouse can induce neuroinflammation independent of peripheral inflammation suggesting that activation of TLR4 at the brain interfaces is sufficient for the inflammatory effect in the CNS (Chakravarty and Herkenham, 2005).

In addition, these compounds can cause alterations in the brain barriers, leading to increased permeability that might facilitate the communication of inflammatory molecules and cells. This is further discussed in the next sections.

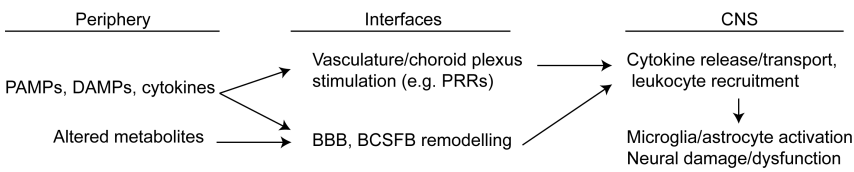

Figure 4. The possible mechanisms of neuroinflammation due to systemic inflammation/infections.

Brain vasculature in mediating inflammation

Inflammatory mediators in the periphery can reach the CNS via an intact, altered or disrupted BBB (Varatharaj and Galea, 2017). Some cytokines can be transported from blood to the brain by specific saturable transporters. The rate of transport is dependent on the cytokine type and the brain region (Banks, 2005). Polarized nature of endothelial cells also enables them to act as communicators between the luminal (blood side) and abluminal (brain side) sides. Therefore, stimulation of the luminal side with PAMPs leads to the release of cytokines to the abluminal side, and vice versa (Banks et al., 2009). Systemic infection/inflammation such as in sepsis is also associated with disruption of BBB that might further enhance the neuroinflammation (Gofton and Young, 2012).

Leukocytes can pass the BBB in various brain pathologies. The transmigration occurs mainly at capillaries and post-capillary venules, however, other parts of brain vasculature may also be a route of entry (Larochelle et al., 2011). Leptomeningeal vessels has been recently shown to be the main route of effector T cell migration to the CSF and brain parenchyma during experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (Schläger et al., 2016).

Choroid plexus in mediating inflammation

There are several studies supporting the role of choroid plexus in mediating peripheral inflammation to the CNS and contributing to neuroinflammation. In response to peripheral LPS administration, the expression of several inflammatory cytokines and chemokines was upregulated in adult mouse choroid plexus (Marques et al., 2007; Marques et al., 2009b; Marques et al., 2009a). Moreover, choroid plexus epithelial cells produce a panel of cytokines and chemokines in response to meninigitis-causing bacteria, such as *Neisseria meningitidis* (Steinmann et al., 2013; Borkowski et al., 2014). Recently, it was demonstrated in two adult mouse models of systemic inflammation (LPS injection or cecum ligation) that extracellular vesicles containing microRNA are secreted by the choroid plexus into the CSF. These microRNAs are taken up by microglia and astrocytes leading to up-regulation of pro-inflammatory genes (Balusu et al., 2016b). The choroid plexus has also been suggested as an invasion route of inflammatory leukocytes into the CNS in many pathologies such as meningitis, multiple sclerosis and brain trauma (Reboldi et al., 2009; Wewer et al., 2011; Schmitt et al., 2012;

Szmydynger-Chodobska et al., 2012). However, recent advances revealed that the choroid plexus could also act as an educational gate for trafficking of anti-inflammatory and beneficial immune cells to the CNS during, for example, spinal cord injuries (Shechter et al., 2013; Schwartz and Baruch, 2014). The role of TLRs in the choroid plexus in the communication between systemic inflammation and the brain has not been investigated. Expression of many TLRs has been shown in mouse choroid plexus and human choroid plexus papilloma cells, but to date no data is available from healthy human choroid plexus (Stridh et al., 2013; Borkowski et al., 2014; Schwerk et al., 2015).

Immune cells in neuroinflammation

As mentioned, microglia cells are the most abundant professional immune cells in the brain parenchyma. Similar to peripheral macrophages, microglia have conventionally been categorized into two phenotypes, M1 and M2, based on expression of specific cell markers. The M1 phenotype is considered pro-inflammatory and is identified by expression of markers such as TNF-a, iNOS, major histocompatibility complex (MHC II), IL-6, and CD86. The M2 anti-inflammatory reparative phenotype is identified by markers such as arginase-1, CD206, transforming growth factor beta (TGF-b) and IL-10. M2 phenotype is involved in phagocytosis of debris and dead cells, resolution of inflammation, remodeling of axons, oligodendrogenesis and neurogenesis (Hu et al., 2015). In response to external stimuli, microglia develop different phenotypes with various functions (Chhor et al., 2013). However, the paradigm of M1/M2 phenotypes of microglia that has arisen from *in vitro* studies has been criticized for oversimplification of the biologically complex immune state of microglia *in vivo* (Hu et al., 2015). In support, neonatal HI induces various microglia phenotypes in the brain, including mixed classical (M1) and alternative (M2) phenotypes (Hellstrom Erkenstam et al., 2016).

There are several studies showing that systemic inflammation induced by peripheral administration of LPS or bacteria leads to microglia activation and upregulation of pro-inflammatory genes such as TNF-a and IL1-b (systematically reviewed in (Hoogland et al., 2015)). Recently, it was discovered that activated microglia cells release TNF-a, IL1-a and C1q, which induce a reactive phenotype of astrocytes termed A1 which is dysfunctional, toxic to neurons and oligodendrocytes and destructive to synapses (Liddelow et al., 2017). Both microglia and astrocytes maintain the activated phenotype long after neonatal HI (Bona et al., 1999). Induction of cytokines by microglia is dependent on expression of Peli1, a regulator of TLR signaling (Xiao et al., 2013).

Microglia and astroglia cells might also mediate leukocyte recruitment to the CNS when there is a local or systemic infection/injury (Babcock et al., 2003). For example, in the adult mouse with inflammatory liver injury, circulating TNF-a activates microglia cells which in turn produce CCL2 (monocyte chemoattractant protein 1, MCP-1) leading to recruitment of peripheral monocytes to the brain (D'Mello et al., 2009). Infiltrating leukocytes can promote the neuroinflammatory milieu by production of inflammatory cytokines and paving the way for further communication between the peripheral immune system and the CNS. For instance, it has been suggested that CNS-invading T cells in a multiple sclerosis animal model, EAE, might mediate recruitment of peripheral myeloid cells to the brain and also activate CNS resident macrophages (Becher et al., 2017). The infiltrating monocytes during EAE contribute to the pathology and differentiate into resident macrophages and dendritic cells. (King et al., 2009). HI in neonatal mice is also associated with long-lasting T cell infiltration to the brain (Winerdal et al., 2012). Leukocyte infiltration to the CSF is also a hallmark of meningitis in newborns and adults (Kim, 2010). Moreover, 9% of preterm infants with abnormalities observed in their brain scans have increased number of leukocytes in their CSF (Viscardi et al., 2004).

Inflammation and brain injury

The notion of inflammation/infection being involved in neonatal encephalopathy stems from several clinical studies. Maternal infections such as funisitis, chorioamnionitis, urinary and respiratory tract infections increase the risk of cerebral palsy in newborns (Grether and Nelson, 1997; Miller et al., 2013; Bear and Wu, 2016). Maternal infections also increase the risk of EONS, pneumonia, asphyxia, intraventricular hemorrhage (IVH) and white matter damage in the newborn (Tita and Andrews, 2010; Nelson and Penn, 2015). Chorioamnionitis is a common pregnancy complication associated with 32% of preterm births (Yoon et al., 2001) and up to 13% of term births (Tita and Andrews, 2010). Maternal inflammation has been associated with increased risk of neurodevelopmental disorders such as schizophrenia and autism (Knuesel et al., 2014). Moreover, preterm infants with FIRS have a higher risk of suffering from severe forms of PVL and IVH (Hofer et al., 2013). Preterm infants with sepsis or NEC have a higher risk of white matter injury and motor impairment at 2 years of age (Shah et al., 2008; Basu et al., 2015).

Term neonates who develop cerebral palsy also show significant elevation of chemokines such as CCL3 and CCL4 (also known as MIP1-a and MIP1-b, respectively), RANTES (regulated on activation, normal T cell expressed and secreted)

and MCP-1 in blood (sampled at 1-18 days of age), suggesting ongoing systemic inflammation (Nelson et al., 1998). Children with cerebral palsy also show an altered immune system with high level of inflammatory molecules in the blood (Grether and Nelson, 1997). Asphyxiated neonates have an increased amount of inflammatory cytokines IL-6 and IL-8 in the CSF that is associated with more severe HIE (Savman et al., 1998) suggesting a neuroinflammatory component of the injury process. Altogether, human studies suggest inflammation as a major risk factor for perinatal brain injury including HIE (reviewed in (Hagberg et al., 2015)).

The underlying mechanisms of inflammation-induced injury have been studied in various animal models. Neonatal mice injected intraperitoneally with various inflammatory cytokines, such as IL1-b, developed larger injuries to subsequent excitotoxic insult (by intracerebral injection of glutamate analogues), an effect that was mediated by prostaglandin production in the brain (Dommergues et al., 2000; Favrais et al., 2007). The inflammation-induced increase in excitotoxicity was due to downregulation of G protein-coupled receptor kinase 2 (GRK2) a key molecule in regulating glutamate receptors (Degos et al., 2013). Systemic activation of TLR4 by LPS before HI sensitizes the brain to the injury through the MyD88 pathway by inducing a systemic inflammation (Eklind et al., 2001; Eklind et al., 2005; Wang et al., 2009a). The mouse pups that received LPS prior to HI had greater brain tissue loss and poor myelination, suggesting aggravated injury in both grey and white matter. Activation of microglial TLR4 leads to axonal and neuronal loss *in vitro* and *in vivo* in a mouse model of neonatal HI (Lehnardt et al., 2003). However, the effect of LPS depends on the interval between endotoxin injection and HI, and the LPS dosage. LPS injection 6 hours or 72 hours before HI increases the brain injury, while LPS administration 24 hour before HI induce preconditioning effect with improved HI outcome (Eklind et al., 2005). The protective effect was associated with up-regulation of corticosterone in rat pups (Ikeda et al., 2006). The link between LPS-induced systemic inflammation and increased vulnerability of the neonatal brain to HI is not fully understood. However, neuroprotective effect of N-acetylcysteine (NAC), a common antioxidant reagent, in a LPS-sensitized HI model suggests a role for ROS in meditating sensitization to the brain injury (Wang et al., 2007). In addition, blocking lymphocyte trafficking in the same model ameliorated the injury, suggesting a contribution of peripheral immune cells to brain damage (Yang et al., 2014), which is consistent with stroke model studies in adult mouse (Liesz et al., 2011; Lee et al., 2014). The LPS sensitization effect was also abolished in neonatal mice lacking TNF gene cluster, suggesting a detrimental role of these cytokines (Kendall et al., 2011).

It has been shown that systemic activation of TLR3 induces neural apoptosis and accelerates prion-induced neurodegeneration (Field et al., 2010) and also nigrostriatal dopaminergic degeneration similar to Parkinson disease (Deleidi et al., 2010). However, TLR3 deficiency is not neuroprotective in an adult mice stroke model (Famakin et al., 2011). Maternal immune activation by TLR3 ligand, Polyinosinic:polycytidylic acid (Poly I:C), causes changes in mouse fetal brain leading to exploratory and social deficits in offspring; an effect that is mediated by IL-6 (Smith et al., 2007). However, the impact of TLR3 activation on neonatal HI has not been studied.

TLR2 activation by intrathecal administration of Pam3CSK4 (P3C) causes leukocyte infiltration to the brain and neuronal loss due to apoptosis (Hoffmann et al., 2007). However, *in vitro* treatment of neurons with the TLR2 ligand did not lead to neural death suggesting the effect is mediated by inflammation rather than direct cytotoxicity of P3C (Hoffmann et al., 2007). Interestingly, TLR2 deficiency exacerbates ischemic brain injury in adult mice (Choi et al.; Bohacek et al., 2012). In contrast, activation of TLR2 and TLR4 of brain infiltrating macrophages by peroxiredoxin proteins released from necrotic neurons increases the ischemic injury, suggesting peroxiredoxin as a potential target in treatment of strokes (Shichita et al., 2012). However, TLR2 deficiency is neuroprotective in a mouse model of HI (Stridh et al., 2011). Whether activation of TLR2 contributes to brain injury in neonatal HI, and the underlying mechanisms, has not been investigated.

Systemic inflammation might also affect the functions of other peripheral organs leading to CNS sensitization to injury. For instance, it might prime or exacerbate respiratory distress, contributing to hypoxia (Speer, 2009, 2011; Forsberg et al., 2016). Furthermore, there is evidence for a detrimental effect of systemic inflammation on mitochondrial function (Jeger et al., 2013) suggesting another potential link between inflammation and HI brain injury .

Aims

The overall aim of the thesis project is to address the question: how is systemic inflammation communicated to the developing brain and affects the hypoxic/ischemic brain injury?

The specific aims are:

- To test the hypothesis that systemic activation of TLR3 signalling has an impact on neonatal HI
- To test the hypothesis that systemic activation of TLR2 signalling has an impact on neonatal HI
- To test the hypothesis that activation of different TLRs in the periphery induce a specific inflammatory profile in the CNS

Methodological considerations

Laboratory animals

Neonatal mice (Paper I-IV) and rats (V) were used as animal models in research projects for this thesis. In Paper III, we also used adult mice in one experiment. The use of mice and rats to model human diseases has revolutionized the biomedical sciences in the last century. Particularly, using transgenic mice has substantially contributed to our understanding of molecular mechanisms of many diseases. However, in spite of remarkable similarity between the human and rodent genome, there are considerable differences at the organ, cellular and molecular level between these species.

The adult mouse brain weighs ca 0.5 gr, almost 3000 times lighter than the human brain (1500 gr). It contains 71 million neurons, which is almost 1200 times less than that of human brain (86 billion) (Azevedo et al., 2009). The mouse brain lacks gyri, the special cortical folding that is highly consistent between human individuals and increases the cortical grey matter surface area and volume (White et al., 2010). Moreover, the white/grey matter ratio and cerebral blood flow regulation is different in rodents compared to humans (Mallard and Vexler, 2015a). Rodents also are born relatively more immature, and many developmental events in the brain occur postnatally compared to humans. We used pups at postnatal day (PND) 8-9, which in terms of brain development roughly corresponds to human infants at near-term age based on several anatomical and functional factors (Hagberg et al., 2002b; Craig et al., 2003; Workman et al., 2013).

The immune system of rodents have many similarities, but also some important differences compared to humans. This has recently been debated after a study published by Seok *et al* claimed that human gene responses to inflammatory conditions are poorly mimicked in mouse models (Seok et al., 2013). Two years later Takao and Miyakawa re-evaluated the same data as Seok et al and concluded that that the gene response to inflammatory conditions in mouse models highly mimics human conditions with 59.5–93.2% of gene changes in the same direction (Takao and Miyakawa, 2015). The difference between human and mouse immune responses in addition to the genetic factors, might be influenced by the laboratory conditions. It has been shown that a pathogen-free environment impedes the full development of the mouse immune system, making them resemble the immature

immune system of the human neonate (Beura et al., 2016). However, normalizing the environment for the mice enhance the similarities to that of the adult human (Beura et al., 2016). The standardization of these "normalized" conditions might be a challenging impediment requiring extensive studies. Therefore in our study, the mice and rats were housed in standard pathogen-free ventilated cages with *ad libitum* access to food and water and 12h light/dark cycle.

The following mice and rat strains were used in this thesis:

- C57Bl6/J is the most used inbred wild type mouse strain (Paper I-V)
- TRIF knock-out (KO) (C57BL/6J–Ticam1Lps2/J; Jackson Laboratory) are the mice lacking the adapter molecule for TLR3, TRIF (Paper I)
- TLR2 KO (B6.129-Tlr2tm1Kir/J; Jackson Laboratory) (Paper II, III)
- Lys-EGFP-ki are mice in which peripheral myeloid cells express the green fluorescence protein (Paper III)
- MyD88 KO (B6.129P2(SJL)-Myd88tm1.1Defr/J; Stock No: 009088; Jackson Laboratory) are mice lacking the adapter molecule downstream of TLR2, TLR4 and IL1R (Paper III)
- MyD88 flox (B6.129P2(SJL)-Myd88tm1Defr/J; Stock No: 008888; Jackson Laboratory) are mice in which two inserted *loxP* genes flanks the MyD88 gene enabling Cre recombinase-mediated gene knock-out (Paper IV)
- Sprague-Dawley and Wistar rats are outbred rats widely used in biomedical research (Paper V)

For experiments on transgenic knockout mice, littermates were used in order to assure that genetic background of the pups and the environment remain comparable (Holmdahl and Malissen, 2012). We bred female and male mice that were heterozygous for the knockout gene, and the pups that were gene knockout or wild type were identified by genotyping and used in the studies.

TLR agonists

Poly I:C (paper I) is a synthetic mimic of viral double-stranded RNA (dsRNA). A class of viruses have dsRNA as their genomic component (e.g. Reoviridae). In single-stranded positive-sense RNA viruses (e.g. picornavirus) and DNA viruses (e.g. Herpesviridae), dsRNA is a bi-product of viral replication (Weber et al., 2006). The TLR3 ecotodomain binds the dsRNA of at least 40-50 base pair length in cytoplasmic endosomes (Liu et al., 2008).

Pam3CSK4 (P3C, paper II-V) is a synthetic lipopeptide constructed of three fatty acid pamitoyl molecules linked to amino acids cysteine, serine and lysine. Lipopeptides are cell wall components of gram-positive and gram-negative bacteria with various roles in bacterial growth, colonization, antigenicity and signal transduction (Kovacs-Simon et al., 2011). The lipid chain of P3C induces heterodimerization of TLR1 and TLR2, which in turn induces dimerization of their TIR domain and initiates intracellular signaling (Jin et al., 2007).

Lipopolysaccharide (LPS, paper III and IV) is the major component of the outer membrane in gram-negative bacteria. It consists of three elements: lipid A, core oligosaccharide and O antigen polysaccharide. The O antigen is structurally variable which gives rise to several serotypes (Maldonado et al., 2016). Lipid A makes a hydrogen bound to MD2 of the TLR4-MD2 complex leading to TLR4-mediated activation (Park et al., 2009). We used ultra-pure LPS from E.coli serotype O55:B5 which is a common enteropathogenic strain (Rodrigues et al., 1996). Due to batch-to-batch variations associated with purified LPS, we used the same batch in all experiments.

All TLR agonists were injected intraperitoneally at a consistent volume of 10 µl/g body weight. After the injection, the pups were held in the hand briefly to assure that there was no leak-out of injected agonsits. The dosages were selected based on our or others' previous studies.

Hypoxic-ischemic brain injury model

The HI brain injury model used in this thesis is induced by permanent ligation of the left carotid artery in mouse pups at PND9 followed by an hour of rest with the dam and then exposure to hypoxia. The length of the surgical operation should be consistent and kept at a minimum since prolonged exposure to the anesthesia compound isoflurane might exert a neuroprotective effect (Chen et al., 2011; Burchell et al., 2013). The operated mice are kept on a warm pad (35°C) to avoid hypothermia. After the rest period, pups are placed in a chamber with a tightly controlled temperature of 36°C. The chamber is first ventilated for 10 minutes with humidified warm air, followed by 50 minutes of hypoxia (air mixed with nitrogen to adjust the oxygen to 10%) and then 10 minutes of air only. The neonatal model, often referred to as the Rice-Vannucci model, was first described in rats (Rice et al., 1981) and then modified for use in mice (Ditelberg et al., 1996). The advantages of the model are its unilateral form of injury that leaves the contralateral hemisphere as an internal control and that the extent of the injury can be controlled by the length of the hypoxia, although the model does produce high inter-animal

variability. The injury pattern is fairly similar between PND9 mouse HI and human term infant HIE. Basal ganglia and thalamus are the main sites of injury in HIE infants; the cortex can be involved if the injury is severe (Miller and Ferriero, 2009). These regions are also affected in the mouse HI model; however, the loss of hippocampal tissue is usually more prominent in the mouse. The damage to white matter is also present in both human and mouse HI brain injury (Miller and Ferriero, 2009; Mallard and Vexler, 2015b).

Immunohistochemistry and histology

Immunohistochemistry (IHC) is a method in which a target protein is detected visually in a tissue by immunolabelling. The tissues in this thesis were processed in different ways prior to IHC: In paper I and II, paraformaldehyde (PFA)-fixed brain tissues were dehydrated and then embedded in paraffin. Paraffin-embedded tissues has the advantage that cutting thin sections (10 μm in paper I and II) is possible and that the tissue morphology is highly preserved. In paper III, PFA-fixed brain tissue were cryopreserved in sucrose and then frozen. Thick sections (40 μm) were cut on a cryostat and were kept in -20°C freezer in a cryoprotectant solution. Free-floating immunostaining has the advantage of better antibody penetration in a thick section but handling the tissue can be troublesome. IHC on thick sections provides more information on the tissues three-dimensional structure and the protein localization. In paper V, whole choroid plexus tissue was fixed in PFA and then immunolabeled, which helped to identify limited numbers of infiltrating cells in a small tissue sample.

The localization of the target protein and an estimation of its expression level can be acquired by IHC. A simple IHC procedure include antigen retrieval (optional), blocking of non-specific binding sites, primary antibody incubation, secondary antibody incubation and visualization. The primary antibody is raised in and purified from an animal species, and usually is an IgG class of immunoglobulins. The secondary antibody is raised against the primary Ig molecule (usually the class-specific heavy chain of Ig) in a different species, therefore it reacts with the primary antibody at several sites and amplifies the signal. The secondary antibody is modified in a way that it can be visualized by microscopy. This modification is commonly a fusion with a fluorescent molecule or an enzyme that reacts with certain substrates, producing a color.

Tissue processing is an important step in IHC. The tissue is usually immediately fixed with a fixative to preserve the protein structures. PFA is the most commonly used fixative, which acts by cross-linking proteins. The cross-linking is initiated

by covalently linking primary amines, purines and thiols (24-48h) and is continued by involving other functional groups such as amides (Thavarajah et al., 2012). The cross-links can be reversed by a procedure called antigen retrieval that exposes the antigenic sites of the protein to the primary antibody. Antigen retrieval is achieved by heating the fixed tissue sections or/and the use of detergents (Fowler et al., 2011). In Paper I, II and III, the antigen retrieval was performed by heating the sections in citric buffer. The non-specific binding site was blocked by incubating the sections with blood serum containing various proteins or a serum purified protein such as albumin. After incubation with primary antibodies, a biotinylated secondary antibody was added. Next, an avidin/streptavidin-conjugated horseradish peroxidase was incubated with the sections. Avidin/streptavidin form a very strong non-covalent binding to biotin. Peroxidase in presence of hydrogen peroxide oxidizes the substrate, 3,3′-Diaminobenzidine (DAB), producing a visible reaction product.

Free-floating sections were stained using fluorescent-conjugated secondary antibodies. In paper V whole choroid plexus was dissected out, briefly fixed in PFA and stained using fluorescent-conjugated antibodies. We used Triton X-100, a strong detergent, to enhance tissue permeability and antigen availability in free-floating brain sections and whole-mount choroid plexus staining.

To visualize the staining, a bright-field microscope was used for DAB stained thin sections and a confocal fluorescence microscope for free-floating or whole-mount samples stained with fluorescent antibodies. The confocal microscope has some advantages over conventional fluorescence microscopes including less out-of-focus blurring, better resolution and greater focal depth.

In an unpublished experiment related to Paper II, we performed IHC to visualize microglia cells. Microglia cells were stained for Iba-1 (Wako polyclonal rabbit anti-Iba1; 1:2000) following a standard protocol used in Paper I and II.

In a histology experiment related to Paper II but not previously published (Fig. 10), brain tissue sections were stained with thionin/acid fuchsin. Briefly, after deparaffinization and rehydrating the sections, slides were dipped in 1% thionin/toluidine solution for 4 minutes, followed by 30 seconds dip in acid fuchsin. After rinsing in water, sections were dehydrated and mounted with coverslips. Neuropathology scoring was performed as described previously (Wang et al., 2009a).

Brain injury assessment

For measuring the extent of injury, we performed IHC to visualize microtubule-associated protein 2 (MAP2) and myelin basic protein (MBP). MAP2 is a specific marker for neurons and thus is commonly used to measure neural loss (Gilland et al., 1998a). MAP2 is involved in stabilizing the microtubule structure in neurons. There are three isoforms of MAP2 (Dehmelt and Halpain, 2005) and the HM2 clone of the antibody used in our study reacts with all of them. MBP constitute 30% of the total protein content of the neural myelin sheath and is one of the most abundant proteins in the CNS (Boggs, 2006). MBP staining is commonly used to detect damage to the white matter.

The neural tissue damage was calculated as a percentage of neural loss in relation to the contralateral (uninjured) hemisphere as:

Tissue loss percentage = $(A_c\text{-}A_i)/ A_c \times 100$

A_c is MAP2-stained area in contralateral hemisphere and A_i is MAP2-stained area in ipsilateral (injured hemisphere).

By normalizing the tissue loss to the area of the uninjured hemisphere, the variability in section/tissue size that might be introduced during tissue processing is avoided. Five coronal sections at five different levels of the brain were analyzed from each animal which enabled us to obtain an overall assessment of the brain injury in different regions of the brain.

The white matter damage or myelin loss (MBP) was calculated using the same formula but only three levels of the brain were analyzed. The MBP-positive area was measured in subcortical white matter. The number of animals used in this experiment is based on our previous studies using the same model.

In an experiment related to Paper II (Fig. 11), we stained for microglia cells in the brain sections. To count the cells in an unbiased manner, we performed stereology which is a method to obtain quantitative information with three-dimensional property from a two-dimensional section (Mühlfeld et al., 2010). A Leica DM600 microscope was used together with a stereology software, Stereo Investigator. A Fractionator method was used for quantification.

Quantitative reverse transcription PCR

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is the most commonly used method for quantification of gene expression. We performed a two-step RT-qPCR in paper I to quantify the mRNA of several genes in the mouse brain. The first step is the reverse transcription of the isolated RNA to DNA using a transcriptase enzyme and oligo-dT and random primers. The next step is the PCR amplification of the target gene using specific primers in a thermocycler. A fluorescent dye (e.g. SYBR Green in our study) is bound to the double-stranded DNA and is detected in real time. When the fluorescence intensity of the DNA product reaches the detection threshold, C_t value or threshold cycle is determined, which reflects the amount of the DNA template when the efficiency of the reaction is optimal (assessed by making a standard curve of serial dilutions of a cDNA sample). The DNA quantity of the sample is determined in comparison to the standard curve of the standard sample. The standard sample is ideally a sample with known quantity of the target gene or a sample estimated to have a substantial amount of the target gene. We used the latter, thus the values we obtained by this method are relative rather than absolute. A key step of the calculations is to normalize the obtained value of gene expression to the expression of the reference gene which should not change in response to the stimuli. This way, the results are presented as target gene/reference gene ratio. In some conditions, finding a stable reference gene might be challenging. Therefore, it is important to use exactly the same amount of RNA from all samples in the cDNA synthesis step with several replicates.

RT-qPCR is an extremely sensitive method for detection of a few copy numbers of DNA. Therefore, accurate pipetting of the reagents and samples in the tube is a crucial step. Moreover, a negative control sample containing the reagents only should be included to verify that the reagents are not contaminated with DNA. The melting temperature analysis is also another critical control step to ensure that obtained signal comes from a single PCR product specific to the target template.

Flow cytometry

The transcribed mRNA of genes does not always translate into a protein product due to post-transcriptional regulation of gene expression by, for example, interfering RNAs (RNAi). Therefore, it is important to also measure the protein expression and not just gene expression. Conventional methods such as Western blot and IHC are very reliable but have some limitations such as being semi-quantitative in

addition to limitation of number of targets that can be detected at the same time. Flow cytometry is a method for identification, characterization and quantification of cell phenotypes by detecting and measuring expression of different proteins labelled with fluorescent molecules (e.g. fluorescent antibodies) in single cells in a flow system. The advantage of this method is that the expression of several proteins can be quantified simultaneously by using an array of antibodies tagged with different fluorochromes, which is particularly powerful when phenotyping cells. In paper I and III we performed flow cytometry to identify and characterize different immune cells in the brain. In paper I, the brain tissue was dissociated mechanically to obtain single cell suspension. In paper III, the enzymatic digestion of the brain preceded the mechanical dissociation in order to enhance the cell viability and integrity. White blood cells in blood and CSF were also analyzed by flow cytometry in Paper III. Prior to flow cytometry, red blood cells were lyzed by a hypertonic salt solution. After tissue dissociation and quantification of the single cells, non-specific binding sites were blocked and cells were incubated with primary and fluorescent secondary antibodies. For the CSF samples that do not contain numerous cells, the washing steps were minimized to avoid cell loss.

The analysis of flow cytometry data is based on a gating strategy to distinguish and quantify different cell populations based on their characteristics. First, cells are gated based on the physical characteristics, their size and granularity. This information is obtained based on how the light is scattered by the cells straight forward or to the sides. Next, the cells are usually gated based on viability and singularity. This is followed by gating based on the expression of a lineage marker such as CD45 which is a surface protein expressed by all leukocytes. The gating continues based on other proteins expressed by specific cells in order to identify and quantify cell subtype specific populations. The results are presented as proportion of subtypes of parent populations.

Multiplex cytokine assay

Multiplex cytokine assay is an immunoassay for simultaneous quantification of multiple target proteins in a sample. There is a significant advantage of this technique for small-volume samples such as CSF from rodents. In paper III and V, we used this method to measure several cytokines in plasma, CSF and the choroid plexus epithelial cell culture medium. In this method, magnetic microbeads (the size of a mammalian cells) labelled with a certain fluorophore is covalently bound to an antibody specific to a target molecule, e.g. a cytokine. The cytokine in the sample thus binds the corresponding antibody-coupled bead and the unbound proteins are removed by washing. Then a biotinylated antibody targeting another

epitope of the cytokine is added followed by addition of fluorescent-tagged streptavidin that binds biotin. By this technique, the final sandwich product emits two fluorescent lights, one coming from the bead identifying the target cytokine and the second originating from the bound antibody-biotin-streptavidin correlating to the amount of the cytokine. The fluorescent intensity is translated to a concentration by relating to the values on a standard curve. The detecting machine is also a flow cytometer (Bio-plex 200 System in our case).

Magnetic activated cell sorting (MACS)

In unpublished work related to Paper II, we isolated microglia cells from PND9 mice by performing MACS. The method involves identifying target cell types in a suspension based on a cell surface marker using a specific antibody conjugated to magnetic beads. The cells then pass through the magnetic columns and the target cells are bound to the magnets and then eluted. To make the cell suspension, brain tissue was enzymatically digested using 0.01% papain, 0.1% dispase, 0.01% DNase and 12.4mM $MgSO_4$ in phosphate buffer saline (37°C for 10 min) followed by mechanical dissociation by triturating. For MACS isolation of microglia cells CD11b magnetic bead-conjugated antibody (Milteny) was used following the manufacturer protocol and using autoMACS Pro Separator (Milteny). Posseld2 program was used in which the cells pass through two magnetic columns instead of one to enhance the purity.

Western blot

In another unpublished work related to paper II (Fig. 12), Western blot (WB) was performed on brain isolated mitochondria. Brain mitochondria isolation was performed according to the previously described Method A in (Wang et al., 2011). WB is a gold standard immunoassay for detection and relative quantification of the target proteins. Isolated mitochondria were frozen and thawed followed by a brief sonication. The lysed mitochondria were mixed with Laemmli Sample buffer (containing 10% beta-Mercaptoethanol, Bio-Rad) and heated at 95°C for 5 min before being loaded to the 8–16% Criterion™ TGX™ Precast Protein Gel. The proteins were transferred to a PVDF membrane in a Trans Blot Turbo system (Biorad). Following blocking in 5% milk for 1h RT, the membranes were incubated with primary antibodies against superoxide dismutase 2 (SOD2, abcam; 1:2000) or peroxiredoxin 3 (PRX3; abcam; 1:2000) overnight at 4°C. After incubation with corresponding secondary antibodies (Vector) visualization was performed using Clarity ECL substrate (Bio-Rad) in a ChemiDoc XRS+ System (Bio-Rad),

and total protein normalization was performed following the manufacturer's protocol.

Mitochondrial respirometry

Mitochondria are the power plants of the cells, producing the majority of the cell high-energy phosphate molecule ATP through their electron transfer chain (ETC) and ATP synthase (Fig. 5) (West et al., 2011). The glycolysis (cytoplasm) and Krebs cycle are other cellular pathways producing small numbers of ATP molecules. The glycolysis by-product pyruvate enters the mitochondria and through the Krebs cycle, the electron carrier molecules nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) are produced and can enter the ETC. The ETC consists of four transmembrane enzyme complexes in the inner membrane (between the matrix and intermembrane space) of the mitochondria, of which three act as proton pumps as well. NADH and $FADH_2$ electrons are transferred to the complex I and complex II of the ETC respectively, and then via the complex II to complex III and complex IV, leading to H^+ protons being pumped out of the membrane into the intermembrane space.

The electrons are received back in the matrix via oxygen molecules and water molecules are formed. As the concentration of H+ protons increases, the gradient forces relay of the protons to complex V, the ATP synthase, and back to the matrix during which ATP is formed from ADP. This is called coupled respiration or oxidative phosphorylation. If the protons do not enter complex V to make ATP but

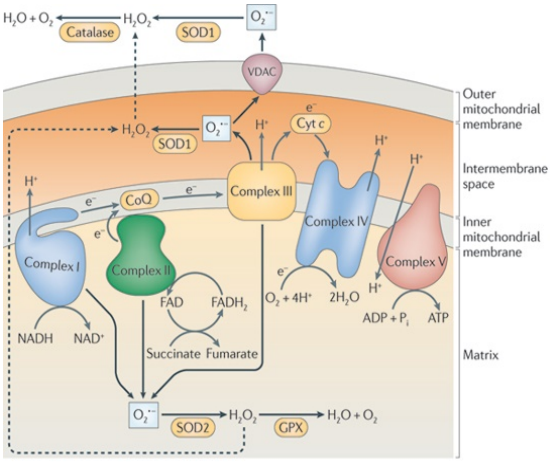

Figure 5. Mitochondria electron transport chain. . Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] (West et al., 2011).

just leak back to the matrix (e.g. in the absence of ADP) or transfer through uncoupling proteins it is called leak or uncoupled respiration, respectively. Throughout the mitochondrial respiration, ROS are also produced of which a proportion are neutralized by mitochondrial antioxidant enzymes such as superoxide dismutase (SOD) (Fig. 5).

Mitochondrial respiration is assessed by measuring the oxygen consumption in a closed chamber. We used a high-resolution low-noise respirometry system, O2K (Oroboros Instruments). In the respirometer, the oxygen amount is first obtained as a current voltage between a gold cathode and silver anode with potassium chloride as the electrolyte. The current is the result of the oxygen reduction at the cathode. The voltage is then translated to O_2 flux ($pmol.s^{-1}.ml^{-1}$). Each of the ETC enzyme complexes can be fed with their substrates, at saturating concentration, and O_2 consumption can be measured in real-time, which is the main advantage of the O2K system, in addition to the highly sensitive low-noise O_2 sensors. The respirometry can be performed on cells or isolated mitochondria. The advantage of respirometry on isolated mitochondria is that the respiration is not affected by other cellular factors, thus easier to interpret the mitochondrial function. Therefore, we isolated mitochondria from the brain using an established method. The complex I substrates pyruvate and malate were added to the respirometer chambers with mitochondria which leads to the leak or basal respiration. Addition of ADP rapidly stimulates oxidative phosphorylation and ATP synthesis. Adding the compound FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) which permeabilizes the inner membrane to the H^+ protons leads to breakage of the proton gradient and uncoupling of ETC from oxidative phosphorylation. The O_2 consumption rate was normalized to the protein content of the sample.

Brain barriers permeability test

The BBB and BCSFB are impermeable to peripheral molecules with certain physical and biochemical properties. In addition, efflux system at the barriers removes undesired molecules. This is a major obstacle in developing drugs that can penetrate into the CNS. Although it is generally believed that molecules larger than 500 Da or/and low lipophilic are not passively diffused to the CSF, several exceptions makes this general assumption irresolute (Banks, 2009). We tested the brain barriers permeability to ^{14}C sucrose, a passive permeability marker, with molecular weight of 340 Da. In paper III, the samples were collected 30 minutes after sucrose i.p. injection and the plasma/brain tissue or plasma/CSF ratio of radioactivity was obtained as permeability values for BBB and BCSFB respectively. The

brain tissue concentrations were corrected for blood residual which is estimated to be ~1% of brain volume (Gregoriadis, 1993). In paper V, first a concentration-time curve was obtained by collecting plasma 3-30 minutes after ^{14}C sucrose i.p. injection and measuring the radioactivity in samples.

The area under the curve (AUC) was used to calculate the ^{14}C sucrose CSF influx constant as:

$$K_{in\ csf} = C_t / AUC_{0 \rightarrow t}$$

The C_t is the sucrose concentration in the CSF at sampling time and $AUC_{0 \rightarrow t}$ is the ^{14}C sucrose concentration in the plasma from time zero to the CSF sampling time.

Cre-recombinase mediated gene targeting in the choroid plexus

Cre recombinase/loxP system has been widely used for tissue-specific knock out of genes in laboratory animals. Cre recombinase is an enzyme that was first discovered in P1 bacteriophages with a function of catalysing the DNA recombination at the recognitions sites of *loxP* (Nagy, 2000). Cre recombinase/*loxP* system in mice involves a mouse that expresses Cre and a mouse that has *loxP* sites flanking a specific gene. The Cre gene is transgenically inserted into the mouse genome and replaces a gene which is specifically expressed in a tissue of interest. The loxP sequence is inserted on the sides of a target gene. Breeding the Cre mouse to loxP mouse for several generations leads to excision of the target gene in that specific tissue. Since the choroid plexus is an epithelial tissue which is abundant in the body, it is challenging to specifically target the choroid plexus using Cre/*loxP* system as described. A solution would be to expose the target cells directly to the Cre recombinase. However, cells are not permeable to the Cre enzyme. To overcome this challenge, a study described how Cre can be fused to the HIV protein Tat (Cre-Tat) to enhance the cell penetration (Wadia et al., 2004). To target the

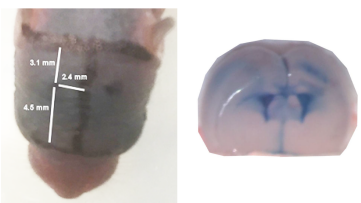

Figure 6. Intracerebroventricular injection sites on a P3 mouse. Left panel the injection site coordinates; right panel the coronal cut of the brain after the dye injection.

choroid plexus cells, we injected Cre-Tat directly into the ventricles to access the epithelial cells of choroid plexus as described by (Spatazza et al., 2013) in the adult mouse. We modified the method for P3 pups. The coordinates of the intracerebroventricular (ICV) injection sites was determined after several test injections of a dye, Evans blue (Fig. 6). The Cre-Tat dosage was also determined in pilot experiments based on the manufacturer data from *in vitro* tests.

RNA sequencing

RNA sequencing (RNA seq) is known as a "revolutionary tool" that has transformed the biomedical science in the past decade by revealing the whole transcriptome of cells in health and diseases and in different animal species (Wang et al., 2009b). RNA seq has some advantages over older transcriptomic tools such as microarray. The microarray transcriptomic techniques are limited to known transcripts of genes, while with RNA seq it is possible to discover new gene transcripts or novel spliced forms. High sensitivity for detection of low number of transcripts and great reproducibility are other advantages of RNA seq (Wang et al., 2009b). Using this method, different species of RNA (e.g. mRNA, miRNA) can be sequenced and quantified. In Paper IV, we performed RNA seq on an Illumina platform. In our study, the RNA was extracted from the choroid plexus and the quantity and quality of the RNA was assessed using a Bioanalyser system. In this system, RNA is electrophoretically separated on a microfabricated chip. All RNA bands, including the ribosomal RNA (18S and 28S) are automatically analyzed (Fig. 7) and a RNA integrity number is obtained (RIN score, scale of 0-10). The average RIN score of the samples in our study was 9.2, suggesting a high quality of RNA.

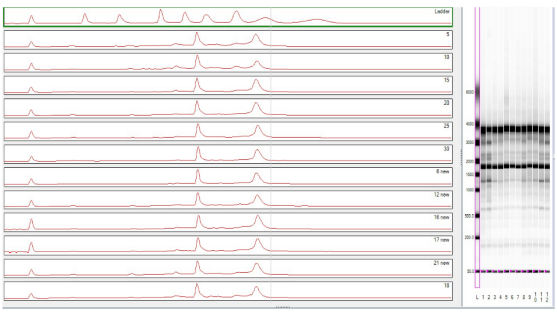

Figure 7. RNA quality assessment by Bioanalyser system on samples in paper V. RNA bands on electrohoretic gel (right panel) are analyzed by the system. The large peaks represent bands corresponding to 18S and 28S ribosomal RNA (left panel).

The sample preparation included removal of ribosomal RNA, fragmentation and cDNA synthesis. During removal of ribosomal RNA, small RNAs were also depleted leaving mRNAs and long non-coding RNAs. We omitted small RNAs since a different sample preparation was required and the RNA quantity was not sufficient. We aimed for 30 million reads per sample which is sufficient for differential expression analysis (Conesa et al., 2016). The length of the read was 100 base pair and was performed in a paired-end manner. The length of the read is important since very short reads cannot be mapped with certainty and are discarded. The read length of 100 base pairs is appropriate for accurate mapping as well as for detection of the splice junctions (Chhangawala et al., 2015). In paired-end sequencing, a DNA fragment is read from both ends in opposite directions, while in single-end sequencing the read is done in one direction. The former enhances the accuracy of the transcript mapping on a chromosome and identification of the splicing isoforms (Katz et al., 2010).

Transcriptome analysis

RNA seq produces a significant amount of data for analysis. Some experimental or clinical conditions change the transcription of thousands of genes. Therefore, the analysis and interpretation of this bulk of data requires the use of sophisticated mathematical models and analysis software. In paper V we compared the transcriptome of choroid plexus in five conditions including control samples. The count number of reads of a transcript is obtained from the sequencer. The basic analysis is differential expression analysis that demonstrates which genes are significantly regulated by conditions and how large is the difference (i.e. fold change). Following this, a gene clustering analysis can be performed where the aim is to find groups of genes that have similar expression patterns. This is per-

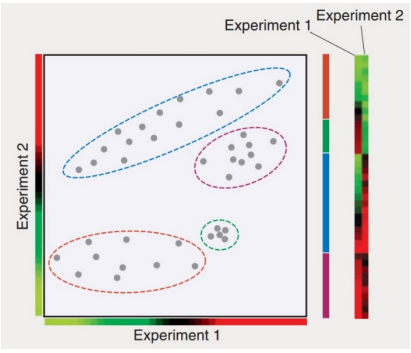

Figure 8. The clustering of genes expressed in two experimental conditions. Each dot represents a gene and x and y axis represent their expression level. The genes with shorter distance from each other cluster together. A heatmap (right panel) is made based on the clusters. Adapted by permission from Macmillan Publishers Ltd: [Nature biotechnology] (D'Haeseleer, 2005)

formed by calculating the mathematical distance between them. As shown in figure 8, the genes that are closer together form a cluster (D'Haeseleer, 2005).
Then, these groups of genes with similar expression patterns can be further analyzed by functionally annotating the gene clusters. This is performed by GO ontology profiling of genes. In the GO ontology database, each gene has been assigned a GO term (e.g. a biological pathway/function such as apoptosis) based on the knowledge or predictive methods (Yon Rhee et al., 2008). By mathematically comparing the number of significantly regulated genes that are related to a GO term in a dataset (e.g. 4 out of 1000 genes related to X term in our dataset) with all genes related to that GO term in a species (e.g. 500 out of 2300 mouse genes related to X term), an enrichment score and a significance value is obtained. We used DAVID Functional Annotation Tool v 6.8 for this purpose (Huang da et al., 2009b, a).

The GO ontology is a useful tool to gain an overall biological interpretation about the regulated genes in a dataset. However, this tool does not take into account the interactions between various gene products in a biological system and directionality of these interactions. Therefore, we used the Ingenuity Pathway Analysis (IPA) which is a knowledge-based platform for analysis of omics data. The IPA knowledge database is constructed and continuously updated by curators, which extract all the information about the interactions between biological molecules from research studies and their relationship to biological pathways and functions. Based on this database and mathematical models, IPA predicts what biological pathways are regulated (up or down) by the gene changes in the dataset. Moreover, upstream or downstream regulators of pathways are also predicted.

In vitro model of neutrophil transmigration through choroid plexus

Modelling choroid plexus *in vitro* has been a challenge for several reasons:

- choroid plexus consists of various cell types and a stroma
- choroid plexus is a highly polarized tissue
- choroid plexus epithelial cell barrier function should be mimicked
- choroid plexus of rodents such as mouse and rat is a small tissue, which means several animals need to be sacrificed for an *in vitro* experiment

The current choroid plexus *in vitro* models include a monolayer of epithelial cells

(choroid plexus epithelial cells; CPEC), the principal cell component of the choroid plexus tissue. To date, there is no co-culture model of choroid plexus epithelial and endothelial cells that comprises the choroid plexus vessels. However, most other properties of the choroid plexus can be recapitulated *in vitro*. For CPEC culture, both primary and immortalized cells have been used. However, the immortalized cell lines fail to reproduce some critical functions and characteristics of the choroid plexus such as the barrier function (Kläs et al., 2010; Lazarevic and Engelhardt, 2016). Therefore, in Paper V we used a method of primary culture of neonatal rat CPEC which was first described by (Tsutsumi et al., 1989) and later was optimized by (Strazielle and Ghersi-Egea, 1999). In this method, the choroid plexus tissue is first enzymatically and mechanically dissociated and then the epithelial cells are separated by sedimentation and differential adhesion to a plastic surface. The isolated cells are grown for approximately one week on a filter coated with extracellular matrix protein, laminin. For leukocyte trafficking studies the filter insert is inverted and cells are seeded before placing it back to its position in the well (Fig. 9). Before the transmigration experiment, the effect of different dosages of P3C on the CPEC permeability was assessed to insure the integrity of the barrier.

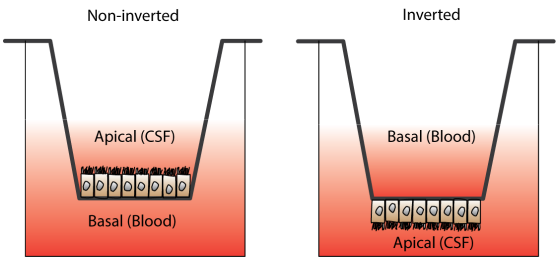

Figure 9. Inverted and non-inverted model of choroid plexus epithelial cell culture on the filter insert.

We isolated leukocytes from neonatal rat blood after hypertonic lysis of red blood cells. The leukocytes were added to the basal side of the choroid plexus and were allowed to transmigrate for five hours. The transmigrated cells were then collected from the apical side by centrifuging the medium and the cells were counted in a Bürker chamber or fixed on a slide for immunocytochemistry.

Statistics

In general, we used Student's t-test when comparing two groups of samples and one-way analysis of variance (ANOVA) together with a post-hoc test when comparing multiple groups. The significance was set at $p<0.05$. These tests are based on the assumption that the sample populations follow a Gaussian distribution.

However, the tests perform well even if the distribution is not ideally normal especially for large sample size (Lumley et al., 2002). Therefore, we did not test for normal distribution. The sample size for the HI experiments was fairly large and based on our previous studies and understanding of the injury variability. The data are presented as mean ± SEM or mean ± SD.

Results summary

Systemic activation of viral (TLR3) or bacterial (TLR2) receptors aggravates HI brain injury in neonatal mice

Infection and inflammation in the perinatal period is a major risk factor for brain injury in newborns (Hagberg et al., 2015). Our group previously showed for the first time that systemic activation of TLR4, a receptor for bacterial LPS, worsens the HI injury in the neonatal rats (Eklind et al., 2001). TLR4 is the main immune receptor for gram-negative bacteria. However, a large proportion of perinatal infections are caused by gram-positive bacteria and mycoplasma (Tita and Andrews, 2010) for which TLR2 plays a key role in recognition and in the inflammatory response (Takeuchi et al., 1999). Moreover, viral infections are another cause of inflammation in the perinatal period (Silasi et al., 2015) with TLR3 being critical for initiating the response. Therefore, we asked whether systemic activation of these immune receptors impacts on the HI brain injury. We used a neonatal mouse model of HI at an age corresponding to brain development in near term infants. Poly I:C and P3C were used as agonists for TLR3 and TLR2 respectively. Systemic administration of TLR3 and TLR2 agonists 14h prior to HI increased the neuronal tissue (MAP2+ tissue loss) loss by 91% (Paper I) and 46% (Paper II) respectively compared to the control group which received saline. Moreover, TLR3 stimulation increased the white matter (MBP+ tissue loss) injury at hippocampal and striatal levels by 430% and 140% respectively (Paper I). TLR2 activation also increased the total subcortical white matter injury by 32% (Paper II). Neonatal mice lacking the TLR3 adapter molecules TRIF were protected against the sensitizing effect of PolyI:C (Paper I). Likewise, TLR2 knockout mice were protected against the sensitizing effect of P3C (Paper II).

We also asked whether specific regions of the brain were more vulnerable to P3C-sensitized HI injury. Neuropathology scoring did not show a regional specificity of P3C sensitization (Fig. 10A). However, TLR2 knock out mice were most protected in the cerebral cortex (Fig. 10B).

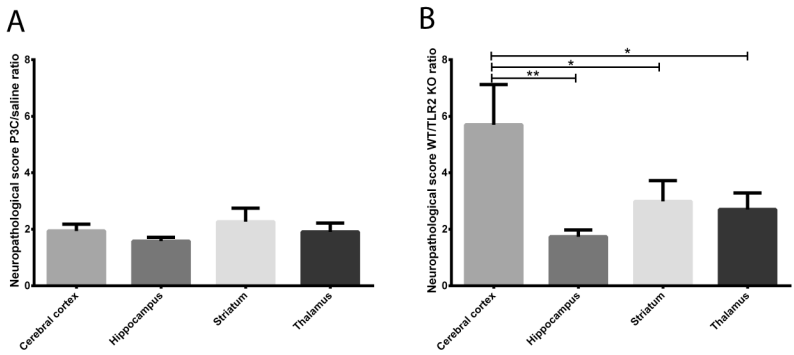

*Figure 10. TLR2 activation homogenously sensitizes all the brain regions to HI (A), however, TLR2 knockout mice are most protected in the cerebral cortex (B). Data presented as mean ± SEM. *P<0.05, **p<0.01, One-way ANOVA, n= (see Paper II).*

TLR3 activation induces neuroinflammation

Next, we asked whether systemic TLR3 activation leads to CNS inflammation. We assessed the CNS inflammation induced by Poly I:C by RT-qPCR (Paper I). Poly I:C significantly upregulated mRNA expression of IL6, TNF-a, IP10, IFN-b and MCP1 at 6h time point compared to the control group. At 14h, TNF-a, IL1-a, IFN-b and MCP1 were significantly upregulated (Paper I). The neuroinflammatory effect of PolyI:C was TRIF dependent as PolyI:C had no neuroinflammatory effect in TRIF KO mice (Paper I).

TLR3 activation suppresses cell survival pathways in the brain

Quantification of mRNA expression of Fas ligand, a pro-apoptotic protein, showed a significant upregulation by PolyI:C compared to the control. Furthermore, the phosphorylation of Akt, a pro-survival signaling molecule was significantly reduced by PolyI:C treatment (Paper I).

TLR3 and TLR2 stimulation alter the microglia phenotype

Microglia cells are the most abundant professional immune cells in the brain parenchyma, and develop different phenotypes in response to stimuli including a M1-like pro-inflammatory and M2-like reparative phenotype. Therefore, we asked whether TLR stimulation alters the microglia phenotype using flow cytometry on the brain single cell suspension (Paper I) and qPCR on isolated microglia. Poly I:C reduced the proportion of microglia with a reparative M2 phenotype. Likewise, neonatal mice treated with Poly I:C and exposed to HI had a significantly lower number of M2 microglia cells in the contralateral hemisphere compared to control group. In the ipsilateral hemisphere Poly I:C+HI mice also had a lower number of M2 microglia than saline+HI mice, although the difference was not statistically significant (Paper I).

On the other hand, microglia cells isolated from brain by magnetic-activated cell sorting at 14h after P3C treatment showed a mixed inflammatory phenotype characterized by upregulation of mRNA expression of both pro-inflammatory cytokines (e.g. IL1a, IL1b, TNFa) and anti-inflammatory cytokines (e.g. IL10) (data not shown).

Moreover, the number of microglia cells increased after HI in the ipsilateral hemisphere, however, prior PolyI:C (Paper I) or P3C (Fig. 11A) treatment did not affect the microgliosis. TLR2 deficiency also had no effect on microgliosis after P3C/saline+HI (Fig. 11B).

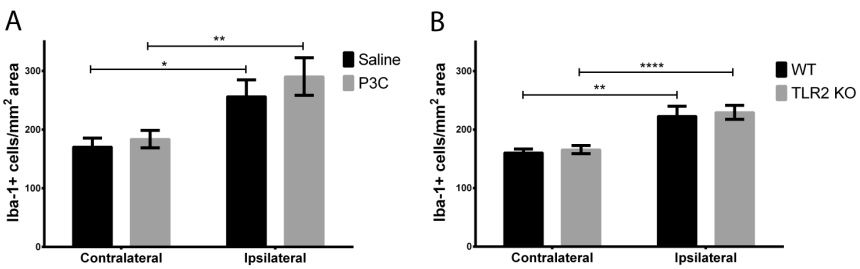

*Figure 11. Number of Iba-1+ microglia cells increase in ipsilateral hemisphere after HI. Microgliosis is not affected by prior P3C treatment (A) or TLR2 deficiency (B).). Data presented as mean ± SEM. *P<0.05, **p<0.01, ****p<0.0001, One-way ANOVA, n= (see in Paper II).*

Systemic TLR2 activation suppresses brain mitochondrial respiration

HI brain injury is primarily induced by general energy failure. Therefore, we asked whether systemic inflammation alters the function of the cellular powerhouse, the mitochondrion. We used a high-resolution respirometry system to assess respiration of mitochondria isolated from the brain 14h after systemic administration of P3C or saline. Oxidative phosphorylation induced by ADP was significantly suppressed by 23% in the P3C group compared to the saline group. Moreover, uncoupled respiration induced by FCCP showed a 21% reduction in the P3C group although the difference was not statistically significant (paper II).

We also asked whether P3C-induced systemic inflammation induces a mitochondrial oxidative stress and change in mitochondrial antioxidant enzymes. Therefore, we performed western blotting to measure the amount of SOD2 and PRX3 from brain-isolated mitochondria 14h after P3C or saline administration. We did not observe any significant change between P3C and control group (Fig. 12).

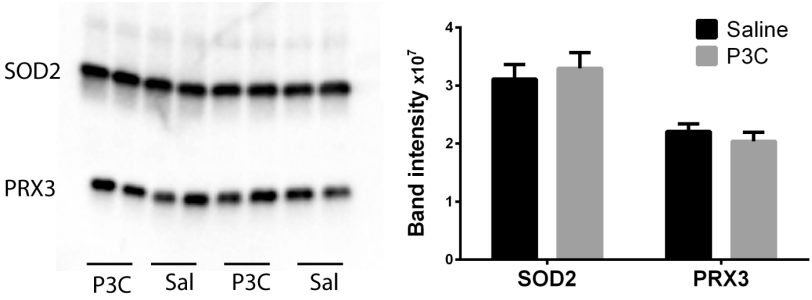

Figure 12. P3C does not change the protein expression of mitochondrial antioxidant enzymes SOD2 and PRX3 analyzed 14h after treatment. Representative image of western blotting for SOD2 and PRX3 (left) and the band analysis (right). Data presented as mean±SEM, n=8. No significant difference was observed between groups as assessed by Student t-test.

Systemic activation of TLR2 but not TLR4 induces neutrophil and monocyte invasion of the CNS

TLR2 and TLR4 are both receptors that are activated by certain bacteria and activation of either leads to phosphorylation and nuclear translocation of transcription factor NF-κB and expression of inflammatory cytokines. Surprisingly, i.p. injection of P3C (TLR2 agonist) but not LPS (TLR4) induced massive leukocyte infiltration to the CSF of neonatal and adult mice (Paper III). Likewise, P3C injection in neonatal rats caused a similar effect (Paper V). Infiltrating leukocytes were detected in the CSF as early as 6h after the P3C administration and peaked at 14h in neonatal mice. The number of cells gradually decreased at 24h and 48h time points (Paper III). Two dosages of P3C (1 mg/kg and 5 mg/kg) had similar effect. At the 48h time point, LPS injected at 1mg/kg (but not 0.3 mg/kg) slightly increased the number of leukocytes in the CSF. Flow cytometry revealed that the P3C-induced CSF-infiltrating leukocytes in neonatal mice were mainly neutrophils at 6h and inflammatory monocytes at 14h and 24h time points (Paper III). However, in neonatal rats infiltrating leukocytes were mainly neutrophils at 14h, as assessed by immunocytochemistry (Paper V).

To understand the striking difference between P3C and LPS, we first hypothesized that P3C at the administered dosages induced a stronger inflammatory response than LPS. We measured 23 cytokines in the plasma and spleen and found that LPS at either high or low dosage induced higher or similar level of cytokines as P3C 6h after injection. Therefore, we asked if P3C induces a specific neuroinflammatory profile. Multiplex cytokine assay showed significant release of IL1a, IL12, KC, MCP1, MIP1a, MIP1b and G-CSF in the brain 6h after administration of 5 mg/kg P3C. High dose LPS, on the other hand, induced significant release of G-CSF, IL6, KC, MCP1 and RANTES in the brain, while cytokine induction by low dose LPS was not significantly regulated (Paper III).

Next, we asked if P3C administration changed the CNS barrier permeability. To achieve this, we injected ^{14}C-sucrose intraperitoneally following P3C treatment and measured the accumulation rates of sucrose from blood to different CNS compartments including the CSF to assess changes in CNS barrier permeability. P3C increased the permeability of both the BBB and BCSFB 10h after administration (Paper III). The effect of P3C on BSCFB permeability was also tested and reproduced in neonatal rats (Paper V).

Choroid plexus is a route of TLR2-induced leukocytes trafficking

Next, we asked where in the brain the infiltrating leukocytes localize. To address this question, we first performed flow cytometry and found that in P3C-injected neonatal mice there were significantly higher numbers of CD11b+CD45^{hi} infiltrating leukocytes (Paper III). Then, we performed immunohistochemistry using LysM-EGFP mice in which peripheral myeloid cells express GFP. The results showed that infiltrating neutrophils and monocytes localize in periventricular regions of the brain including the choroid plexus and median eminence as well as in the subarachnoid space (Paper III). Therefore, we hypothesized that choroid plexus is a route of leukocyte entry. We tested this hypothesis *in vivo* and *in vitro*:

We first asked whether knocking down TLR2 adapter molecule MyD88 specifically in choroid plexus would inhibit the P3C-induced leukocyte infiltration to the CSF. To address this question, we injected Cre-Tat or saline intracerebroventricularly to P3 MyD88-flox mice. In response to P3C injection at P8, mice that had received Cre-TAT demonstrated 59% reduction in the number of infiltrated leukocytes in the CSF compared to the mice that received saline (Paper V).

To investigate whether leukocytes can transmigrate through the choroid plexus we performed studies *in vitro*. Leukocytes were isolated from neonatal rats after P3C injection and were added to the monolayer of CPEC pre-treated with P3C or saline. Neutrophils could transmigrate through the CPEC pre-treated with P3C but not saline (Paper V). Altogether, the results showed that the choroid plexus is a route of leukocyte entry after P3C administration.

TLR2 induces specific chemotaxis and cytoskeleton regulating pathways in the choroid plexus

To understand the mechanism of leukocyte transmigration through choroid plexus we performed RNA sequencing on choroid plexus isolated from mice 6h and 14h after injection with P3C or LPS. We hypothesized that both P3C and LPS induce an inflammatory response in the choroid plexus; however, genes that are specifically induced by P3C might be responsible for regulating the leukocyte transmigration. We selected the dosages of LPS and P3C that induced a similar systemic inflammatory response (Paper II).

Differential expression analysis of RNA seq data revealed that both P3C and LPS significantly regulated expression of thousands of genes in the choroid plexus (Fig. 13, paper IV). The number of genes regulated by LPS was higher than by P3C at both time points. There were 832 and 241 genes specifically regulated by P3C at 6h and 14h, respectively (Fig. 13). Gene cluster analysis and Ingenuity Pathway Analysis revealed P3C-specific effects on chemotaxis and cytoskeleton-regulating pathways (Paper IV).

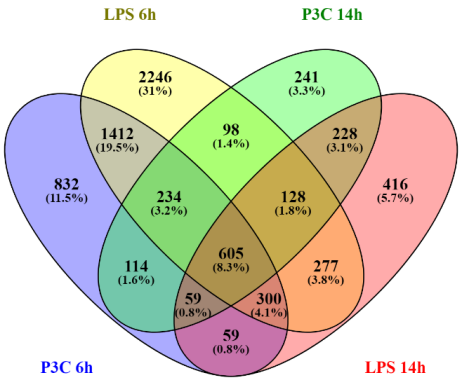

Figure 13. A Venn diagram showing the number and percentage of genes significantly regulated by P3C or LPS in the choroid plexus at different times after treatment.

N-acetyl cysteine blocks TLR2-mediated leukocyte infiltration to the brain

Reactive oxygen species (ROS) have a profound role in immune response as well as in cytoskeleton remodeling (Staiculescu et al., 2014). We asked whether targeting ROS has an impact on TLR2-mediated leukocyte migration to the CSF. We used N-acetyl cysteine (NAC) to target ROS as it has previously been shown to be effective in neonatal LPS-induced inflammation (Wang Ann Neurol 2007). NAC treatment almost completely blocked leukocyte infiltration to the CSF in neonatal rats treated with P3C (Paper V). Likewise, NAC treatment of CPEC significantly diminished P3C-mediated neutrophil transmigration. Multiplex cytokine assay revealed that the effect of NAC was largely independent of peripheral and central cytokine induction (Paper V).

Discussion

Sensitization of the developing brain to HI by systemic inflammation

Infection and inflammation are known risk factors for human neonatal brain injury. Previous experimental studies also support a contributory role of inflammation in neonatal HI injury (Hagberg et al., 2012; Hagberg et al., 2015). These studies have also shown that the inflammation-induced sensitization of the developing brain to the injury is time-dependent and dose-dependent. In other words, the effect of the inflammation on the brain injury depends on the temporal stage of the inflammation and its magnitude. For example, when LPS is injected 4h, 6h, or 72h before HI in neonatal rats the brain injury is increased (Eklind et al., 2001; Eklind et al., 2005). Likewise, LPS injection 14h before HI on P9 mice exacerbated the brain injury (Wang et al., 2009a). However, LPS injection 24h before HI leads to a pre-conditioning effect and reduced the injury (Eklind et al., 2005). Furthermore, low dose LPS injected 24h before HI reduces the neuroinflammation and protects the neonatal rat brain while high dose did not (Lin et al., 2009). The preconditioning is a general effect following a subtle insult that protects against a major insult. For example, a period of hypoxia 24h before the HI protects the brain of neonatal rats and improve the long-term outcome of sensory and cognitive functions (Gustavsson et al., 2005). The mechanism of the LPS preconditioning is yet to be elucidated; however, some components of cell death/survival pathways such as Bcl-2 family, JNK pathway, trophic factor pathways and inositol triphosphate kinase/Akt pathway has been suggested to be involved (Gustavsson et al., 2007; Mallard and Hagberg, 2007).

Overall, previous experimental studies suggest that inflammation with sufficient strength during an acute phase can sensitize the brain to HI injury. However, less is known about how different inflammatory stimuli sensitize to HI injury. As mentioned, the effect of the TLR4 agonist LPS on neonatal HI brain injury has been studied. LPS is the major component of the cell wall of gram-negative bacteria. However, gram-positive bacterial infections and viral infections are other important causes of perinatal inflammation that stimulate an inflammatory response via TLR2 and TLR3 respectively. In the present thesis we demonstrated that systemic activation of either TLR3 (Paper I) or TLR2 (Paper II) exacerbated the HI brain injury both in the grey and white matter.

While preparing this thesis a study was published by (Falck et al., 2017) showing that systemic activation of TLR2 in neonatal rats also sensitize the brain to HI

injury. Interestingly, the authors found that hypothermia was neuroprotective in P3C+HI model while it had no effect on the LPS+HI model suggesting a pathogen-dependent mechanism of injury and protection.

How does systemic inflammation sensitize the brain to HI injury?

We showed that systemic activation of TLR3 and TLR2 sensitizes the developing brain to HI injury. Both grey matter and white matter were affected. The grey matter tissue loss is the result of neural cell death. White matter loss was examined by loss of area of MBP staining. This may reflect white matter injury due to damage of the myelin sheath, axons and/or the oligodendrocytes (Hagberg et al., 2002a; Back and Rosenberg, 2014). In the present thesis detailed anatomical changes of the brain injury were not examined. However, potential mechanisms that may be involved in the injury progression was examined by addressing mainly three aspects:

1. TLR-mediated neuroinflammation

We found that both systemic TLR3 and TLR2 stimulation caused inflammation in the brain represented by expression of various inflammatory cytokines and chemokines, although, the neuroinflammatory profile was highly different between the two TLR agonists. PolyI:C induced interferon-related genes as well as genes regulated by NF-κB transcription factor such as IL1b. On the other hand, P3C induced release of several cytokines and chemokines in the brain leading to the CNS leukocyte invasion. One way to investigate experimentally whether these inflammatory changes in the brain are essential for sensitization, is to block the communication of systemic inflammation to the brain. We did found that inhibiting MyD88 activity in the choroid plexus ameliorated the P3C-induced neuroinflammation, however, the effect on HI injury remains to be investigated. Likewise, in paper V, we showed that targeting ROS activity by NAC reduced the neuroinflammation induced by P3C. However, we did not assess the effect of NAC on the HI injury.

What factors other than neuroinflammation may underlie the sensitization effect? One possible mechanism could be inflammation-induced effects on the cerebral blood flow. However, a previous study did not find that LPS affects the cerebral blood flow in an LPS+HI model in neonatal rats (Eklind et al., 2001). Another possible mechanism might be the changes in body temperature induced by systemic inflammation, since hyperthermia is associated with activation of apoptotic

pathways in the developing brain (Khan and Brown, 2002) and increased brain injury in adult stroke (Ginsberg and Busto, 1998). Conversely, hypothermia is neuroprotective in human neonates with HIE and animal models (Davidson et al., 2015). The neuroprotective effect of hypothermia might also affect neuroinflammation. However, P3C administration did not change the rectal temperature during HI, ruling out the possibility of hyperthermia-induced sensitization (Paper II). This is in agreement with the study by Falck *et al* where P3C had no effect on neonatal rat rectal temperature, however, hypothermia protected against the sensitized brain injury. Other systemic effects of inflammation on lung, kidney and liver function might also affect circulation metabolites, nutrients and gases that can play a role in sensitizing HI injury. However, it can still be assumed that neuroinflammation is the key component.

2. Neuroinflammation and cell death pathways

Does neuroinflammation sensitize neurons to injury? First, it should be considered that microglia and astrocytes play key roles in inducing a neurotoxic inflammatory milieu. We found a switch in microglia phenotype by PolyI:C and P3C. It was recently discovered that microglia release of TNF, IL1a and C1q induces a neurotoxic phenotype of astrocyte (called A1), although the secreted neurotoxin by A1 astrocytes remain to be identified. (Liddelow et al., 2017). In our study, PolyI:C induced TNFa mRNA upregulation in the brain. P3C, on the other hand, increased the mRNA expression of both TNFa and IL1a in the microglia and IL1a protein in the whole brain. Therefore, it is plausible that the neuroinflammation-induced sensitization might be partly due to microglia-dependent induction of A1 astrocytes. In support, it has been shown that microglia are required for LPS-induced oligodendrocyte injury and hypomyelination in the developing rat brain (Lehnardt et al., 2002). In addition to aforementioned hypothesis, neuroinflammation might directly affect the cell death pathways. Generally, cell death occurs through three major mechanisms: necrosis, apoptosis and autophagy. The necrotic and apoptotic pathways have some overlaps during HI leading to a "continuum" form of cell death (Northington et al., 2011a). Necrosis was previously considered as a non-programmed accidental cell death mechanism. However, recent advances revealed several regulated forms of necrosis including necroptosis, ferroptosis and parthanatos (Conrad et al., 2016). Necroptosis and parthanatos has been implicated in neonatal HI injury (Hagberg et al., 2004; Chavez-Valdez et al., 2012). Inhibiting necroptosis pathway by necrostatin reduced neuroinflammation and protected neonatal mice against HI brain injury (Northington et al., 2011b). Necroptosis is induced by TNF, FAS ligand, TNF-related apoptosis-inducing ligand (TRAIL) as well as TLR3 and TLR4 ligands (Weinlich et al., 2017). We showed that PolyI:C

upregulated the Fas ligand and TNFa expression in the brain (Paper I). P3C also upregulated the expression of TNF mRNA in the choroid plexus, however, it did not significantly change the TNFa protein in the brain. Therefore, there might be a link between TLR3 and TLR2 activation and the necroptosis pathway.

Parthanatos is a caspase-independent mechanism of cell death mediated by poly (ADP-ribose)-polymerase (PARP) and involving mitochondrial protein AIF (Fatokun et al., 2014). Interestingly, in EAE mice a cholesterol derivative activates PARP1 in microglia and astrocytes in a TLR2-dependent manner suggesting a link between these signaling pathways (Farez et al., 2009). Therefore, TLR2 activation affecting parthanatos in the neonatal brain might be plausible and warrants further investigation.

Both TNF and Fas can also trigger apoptosis. Triggering apoptosis by these death receptor ligands as well as by excitotoxicity leads to oligomerization of BAK and BAX mitochondrial proteins which mediates permeabilization of mitochondria outer membrane, release of cytochrome c to the cytosol, formation of apoptosome and activation of the executioner caspases (Fuchs and Steller, 2015). Inhibition of Bax activity by intracerebroventricular injection of a Bax-inhibiting peptide is neuroprotective in neonatal mouse HI (Wang et al., 2010). Therefore, mitochondria plays a central role in HI brain injury (Hagberg et al., 2014). TLR3 activation has been associated with apoptosis mediated via death receptors and mitochondria in an endothelial cell line (Sun et al., 2011). Likewise, intrathecal administration of P3C is also associated with neural apoptosis that is mediated by microglia cells (Hoffmann et al., 2007). Therefore, apoptosis priming might be one of the sensitizing mechanisms of PolyI:C and P3C. In support, we showed that PolyI:C inhibits the activation of anti-apoptotic kinase Akt (Paper I). In addition, PolyI:C activated NF-κB signaling (Paper I) that might in turn induce the pro-apoptotic p53 signaling (Fleiss et al., 2015). We also showed mitochondrial dysfunction by P3C in paper II which might prime the HI-induced apoptosis. As it is shown in some conditions, mitochondrial dysfunction precedes apoptosis probably via induction of ROS (Green and Leeuwenburgh, 2002; Griffiths et al., 2008; Zanotto-Filho et al., 2011). Moreover, reduction in mitochondrial respiration in cancer cells is associated with increased apoptosis mediated by ROS (Pelicano et al., 2003; Kwong et al., 2007). We did not measure ROS induction in the brain after TLR3 or TLR2 stimulation, and assessment of brain mitochondrial antioxidant enzymes did not show any changes in response to P3C administration which may or may not reflect the oxidative status of the brain. Finally, it is known that HI-induced excitotoxicity is enhanced by prior IL1b exposure (Degos et al., 2013). The expression of this cytokine was significantly upregulated by PolyI:C in the

brain and by P3C in the choroid plexus (Paper I and II) suggesting another mechanism of sensitization.

Autophagy is a cellular intrinsic mechanism for homeostasis by which undesired macromolecules and organelles undergo lysosomal degradation. Whether autophagy is a mechanism or a sequela (or both) of cell death has been a matter of debate in the last few years. However, growing evidence support the hypothesis of autophagy as a cell death mechanism (Kroemer and Levine, 2008). Autophagy has been detected in dying thalamic neurons of asphyxiated newborns (Ginet et al., 2014). In adult ischemic brain, however, autophagy has been suggested as a neuroprotective mechanism (Papadakis et al., 2013). Autophagy also plays a key role in inflammation and immunity (Levine et al., 2011). Although, we did not investigate the effect of TLR3 and TLR2 activation on brain autophagy-related proteins, there is evidenc for a link between TLR signaling and autophagy (Shi and Kehrl, 2008). Altogether, neuroinflammation might prime cell death pathways leading to sensitization of the brain to HI.

3. Neuroinflammation and cerebral energy metabolism

HI is essentially an energy failure condition. The energy failure occurs in two phases. During the primary insult a transient reduction in cerebral blood flow causes primary energy failure and is followed by a latent phase where metabolism recovers, as assessed by measurement of high-energy phosphates by magnetic resonance spectroscopy in asphyxiated human newborns (Hope et al., 1984) and piglets (Lorek et al., 1994). The latent phase is followed by secondary energy failure, the mechanism of which is not fully understood; however, excitotoxicity, mitochondrial dysfunction, inflammation and oxidative stress have been implicated (Allen and Brandon, 2011; Hagberg et al., 2014). In paper II we showed that systemic activation of TLR2 impairs complex-I mediated mitochondrial respiration. The respirometry was performed on mitochondria isolated from the brain, at the time when HI would have been induced. In other words, our results suggest that mitochondria are already dysfunctional at the time of primary energy failure. Therefore, TLR2 activation might have an accumulative effect on the HI-induced primary energy failure. Moreover, TLR-2 mediated mitochondrial dysfunction might potentiate the secondary energy failure as well. These hypotheses could be tested by measuring e.g. ATP at several time points after P3C/saline+HI. Furthermore, we did not investigate the underlying cause of mitochondria dysfunction, nor did we test the respiratory function of other complexes in the ETC. The enzymatic activity measurement of different ETC complexes would be a complementary experiment to elucidate the mitochondrial status change by systemic TLR2

activation. Several studies have shown a link between systemic inflammatory conditions such as sepsis and mitochondrial dysfunction in the brain (Brealey et al., 2002; d'Avila et al., 2008; Jeger et al., 2013) supporting a potential link between inflammation, mitochondrial function and HI injury.

TLR2-mediated leukocyte trafficking to the CNS through choroid plexus

We showed that systemic administration of the TLR2 ligand P3C induces marked infiltration of neutrophils and monocytes into the CNS. By performing several *in vivo* and *in vitro* experiments in two animal species, we found that choroid plexus is the most likely route of leukocyte entry into the CNS. Here I discuss some aspects of these results:

1- What is special about TLR1/2 signalling?

We found that systemic activation of TLR1/2, but not TLR2/6 and TLR4 results in significant leukocyte infiltration to the CSF and periventricular regions of the brain. Previously, we reported that TLR3 activation does not cause the same effect in leukocyte trafficking (Stridh et al., 2013). What is special about activation of TLR1/2? Both TLR2 and TLR4 signal through adapter molecule MyD88 leading to activation and translocation of transcription factor NF-κB leading to the expression of different inflammatory cytokines. TLR4, in addition, signals through another adapter molecule, TRIF, leading to activation of interferon-regulating factors (IRFs). IRFs are transcription factors that when activated result in an induction of interferon responses. We showed that LPS caused higher or similar level of systemic inflammation as P3C. However, the neuroinflammatory profile was strikingly different. One potential mechanism underlying this difference might be the differential expression of TLRs on immune cells and at the brain interface. TLR2 and TLR4, but not TLR3, are expressed in neutrophils and monocytes of neonatal and adult human and mice (Levy, 2007; Melvan et al., 2010; Dasari et al., 2011; Kollmann et al., 2012; Thomas and Schroder, 2013). In adult human monocytes the expression of TLR2 is higher than TLR4 (Hornung et al., 2002). TLR2-4 mRNA are also expressed on brain endothelial cells as well as in the choroid plexus (Nagyőszi et al., 2010; Mallard, 2012; Stridh et al., 2013). Therefore, it is not likely that different expression patterns of TLRs underlie the different neuroinflammatory effects. A TLR2 specific molecular mechanism must be behind its special effect on leukocyte transmigration. There are some studies comparing the gene expression and metabolic profiles induced by TLR4 versus

TLR2 agonists in leukocyte subtypes (Blankley et al., 2014; Lachmandas et al., 2016). The principal difference observed in the transcriptomic studies was that both TLR2 and TLR4 induce NF-κB-related genes but only TLR4 activation leads to expression of interferon-related genes as expected. Lachmandas *et al* recently reported that TLR2 and TLR4 stimulation induce different metabolic programs in human monocytes (Lachmandas et al., 2016). However, when the comparison was made between dosages of agonists which induced a similar level of inflammatory response, no significant difference was observed in the metabolic programs. Therefore, it is crucial to consider the dose-dependent nature of responses to different TLRs. Hence, in the RNA seq study (Paper IV) we used dosages of LPS and P3C that induce comparable inflammatory responses in the circulation. To our knowledge, no other studies have compared the effect of different TLR agonists on the brain interfaces at whole transcriptome level. However, recently, a qPCR-based study showed that lipoteichoic acid, a TLR2 agonist, downregulated mRNA expression of tight junction gene claudin 5 while LPS upregulated it (Mayerhofer et al., 2017). Moreover, the study showed that both agonists downregulated occludin which is in agreement with our transcriptomic results.

We demonstrated that P3C induces a specific cytokine profile in the choroid plexus although expression of many cytokines was similar between the two agonists. Moreover, a cytoskeleton-related pathway was also induced specifically by P3C. Therefore, P3C induces changes at the choroid plexus that potentially are required for the recruitment of leukocytes and facilitation of transmigration.

Reactive oxygen species influence many functions in cells including inflammatory responses (Mittal et al., 2014) and cytoskeleton/tight junctions rearrangement (Schreibelt et al., 2007). We showed that targeting ROS with NAC inhibited P3C-induced leukocyte migration through choroid plexus, which was independent of the peripheral and central cytokine response. Therefore, the results suggest that ROS activity within the migrating cells or epithelial cells at the choroid plexus is required for TLR2-mediated cell trafficking. Altogether, the results provide evidence for specific molecular mechanisms downstream of TLR2 such as chemotaxis and cytoskeleton pathways that might be involved in leukocyte trafficking to the brain.

2- Why does TLR2 activation results in leukocyte migration to the CNS?

Not all bacterial infections in the periphery leads to a massive leukocyte migration into the CNS. In fact, the effect of P3C in inducing leukocyte infiltration to the CSF best resembles clinical bacterial meningitis. P3C is a synthetic mimic of lipoproteins that are present in the bacterial cell wall of both gram-positive and gram-

negative bacteria. Localization and surface projection of these lipoproteins and therefore their associated TLR2 activation is strain-dependent (Kovacs-Simon et al., 2011). Moreover, it was recently demonstrated that bacteria-induced activation of TLR2 is dependent on the shedding of lipoproteins from the bacterial cell wall (Hanzelmann et al., 2016). However, many bacterial strains that activate TLR2 do not cause leukocyte infiltration to the CSF. This difference in response might be due to the diversity of lipoprotein structure (Kovacs-Simon et al., 2011; Nakayama et al., 2012) in different bacterial strains and whether they bind TLR1/2 or TLR2/6 at the brain interfaces, especially at the choroid plexus. Interestingly, some bacterial strains can hijack the TLR2 signaling pathway in order to alter the epithelial barrier function and access the target tissue (Chun and Prince, 2009; Clarke et al., 2011). Therefore, there might be an evolutionary competition between the immune system and pathogenic bacteria related to the function of TLR2.

Conclusion and future perspective

In this thesis, I investigated the effect of systemic inflammation on the developing brain in the context of HI injury (Fig. 14)

We showed for the first time that systemic inflammation induced by intraperitoneal injection of agonists for TLR3 (PolyI:C) and TLR2 (P3C) sensitize the neonatal mouse brain to subsequent HI injury. TLR3 activation augmented pro-apoptotic and suppressed pro-survival pathways. TLR2 activation, on the other hand, suppressed mitochondrial respiration in the brain. Both agonists induced a phenotypic switch in microglia cells that might compromise their reparative function. We also demonstrated that P3C induces a substantial infiltration of leukocytes to the CNS of neonatal mice and rats. Several *in vivo* and *in vitro* experiments suggested choroid plexus as the route of the leukocyte entry. By transcriptome analysis of choroid plexus, we demonstrate potential mechanism underlying the P3C-induced leukocyte trafficking including a chemotaxis signature and cytoskeleton reorganization.

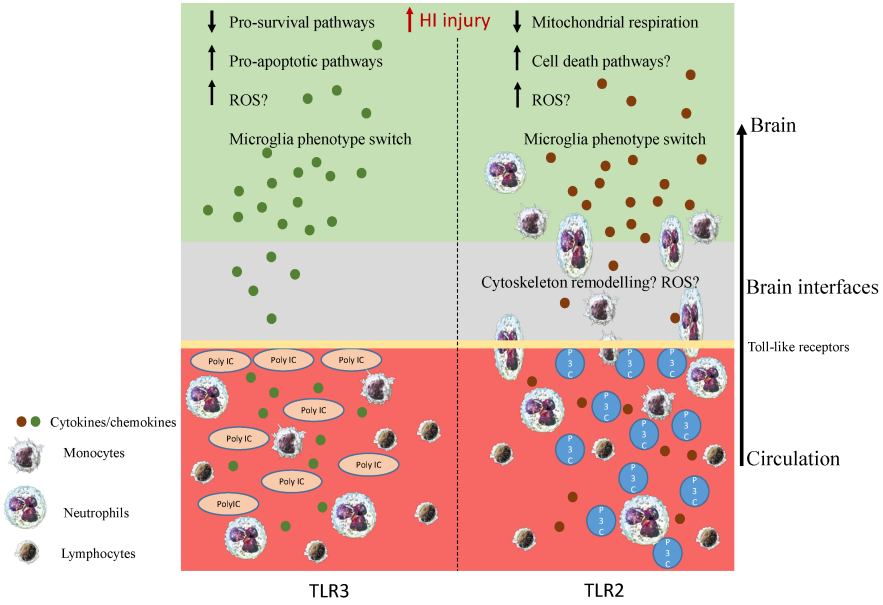

Figure 14. Schematic figure summarizing the mechanism of TLR3- (left) and TLR2-induced (right) sensitization of developing brain to HI injury.

Finally, we demonstrated that the antioxidant compound, NAC, inhibits the leukocyte infiltration without substantially affecting the cytokine/chemokine response. Infection and inflammation are major risks factors for neonatal HI injury, and this study reveals some mechanisms underlying this association and proposes novel drug targets.

In treatment of pregnancy complication such as perinatal viral infections and asphyxia, TLR3 might be a potential drug target to attenuate neonatal brain injury. Currently, no TLR3 inhibitor is clinically in use. However, some small-molecule probes have been described with antagonistic effect on TLR3 (Cheng et al., 2011). Moreover, this thesis and a previous study (Stridh et al., 2011) suggest TLR2 as a potential drug target, and some progress has been made in recent years in developing an inhibitor of TLR2 (Mistry et al., 2015; Patra and Choi, 2016). Therefore, the next step in translating our results into a therapy is to test the efficacy of these TLR inhibitors in pre-clinical neonatal models of HI brain injury. Moreover, we showed that NAC blocks the TLR2-mediated leukocyte invasion of the CNS. However, whether NAC diminishes the TLR2-sensitized HI injury remains to be investigated. We previously reported that NAC reduced the brain injury in an LPS+HI model in neonatal mouse, supporting its potential use in inflammation-sensitized brain injury (Wang et al., 2007). Fortunately, the pharmacokinetics of NAC in mothers with chorioamnionitis has been described and its safety has been confirmed in two pilot trials that paves the way for further clinical investigations (Wiest et al., 2014; Jenkins et al., 2016). We also demonstrated that the choroid plexus communicates the peripheral inflammation to the brain. Whether inhibiting this communication alleviates the brain injury is a question that needs to be addressed in future experimental studies. Hence, in the future, choroid plexus-targeted therapy might be considered as a strategy in treatment of inflammation-sensitized brain injuries.

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